

# Highly Potent Multi-Valent Nanobodies Against Chikungunya with VHH Screened from Alpaca Naïve Phage Display Library

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## Research Article

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# Abstract

**Background** Chikungunya virus (CHIKV) is a re-emerged mosquito-borne alphavirus that can cause musculoskeletal disease and impose a substantial threat to public health globally. It would be desirable to develop a high-affinity antibodies for the diagnosis and therapy of CHIKV infection. As potential diagnostic and therapeutic agents, multivalent nanobodies hold a significant promise towards nanomedicine. Here, we developed the highly potent multivalent nanobodies from an alpaca naïve phage display library targeting the E2 glycoprotein of CHIKV virus. Multivalent nanobodies play an important role in the promotion of high-affinity binding to E2 protein.

**Results:** In the present study, we generated 20 nanobodies using a naïve phage display library for binders to the CHIKV E2 glycoprotein. Of which, multivalent nanobodies of Nb-2E8 and Nb-3C5 had specific high-affinity binding to E2 protein with nanomolar range, showing the equilibrium dissociation constant (KD) of 2.59-20.7 nM, which is 100-fold stronger than monovalent nanobodies' affinity. Moreover, epitope mapping showed that the Nb-2E8 and Nb-3C5 recognized different linear epitopes located on the E2 glycoprotein domain C and A, respectively. A facile protocol of sandwich ELISA was established using the BiNb-2E8 as a capture antibody and HRP-conjugated BiNb-3C5 as a detection antibody. A good linear correlation was achieved between the *OD450* value and the E2 protein concentration in the 5-1000 ng/mL range ( $r=0.9864$ ,  $P<0.0001$ ), indicating that it can be used for the quantitative detection of E2 protein.

**Conclusions:** Multivalent nanobodies Nb-2E8 and Nb-3C5 exhibit functional features and high affinity distinct from monovalent nanobodies, showing new candidate diagnostic applications to detect sera binding protein and/or virions.

## Background

Chikungunya virus (CHIKV) is the causative agent of chikungunya fever (CHIKF), generally causes febrile, arthralgia/arthritis and rash with transmission from person to person mainly by *Aedes aegypti* and *Aedes albopictus* mosquitoes. Human CHIKV infection was not reported in Tanzania until 1953 when the pathogen was first major outbreak [1]. In 2007, the *World Health Organization* (WHO) launched the "Chikungunya Fever, a re-emerging disease in Asia" as a call for an increased focus of worldwide attention on Chikungunya [2]. In the past decades, it is prevalent in Pacific region and Latin America, affects >100 countries have been reported in millions of cases [3, 4]. Recent outbreak occurred in the China-Myanmar border in 2019, which was imported from Myanmar to Ruili City, Yunnan Province, China. This disease is characterized by a low mortality rate, but it could be easily misdiagnosed. Due to the strong infectivity, it is considered to be the rapidly spreading mosquito-borne diseases worldwide following malaria and dengue. While there are no licensed antiviral treatments or vaccines available for CHIKV infection, early diagnosis is nevertheless important.

CHIKV is an enveloped single-stranded RNA *Alphavirus* [6]. The mature infectious CHIKV is a ~70 nm icosahedral enveloped particles in diameter, composed of transmembrane glycoproteins E1, E2 and E3.

E1 and E2 glycoproteins assemble into heterodimers with 80 trimeric spikes of the virions [7, 8]. E1 is a type II transmembrane glycoprotein which mediates fusion of virus and the host cell membrane [9, 10]. E3 is responsible for the folding of the E2-E1 heterodimer but is cleaved during post-translational maturation [11]. E2 is composed of three immunoglobulin (Ig)-like domains located at the center of an N-terminal domain A, the lateral tip of domain B, and at the lateral tip of a C-terminal domain C. Domain A and domain B is considered to be the binding site of the Mxra8 receptor on the target cell [12]. Because of this structural advantage, CHIKV glycoprotein E2 appears to be immunodominant with multiple identified epitopes which induce neutralizing antibodies and has a higher serodiagnostic potential.

VHHs, are single-domain antibodies of molecular weight ~15 kD that are derived from camelid and cartilaginous heavy-chain only antibodies [13, 14]. As the smallest functional antibody (nanobody), it is widely studied both in *vitro* and in *vivo* and in diagnosis. Compared to conventional antibodies, VHHs are highly stability and solubility, easily purified from *E. coli*, and typically have nanomolar binding affinities [15–17]. Specifically, the CDR3 of nanobodies plays a key role in recognizing cavities or hidden epitopes such as the virus-binding site of a cell surface receptor [18] and the active site of an enzyme [19, 20]. For fast diagnosis and efficient treatment, screening of nanobodies from naïve library is the most straight forward way. Dong, et al [21] and Huo, et al [22] identified the high-affinity nanobodies against SARS-CoV-2 from naïve llama VHH libraries that binds to the S/RBD protein and block its interaction with ACE2. Yan, et al [23] recognized two human procalcitonin (PCT) nanobodies from naïve phage display library and successfully applied to develop a sandwich enzyme-linked immunosorbent assay, which showed a linear working range from 10-1000 ng/mL of PCT.

