



Efficient Capture and Detection of Zika Virion by Polyclonal Antibody Against Prokaryotic Recombinant Envelope Protein

Liding Zhang¹, Congjie Chen¹, Li Dai¹, Li Zhang¹, Keqing Xu¹, Yuzhu Song¹, Xueshan Xia¹, Qinqin Han¹, Qiang Chen¹ and Jinyang Zhang^{1,*}

¹Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, China

*Corresponding author: Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, China. Tel: +86-87165939528, Email: jy Zhang@kmust.edu.cn

Received 2018 March 23; Revised 2018 August 28; Accepted 2018 September 03.

Abstract

Background: The outbreak of Zika virus (ZIKV) in many countries caused alarming numbers of babies born with microcephalus and severe neurologic disorders, however, the methods for the detection of the ZIKV are limited at present.

Objectives: The aim of this study was to produce polyclonal antibody against full length envelop protein of ZIKV (ZIKV-E protein) in prokaryotic system and to evaluate its efficacy to capture ZIKV virion.

Methods: The recombinant full length ZIKV-E protein was purified and then used as an immunogen to vaccinate BALB/c mice to produce polyclonal antibody. Protein A/G coated magnetic beads were coated with the polyclonal antibody to capture the ZIKV virion in the culture medium of Vero cells infected with ZIKV. The products of immunoprecipitation were further used for Western Blot, Loop-mediated isothermal amplification (LAMP), and PCR assay.

Results: Western Blot and indirect immunofluorescence analysis showed that the mouse polyclonal antibody could react specifically with the native E protein in Vero cells infected with ZIKV. The results of Western Blot, Immunocaptured-LAMP (IC-LAMP), and Immunocaptured-PCR (IC-PCR) showed that polyclonal antibody of ZIKV-E recombinant protein can capture ZIKV virion specifically, however, the polyclonal antibody against the ZIKV-NS1, *Shigella*, and *Salmonella* without such a function.

Conclusions: These findings may provide the basis for the development of the rapid diagnostic kits such as, IC-PCR, IC-LAMP, and sandwich ELISA. The serum virion capture activity elicited by prokaryotic expressed recombinant ZIKV-E protein may also provide a basis for further preparation of neutralizing antibody and protein subunit vaccine candidate against ZIKV.

Keywords: Zika Virus, E Protein, Polyclonal Antibodies, IC-LAMP

1. Background

Zika virus (ZIKV) is an insect-borne virus, belonging to the *Flaviviridae* family, genus, which is similar to dengue fever, Japanese encephalitis, West Nile fever, and chikungunya virus (1). Recently, studies showed that it has Asian and African lineages (2, 3). Many symptoms such as rash, joint pain, and fever were caused by ZIKV, which is similar to many other diseases (3). Nevertheless, ZIKV is not widely concerned due to the fact that symptoms infected with ZIKV were actually asymptomatic (4). However, the outbreak of ZIKV in the northeast of Brazil, in 2015, caused alarming numbers of babies born with microcephalus (5). The *Aedes* species mosquitoes are the main way, however, the blood, perinatal, and sexual exposures are also allowed ZIKV transmission (2, 6). In 2015, ZIKV was first discovered in Brazil, causing characteristic birth defects, which

were associated with microcephaly and Guillain-Barre syndrome. Then, ZIKV was spread rapidly and the large-scale outbreak happened in other continents (7). As of April 2016, there were about 4,800 people who confirmed being infected with ZIKV. Over 46 countries have been reported for the cases of natural ZIKV infections. In China, 13 ZIKV cases have been documented, with the possibility of potential outbreaks still expected. The genetic diversity of ZIKV increased gradually with geographic expansion since 2013 (8). On Feb 1, 2016, ZIKV has been recognized as a public health emergency (9). Recently, it was reported that the ZIKV infection may result in deleterious cardiac effects in some patients (HealthDay News).

Now, ZIKV exhibits the potential to be transmitted to other areas and plausibly results in a future epidemic. Hence, it is needed to detect the virus accurately and rapidly. However, methods for the detection of ZIKV are

limited at present (10). The rapid, efficient, and user-friendly test kits are unavailable (11). In addition, there were less reported regarding the highly specific diagnostic reagents for the detection of ZIKV antibodies and antigens. Thus, an antibody based ZIKV fast detection kit would greatly improve the current approaches (12). E (envelope) protein is inlaid on the glycosylated viral envelope protein, which is the key element of the virus surface (13). It determines virulence, virus adsorption, and penetration, which is closely related to the viral disease pathogenesis.

The high N-mannose side chain in E protein plays an important role on the antigenicity of viral proteins (14, 15). Besides, the E protein is a kind of receptor protein, which plays an important role for host range, virulence, and cell tropism. E protein is a main antigen that can produce neutralizing antibody in the process of immune response (16). N-linked glycosylation of E protein is an important factor for ZIKV virulence and neuroinvasion (17). The losing of E N-linked glycosylation leads to compromised expression and the secretion of E ectodomain from mammalian cells (18). This indicates the importance of the E protein. However, it is not clear whether the total length of the E protein can be produced by prokaryotic expression and produced effective epitopes to recognize the ZIKV virion.

2. Objectives

In this work, a prokaryotic expression vector was used to express the ZIKV-E protein. ZIKV-E protein was purified and used as an immunogen to inoculate BALB/c mice (19). Western Blot and indirect immunofluorescence analysis showed that the mouse polyclonal antibody could react with the native E protein in Vero cells specifically infected with ZIKV. The immunoprecipitation, Immuno-LAMP (IC-LAMP), and Immuno-PCR (IC-PCR) technology were used to assess the ability of the polyclonal antibody capturing ZIKV virion.

3. Methods

3.1. Ethics Statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

3.2. Virus Strain, Culture Media, Cell Lines and Antibodies

The *Escherichia coli* host strain DH5 α and plasmid vector pET-32a (+) and BL21 (DE3) were purchased from Novagen (Darmstadt, Germany). The ZIKV strain SZ-WIV01 isolated from people in China was amplified in Vero cells, and

the supernatant was collected and stored for further use. The viral DNA/RNA extraction kit was purchased from TIANGEN BIOTECH (TIANGEN, China). DMEM medium and FBS were purchased from GIBCO. HRP-conjugated goat anti-mouse IgG antibody (Affinity Biologicals, USA) and goat anti-mouse FITC-conjugated antibody (Abcam, Cambridge, UK) were used in this study.

3.3. Generation of Prokaryotic Expression Plasmid for the ZIKV E Protein

Vero cells infected with ZIKV were cultured in DMEM Media (Gibco™, USA), containing 1% (v/v) FBS. Then, the RNA of ZIKV was extracted using the TIANamp Viral DNA/RNA kit. The extracted RNA was reverse-transcribed into cDNA using HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme, China). The ZIKV-E gene was amplified by PCR with the specific primers F 5' GGAATTCATCAGGTGCATAGGAGTC 3' (underlined, EcoR I site) and R 5' CCGCTCGAGAGCAGAGACGCTGT 3' (underlined, Xho I site). The EcoR I and Xho I were used to digest the PCR products. Then, the target fragment was gel-purified and ligated into the pET-32a (+) vector to generate the pET-32a (+)-E.

3.4. Expression of the ZIKV-E Protein

The pET-32a/ZIKV-E was transfected into *E. coli* Rosetta (DE3) cells and cultured in LB liquid medium. Next, 1.0 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to induce the ZIKV-E protein at 20°C for 12 hours (20). The total bacterial lysate was collected