Here, we generated 20 nanobodies using a naïve phage display library for binders to the CHIKV E2 glycoprotein. Considering the advantages of activity and affinity, Nb-2E8 and Nb-3C5 were selected as the focus of the present study. Our results suggested that both nanobodies could bind to CHIKV E2 protein and virus particles as well as recognizing different epitopes. In addition, a 100-fold increase in its affinity for the E2 following two nanobodies were further constructed into multivalent formats. It can be used for diagnostic applications to detect sera binding protein and/or virions, either as single VHHs or in combination.

## Materials And Methods

### Cell lines and viruses

Vero cells were obtained from the ATCC and cultured in DMEM supplemented with 10% FBS (Thermo Fisher Scientific) and 1% penicillin and streptomycin at 5% CO<sub>2</sub>, 37°C.

Chikungunya/human/China/GD134/2010 (GenBank Accession: HQ846359) was isolated from serum of an infected female patient at Guangdong Provincial Center for Disease Control and Prevention.

Zika/human/China/GD01/2016 (GenBank Accession: KU740184) was isolated from Guangdong inbound passengers. All experiments involving the CHIKV and ZIKA authentic virus were conducted in Biosafety Level 3 and 2 laboratories, respectively. Virus virions were inactivated using β-propiolactone

(Sigma-Aldrich) when its concentration was 1:2000 at 4°C for 24 hours. Complete inactivation of virus was confirmed by the lack of replication in a Vero infection experiment. Supernatants containing virus particles concentrated using PEG-it (SBI Biosciences) overnight at 4 °C and resuspended in PBS for further use.

## Nanobodies screening from naïve phage display library

A VHH phage display library (AlpaLife) constructed in the vector pADL-10b and containing  $\sim 2 \times 10^9$  cfu was inoculated into 2×YT supplemented with 100µg/mL ampicillin (2×YTA) and infected with M13K07 helper phage to obtain a library of VHH-presenting phages. Biopanning of phages displaying VHHs specific for the Chikungunya/SL-CK1 E2 (Sino Biological) were performed as described previously [24-25]. After the three rounds of panning, 384 individual clones were picked to inoculate 2×YTA and were grown overnight at 37 °C, supernatant of clonal phage were detected by phage-ELISA with HRP-conjugated goat anti-M13 IgG antibody (Sino Biological).  $OD_{450}$  values  $\geq 0.5$  and  $P$  (positive  $OD_{450}$ )/ $N$  (negative  $OD_{450}$ ) greater than 3  $P/N$  ratios was determined as positive clones. Positive candidates were sequenced (Sangon Biotech) and aligned with complementary determining regions (CDRs) amino acid sequence. Five percent BSA was used as a negative control for each round.

## Expression and purification of VHHs in *Escherichia coli* (*E.coli*)

For monovalent nanobodies, sequences were synthesized (Generay Biotech), and subcloned into pET-SUMO with a tandem N-terminal His-tag, SUMO-tag, and plasmids were subsequently transformed into *E. coli* BL21(DE3) cells. All protein expression overnight at 18 °C was induced at  $OD_{600}$  of 0.6 by addition of 0.5 mM IPTG. The His-tagged protein was purified by Talon Metal Affinity Resin (Clontech) according to the manufacturer. The eluates were concentrated using a 1.5 mL Microsep advance centrifugal devices (PALL) with a molecular weight cut-off of 3 kDa. Purity quality was analyzed by Coomassie-stained SDS-PAGE.

## Expression and purification of Fc conjugated multivalent nanobodies in HEK293 cells

Bivalent and trivalent VHHs were fused to the Fc region of human IgG1 and cloned into the pcDNA 3.4. Multivalent nanobody units were connected through (GGGS) $\times 3$  flexible linkers. The Fc-fusion constructs were expressed in HEK 293 cells at 8% CO<sub>2</sub> 37°C for 1 week. Nanobodies in the supernatant were purified using protein A. Purity of all samples was analyzed by SEC-HPLC.

## Indirect ELISA to quantitate initial binding

Microtiter plates (Corning) were coated with 2µg/mL Chikungunya E2, 10µg/mL purified virus virions, or the control protein SUMO in carbonate buffer (CBS, pH 9.6) overnight at 4 °C, and blocked with 3% BSA(Sigma-Aldrich) PBS pH 7.4 at 37 °C. Serial 10-fold dilutions of SUMO-tagged VHHs in 3% BSA were incubated with the immobilized antigen, followed by incubation with HRP-conjugated goat anti-SMT3 (1:2000, CUSABIO). After wash, 100 µL of TMB substrate (TIANGEN) was added the wells and reactions were stopped with 100µL of 1M HCl. Absorbance was measured at 450 nm on a Epoch<sup>TM</sup> microplate reader (BioTek Instruments Inc., Winooski, VT, USA). EDIII protein of ZIKA was used as the negative control.

## **Nanobodies were validated by western blotting**

Purified Chikungunya E2 protein (20µg) and purified Chikungunya virion (50µg) were loaded onto 12% SDS-PAGE and electro-transferred to a PVDF membrane (Millipore). Following blocking of membranes with 5% BSA in 0.05% TBST and incubated overnight with 1:500 dilution of nanobodies. Membranes were probed to a SMT3-HRP conjugated antibody (1:1000, CUSABIO) and revelation with SuperSignal West Dura ECL reagent (Thermo Fisher Scientific). Chemiluminescence images were generated with FluorChem E scanner system (ProteinSimpleSan, Jose, CA, USA).

## **Localized Surface Plasmon Resonance (LSPR) assay**

LSPR measurements were performed using a OpenSPR<sup>TM</sup> instrument (Nicoyalife) to determine the affinity of monovalent nanobodies to E2 protein. The COOH chip (Nicoyalife, Canada) was loaded onto the OpenSPR<sup>TM</sup> instrument following the standard OpenSPR<sup>TM</sup> procedure. Run with PBS (pH 7.4) at maximum flow rate (150 µL/min) to reach signal baseline. Sample 200 µL of isopropanol and run for 10 s to evacuate air. After baseline is reached, the PBS buffer is rinsed through the sample loop and evacuated with air. Slow down the flow rate of PBS (pH7.4) to 20 µL/min, and then load 200 µL of EDC/NHS (1:1) solution to activate the COOH sensor chip. Dilute 200 µL of ligand E2 protein (0.4 mg/mL) for 4 min and rinse the sample loop with PBS (pH7.4). Sample with 200 µL blocking solution, the sample loop is rinsed with PBS and evacuated with air. Baseline was observed for 5 min to ensure stability. Selected nanobodies were diluted into a series of different concentrations and sampled at 20 µL/min. Both nanobodies and ligand binding times were 240 s and natural dissociation was 360 s. Kinetic parameters for the binding reactions were calculated using Trace Drawer software (Ridgeview Instruments AB), One to One analytical model.

## **ELISA-based Mxra8-Fc binding assay**

A fragment of cDNA encoding the Mouse-Mxra8 extracellular domain (residues 23-336, GenBank accession number NM\_024263.4) or Human-Mxra8 extracellular domain (residues 24-337, GenBank accession number NM\_032348.3) was appended with a TEV enzyme site and a human IgG1 Fc at the C-

terminus as well as the IL-2 signal peptide at the N-terminus in pcDNA 3.4 expression vectors and transiently transfected into HEK293 cells followed by media collection and purification using protein A sepharose. Mxra8-Fc binding assay was adapted from previously described [26]. MaxiSorp ELISA plates (Corning) were coated with 2µg/mL anti-mouse CHIKV E2 monoclonal antibodies in CBS (pH 9.6) overnight at 4 °C. Washed four times with PBS and blocked with 4% BSA(Sigma-Aldrich) for 1h at room temperature (RT). CHIKV virions (1µg/ml) were diluted and added for 1 h at RT. After washing, MoNb-2E8 and MoNb-3C5 or Mouse Mxra8-Fc fusion protein (all at 10 µg/ml) were incubated for 30min. Plates were washed and Human Mxra8-Fc (10-fold serial dilutions) were added to the plates and incubated for 1h at RT. Plates were washed again and incubated with secondary Rabbit Anti-Human IgG-Fc (1:5000, Bioss). After washing, the plates were developed with TMB substrate (TIANGEN) and 2N H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 450 nm.

## Indirect immunofluorescence assay

An indirect immunofluorescence assay was developed on CHIKV-infected Vero cells, as previously described [27]. Briefly, infected-cells (MOI, 1) were cultured for 72h and then fixed in 4% paraformaldehyde (Biosharp) for 20min. PFA-fixed cells were permeabilized and blocked with 1% Triton X-100(MP)/1% BSA(Sigma-Aldrich)/PBS for 1h at room temperature. The cells were incubated with nanobodies (10µg/mL) at 37°C for 30 min, followed by Alexa Fluor 488 Anti-6×His tag antibody (Abcam) or Cy3-labeled Goat Anti-Rabbit IgG (H+L) (Beyotime) for 30 min at 37°C. After washing with PBS, cells were observed under a fluorescence microscope (Mshot). Anti-CHIKV rabbit serum was obtained from a rabbit immunized with β-propiolactone CHIKV GD134 virion and used as a positive control. Anti-CHIKV E1 mouse IgG antibody (R&D Systems) was used as a control antibody.

## Flow cytometry assay

The binding of nanobodies to virus on cell surface was assessed by flow cytometry assay. Monolayers of Vero cells were infected with CHIKV and ZIKA at an MOI of 0.1 and harvested at 48h post infection. Cells were then fixed with 4% paraformaldehyde, and permeabilized with permeabilization buffer (0.05% Triton-X in PBS). 10µg/ml nanobodies were added into cells and incubated for 60 min on ice. Mouse anti-E2 monoclonal antibody was used as a positive control. After washing twice with PBS, cells were stained with 1:1000 diluted goat anti-human H&L (FITC) or FITC Rabbit polyclonal to 6×His tag (Abcam) for 45 min and analyzed using flow cytometry (Beckman CytoFlex, Brea, CA, USA).

## Human sera samples were detected in a sandwich ELISA

HRP-coupled bivalent VHHs performed using HRP Conjugation Kit (Abcam) following manufacturer instructions. 2 µg/mL of BiNb-2E8 in CBS (pH9.6) was added per well of a coated 96-Well Plate at 4°C overnight. After washing with PBST and blocking with 5% BSA in PBST for 1 h, 100 µL of E2 protein

diluted in a 2-fold dilution series (starting dilution 5 in 10240) or human sera samples (dilution 1:10) were added and incubated at room temperature for 1h. Wells were washed 3×5 min, and 100 µL of HRP labeled BiNb-3C5 (1 µg/mL) was added to each. After 1 h of incubation, plates were washed 5 times with PBST. TMB was added to the wells for colorimetric development and the absorbance was read at 450 nm. Human Mxra8-hFc served as the control. Samples were considered seropositive if *OD450* values higher than the mean obtained for the negative samples plus 3 standard deviations. The CHIKV positive sera samples were sourced from Center for Disease Control and Prevention of Southern Theater Command. Twenty CHIKV negative sera from healthy subjects were used for the calculation and validation of the cut-off value and excluded the sample matrix.

## Epitope-binding using peptide-based ELISA

A pool of 10-mer peptides with 5 amino acid overlap spanning the Chikungunya/SL-CK1 E2 glycoprotein were generated by chemistry to a purity of 90% (GL Biochem). All peptides were provided as lyophilised power, reconstituted in DMSO to a concentration of 1mg/mL, and stored at -80 °C. Each peptide was coated at 1ug/mL in CBS (pH 9.6) overnight at 4 °C and then blocked with 5% BSA(Sigma-Aldrich) in PBST. An irrelevant SARS-CoV-2 peptide was used as the negative control. Binding of the coated peptide was characterized by incubation with 2µg/mL nanobodies. HRP Mouse monoclonal to 6× His tag (Abcam) used at 1:5000 dilution in blocking buffer, and further absorbance measurement of the enzymatic reaction in TMB substrate (TIANGEN), were used to detect the bound epitopes.

## Prediction of the conformation of nanobodies complex with CHIKV E2 protein

The homology model of protein structure was built on the DeepMind algorithm AlphaFold system (<https://deepmind.com/>). The complex model of Nb-2E8 or Nb-3C5 to CHIKV E2 protein were generated by pyDOCK (<https://life.bsc.es/pid/pydockweb>). All structural data of the docking models were visualized using PyMOL software (<https://pymol.org/2/>).

## Statistical analysis

All experiments were performed at least two times in duplicate, and representative data or pooled data from repeat experiments were recorded. Statistical analyses were carried out using GraphPad Prism Software (San Diego, CA, USA). Data were presented as the mean ± SD. Students t-test was used for two groups comparison. Two-sided *P*-values<0.05 were considered statistically significant.

## Results



# Screening and characterization of nanobodies targeting the E2 Protein

Nanobodies have become increasingly accessible using high-throughput naïve libraries to rapidly recognize multiple target antigens while resisting adverse biochemical properties such as instability, multi-reactivity and aggregation during affinity maturation [28, 29]. To obtain active protein-binding clones from enriched library, we conducted three rounds of screening (Fig. 1a) and examined for specific protein-binding using a phage ELISA. Table S1 showed that the phage clones specific to the CHIKV E2 protein were effectively enriched through consecutive selection rounds. A total of 119 positive clones were included for the genomic sequencing and data analyses (Table S2). After complementarity determining regions (CDRs) sequence analysis, 20 unique CHIKV E2 specific nanobodies were identified (Fig. 1b and 1c). These sequences were clustered into several discrete groups based on VHH similarities (Fig. 1d).

## Soluble expression and purification of the nanobodies using the *E.coli* system

Using the prokaryotic system in *E.coli* strain BL21, we expressed recombinant nanobodies with N-terminally 6×His- and SUMO-tagged protein (Fig. 2a). The expressed nanobodies can exist as intracellular soluble and active proteins mostly found in the lysate supernatant (Fig. S1). However, Nb-4C1 and Nb-4G10 were aggregated into inclusion bodies in *E.coli* cytoplasm (Fig. S1). Soluble nanobodies were further purified by cobalt-chelating Sepharose columns using 250 mM imidazole for elution. Coomassie-stained SDS-PAGE showed the recombinant proteins were detected at the expected apparent molecular weights, ~28kDa (Fig. 2b). To confirm the specificity of binding of 20 candidate nanobodies, CHIKV E2 as coating antigen were carried out by indirect ELISA (Fig. S2). Of these, Nb-2E8 and Nb-3C5 can specifically bind with E2 protein as well as CHIKV virion (Fig. 2d). Moreover, western blot showing Nb-2E8 and Nb-3C5 were also able to recognize the purified CHIKV virion and E2 protein (Fig. 2c). The results suggest that both nanobodies recognize linear epitopes on CHIKV E2.

## Binding and identification profiles for MoNb-2E8 and MoNb-3C5

To validate the binding capabilities of nanobodies targeting E2 protein, we measured the binding kinetics of nanobodies by LSPR. While MoNb-2E8 and MoNb-3C5 have an affinity of 101 nM and 368 nM (Fig. 3a), showed a weak affinity with E2 protein. We also evaluate whether Nb-2E8 and Nb-3C5 compete with Mxra8-Fc to bind with CHIKV. Both Human Mxra8-Fc and Mouse Mxra8-Fc could noncompetitively bind to CHIKV (Fig. 3c). This would explain that, Nb-2E8, Nb-3C5 and Mxra8 interact at different sites with virions and most likely do not compete for the same epitope. Nb-2E8, Nb-3C5 and anti-rabbit serum were used as

primary antibodies to detect the expression of viral E2 protein in Vero cells at 72h post-infection. Fig. 3b showed that specific green and red fluorescence from were observed in the virus-infected cells, but not virus-uninfected cells. In order to further confirm the binding specificity of the nanobodies, CHIKV infected cells were labeled with Nb-2E8 and Nb-3C5 and subjected to flow cytometry (Fig. 3d). Blue boxes, the proportion of cells in which the fluorescence value exceeded the gating threshold. Quantifications presented in ratios of fluorescence-positive cells to the total number of cells, revealed that the FITC-positive values of CHIKV infected-cells were visibly higher than ZIKA infected-cells ( $P<0.05$ ).

## Establishment of the sandwich ELISA

To further improve the affinity of nanobodies against CHIKV E2, Nb-2E8 and Nb-3C5 were fused to an Fc domain of IgG1 to generate multivalent fusion proteins and obtain milligram quantities of highly pure (>95%) recombinant proteins (Fig. 4a, 4b, and Table S3). BiNb-2E8, BiNb-3C5, TriNb-2E8, and TriNb-3C5 showed high binding affinities to CHIKV E2 with equilibrium dissociation constants (KD) of 2.59, 20.70, 4.52, and 10.40, respectively (Fig. 3a). Cytofluorimetric quantification of BiNb-2E8 stained cells to the total number of cells was 95.81% and 95.20% for BiNb-3C5 (Fig. S3). We developed a sandwich ELISA protocol, purified bivalent nanobodies was used as the capture and different conjugated bivalent nanobodies was used as the detection, to quantify relative antigen response levels. From 4 independent experiments, the linear equation of  $Y = 6.908e-004 X + 0.7684$  ( $r = 0.9864$ ,  $P < 0.0001$ ) was found to be the optimal linear fit, which demonstrated good linearity over the concentration range (5-1250 ng/mL) (Fig. 4c). The BiNb-2E8 was combined as capture and the HRP-labelled BiNb-3C5 as detection to reveal combination with good performance.

## Validation with human sera samples

To establish a baseline for the evaluation of the E2 antigen capture test, 20 CHIKV seronegative samples were analysed. Cutoff was defined as the mean  $OD_{450}$  value of the negative controls + 3 SD:  $0.0696 + (3 \times 0.0128) = 0.108$ . Hence, sera samples giving  $OD_{450}$  values higher than 0.108 were recorded as positive, and negative when  $OD_{450}$  values lower than 0.108. By this criteria, all 15 serum of patients infected with CHIKV had OD values greater than that of the cut-off value, which were judged as positive. Also, the difference between CHIKV seronegative and seropositive samples were highly significant statistically ( $P < 0.0001$ ) (Fig. 4d). Therefore, the sandwich ELISA established in this study can be used in the early diagnosis of CHIKV viral infection.

## Mapping linear epitopes of the E2 protein using Nb-2E8 and Nb-3C5

To characterize specific linear epitopes of CHIKV E2 protein, 68 peptides with an offset of ten amino acids were used to assess responses (Table S4). As shown in Fig. 5b, P61- GEEPNYQEEW and P10-

LKIQVSLQIG strongly reacted with Nb-2E8 and Nb-3C5, which correspond to the amino acid residues 331 to 340 and 46 to 55 of CHIKV E2 domain C and A, respectively (Fig. 5a). Comparison of epitopes of the Nb-2E8 and Nb-3C5 with sequences from representative strains of each CHIKV genotype (SL-CK1, BR33, CU-Chik683 and Ross: East, Central, and South African (ECSA); RSU1: Asian; Senegal: West African) (Fig. 5c). Of note is that Nb-3C5 recognised epitope which is highly conserved across the six-representative CHIKV genotype. And two amino acids at sites were fairly well conserved among other alphaviruses genus. The Nb-2E8 epitope had two residue variation at positions 302 (E-Q) and 307 (Q-H) in West African genotype of CHIKV and was not identical among all representative of alphaviruses. For visualization, computer docking simulation was used to map the binding of nanobodies to the E2 protein. Similar to peptide-based ELISA, the formation of complex demonstrates Nb-2E8 and Nb-3C5 bind different epitopes on E2 (Fig. 5d). In the complex, residues L98, G99, W100, T101, and L108 from CDR3 loop of Nb-2E8 have made contacts with the P61 epitopes of E2. The CDR1 and CDR3 region of Nb-3C5 has contributed to the P10 epitopes of domain C.

## Discussion

Nanobody opens important possibilities for biomedical applications, but so far most generated by immunized Camelidae, which is time-consuming and expensive for laboratories. Naïve phage display library, a technique for obtaining genetically engineered nanobodies against an antigen without immunization[30], allow the isolation of antibodies against autoantigens, non-immunogenic or toxic antigens. High affinity antibodies can be obtained in a short time when the library capacity is sufficiently large. VHH, also namely nanobody, is 10-fold smaller than conventional antibodies with unique advantages[31]. As a result of their small size and stability, it is easily incorporated into the body's natural metabolic pathways. More importantly, VHH domains have longer complementary antigen-binding regions CDR3, providing greater antigen-binding capacity, and the affinity of nanobodies can be further improved by genetic engineering techniques[32]. In this study, we performed genetically engineered nanobodies targeting CHIKV by using phage display technique based on a naïve antibody library derived from 103 healthy adult alpaca lymphocytes with a capacity of  $2 \times 10^9$  cfu, which was able to screen for specific binding nanobodies. A total of 20 VHH sequences specifically binding CHIKV E2 were obtained, containing CDR1, CDR2 and CDR3. The amino acid sequence length of CDR3 region varies from 17-20, with 85% of the sequences greater than 17 amino acids, indicating typical characteristics of a nanobody. In the FR2 region, high stability and solubility of VHH, attributing to the typical substitutions of hydrophobic residues. Altogether, nanobody was a convenient monovalent scaffold, with the single chain easily expressed in *E. coli*.

The pET-SUMO expression system, which combines the pET plasmid with SUMO partner together, linking the target gene to SUMO via homologous recombination cloning strategy, and is efficiently and stably expressed under the control of strong T7 promoter[33]. The SUMO protein was an ideal candidate tag for enhancing expression, folding and stability of proteins[34, 35], especially for heterologous proteins like antibody fragments[36]. Several studies have reported recombinant proteins have been efficiently

produced using the SUMO fusing system in *E. coli* [37–39]. The results from this study agree with the previous study. Nanobodies were secreted to the culture medium in a soluble form. An N-terminal 6×His Tag was added to the express vector to aid purification and, SDS-PAGE analysis revealed a single band at approximately 28kDa. However, two nanobodies Nb-4C1 and Nb-4G10 were expressed in *E. coli* and refolded from inclusion bodies. We speculated that it can potentially be associated with VHH sequences.

For the purified nanobodies, after identified by ELISA, Western blot, and IFA mediated overlap experiment, Nb-2E8 and Nb-3C5, which we chose for further validation. SPR technology is widely known as a golden standard for capturing antibody-antigen interactions[40, 41]. To determine the kinetic rate and affinity constants, binding analysis of E2 and monovalent nanobodies were carried out by LSPR. SPR results demonstrated that the binding affinity of MoNb-2E8 and MoNb-3C5 is a relatively low. Obviously, recombinant fusions of monovalent nanobodies are limited by weak binding affinity due to the lack of multivalent affinity benefits, poor production capacity and potential immunogenicity[42–44]. To further improve the affinity of nanobodies against CHIKV E2, homo-dimer (BiNb-2E8, BiNb-3C5) and homo-trimer (TriNb-2E8, TriNb-3C5) were fused to an Fc domain of IgG1 to generate multivalent fusion proteins. We surprisingly found that compared with the corresponding monovalent nanobodies, both homo-dimer and homo-trimer show a 100-fold enhancement towards E2 protein. Among them, BiNb-2E8 was confirmed by LSPR analysis, with a binding affinity of ~2.59 nM. Developing highly affinity and specific nanobodies for capturing viral antigens is crucial for any point-of-care testing (POCT) such as ELISA to be successful. Thus, we capitalize on the strengths of the selected nanobodies to establish a rapid, convenient and reliable screening test.

A major advantage of the ELISA method is that it can detect viruses at a concentration of 1-10µg/ml and require little antibodies[45]. Traditional antibodies considered for developing ELISA have been polyclonal and monoclonal antibodies. However, these highly specific antibodies are cost-effective and have poor stability under adverse environmental conditions[46]. Nanobodies are powerful tools for a wide application in molecular biology, providing high affinity and antigen specificity. In this study, we prepare specific multivalent nanobodies against E2 protein and develop a double-antibody sandwich ELISA for the detection of antigen quality using BiNb-2E8 as capture antibodies and BiNb-3C5 as detection antibody. A good linear correlation was achieved in the 5-1000 ng/mL range. ELISA results showed that patients with CHIKV had significantly higher serum E2 levels than CHIKV seronegative ( $P<0.0001$ ). Furthermore, the best choice for coated antibodies is to recognise only a single antigenic determinant[47–49]. Two types of diagnostic and protective antigens, ZIKA and SARS-CoV-2, were tested by indirect ELISA. Nb-2E8 and Nb-3C5 were found no binding activity to react with ZIKA and SARS-CoV-2 protein without any dose dependent manner. Additionally, flow cytometry analysis the two nanobodies of Nb-2E8 and Nb-3C5 positively stain CHIKV-infected cells but negative on ZIKA-infected cells. Overall, Nb-2E8 and Nb-3C5 were specific for CHIKV and had no cross-reactivity with ZIKA and SARS-CoV-2. These findings indicated that the ELISA protocol could be performed in any laboratory and served as the foundation of an inexpensive tool for the early diagnosis of CHIKV.

An understanding of the interaction between the antibody and its targeted antigen and knowing of the epitopes are critical for developing epitope based diagnostic reagents. The epitopes recognised by the nanobodies were identified by peptide-based ELISA, and the epitopes sites were located. Previous studies have reported that several monoclonal antibodies targeting the structural of domain A and B could block fusion and inhibit interaction with cellular receptor [8, 50]. Epitope mapping revealed that Nb-2E8 and Nb-3C5 bound the different linear epitope distributed in domain C and domain A, respectively. In particular, the P10-<sup>46</sup>“LKIQVSLQIG”<sup>55</sup> has not been reported. But we had previously predicted that the peptide “QVSLQIGIK” was the immunodominant B- and T-cell epitope using in *silico* techniques (unpublished data). Several studies suggested that the P61-<sup>301</sup>“GEEPNYQEEW”<sup>310</sup> was found to be B-cell epitopes shared by human and mice [51, 52]. Unfortunately, the selected two nanobodies that focus on the domains of the target linear epitopes are non-neutralizing. The main neutralization sites on the CHIKV E2 protein are likely to be available in a conformation-dependent manner, rather than as linear epitopes.

Based on sequence alignment analysis, the P10 was completely conserved among all CHIKV genotype strains analyzed here, indicating that the Nb-3C5 are potentially capable of recognising three CHIKV genotypes (ECSA, Asian, and West African). It also found that the P61 epitope sequence in West African lineage CHIKV strain had two single two amino acid difference from the other genotypes. This may limit the potential diagnostic applications of Nb-2E8 in countries where West African lineage strains of the virus circulate. However, all epitopes had significant amino acid mutations across other alphaviruses (Mayaro virus, O'nyong'nyong virus, Semliki Forest virus, Ross River virus, Sindbis virus, etc.). These features enable the differential diagnosis of CHIKV. Taken together, these nanobodies, Nb-2E8 and Nb-3C5, specifically recognize different epitopes of the CHIKV E2 protein, which are the optimal pair of nanobodies for the development of a double antibody sandwich ELISA.

There are several potential limitations to the study. First, our study is only focused on highly potent antibodies against CHIKV. Further studies including are need to demonstrated other nanobodies could interact with E2 protein and to evaluate the diagnostic or treatment merit of specific nanobodies. Second, the present study illustrates a strategy to mine the valuable nanobodies by naïve phage display library. The results support the feasibility of nanobodies for a rapid diagnostic tool. However, the diagnostic accuracy parameters and procedures in this study have not been directly optimized. To establish a standardized CHIKV diagnostic process, the optimization of antibody reagents is required. Third, the binding site prediction of nanobody-E2 complex rely on peptide-ELISA and homology-modeling applications, lacking X-ray diffraction (XRD) or cryoelectron microscopy to investigate the crystal structure parameters.

## Conclusions

In summary, we developed nanobodies from naïve phage display library that addresses the specific needs for CHIKV serodiagnosis. The selected nanobodies of Nb-2E8 and Nb-3C5 could bind CHIKV E2 protein with high specificity and affinity in a multivalent platform. These results suggest that the selected

nanobodies are used for diagnostic applications to detect sera binding protein and/or virions, either as single VHHs or in combination.

## **Declarations**

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## **Competing interests**

The authors declare that there are no conflicts of interest.

## **Ethics statement**

The serum samples of CHIKV patients were obtained from Center for Disease Control and Prevention of Southern Theater Command. All patients have agreed to sharing their serum samples by informed consent, and the study was approved by the ethics committee of Sun-Yet Sen university.

## **Availability of data and materials**

All the data analyzed throughout this study are included in the article.

## **Authors' contributions**

QLL participated in the experiments and data analyzes, and drafted the manuscript. HH and QD developed the experimental approaches, generated the majority of the reagents, and conducted preliminary experiments. YL and JW contributed to discussion and reviewed the manuscript. FQZ and TSH contributed to sample and data collection. CG provided support with data analysis and validation. CHL and QW facilitated the organization and coordination between partners. ZLC conceived the study and designed the experiments. JHL supervised and directed the project to ensure its progress. All authors read and approved the final manuscript.

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## Figures

### Figure 1

Screening the CHIKV E2 specific nanobodies from a VHH phage display library. **a** Biopanning procedure of candidate VHH domain. **b** Reactions from the 20 clones specifically binding with CHIKV E2 protein. **c** Alignment of the amino acid sequences of the isolated anti-CHIKV E2 VHH nanobodies. **d** Overall similarity of VHH regions anti-CHIKV E2 nanobody sequences.

### Figure 2

Expression, purification and binding activity detection of nanobodies. **a** Schematic representation of the expression vector pET-SUMO for nanobody. **b** Soluble prokaryotic expression and purification of recombinant nanobody using an N-terminally 6×His- and SUMO-tagged protein (~28kDa). **c** Binding of SUMO-tagged VHHs to immobilized CHIKV E2 (~40kD) or Chikungunya virion was evaluated by western blot. **d** Binding of SUMO-tagged VHHs to immobilized CHIKV E2 or CHIKV virus was quantified by indirect ELISA.

### Figure 3

Biophysics of the nanobodies binding to E2. **a** Binding kinetic for nanobodies were obtained by LSPR. **b** CHIKV E2 protein expression in infected cells was analyzed by immunofluorescence staining using Nb-2E8 and Nb-3C5. **c** The abilities of Nb-2E8 and Nb-3C5 to block CHIKV E2 interaction with Mxra8 were determined by a competition ELISA. **d** Cell binding of the nanobodies were quantified by flow cytometry. Left: representative flow cytometry plots; right: bars show mean  $\pm$  S.E.M.

### Figure 4

Features and applications of multivalent nanobodies. **a** A schematic of multivalent nanobodies. **b** SDS-PAGE of multivalent nanobodies under non-reducing condition. **c** Establishment of standard curve using HRP-labeled bivalent nanobodies. **d** Validation of the sandwich ELISA using CHIKV-positive serum samples.

### Figure 5

Distribution profiles of components of E2-Nb complexes and their corresponding epitopes. **a** Schematic diagram of domains and positions of the CHIKV E2 Glycoprotein. **b** Potential binding peptides response to the Nb-2E8 and Nb-3C5 as measured by a peptide-ELISA. **c** Alignment of the Nb-2E8 and Nb-3C5 epitope region with E2 sequences of different genotype strains and alphaviruses. **d** Structure docking model of Nb-2E8 and Nb-3C5 bound to CHIKV E2 protein.

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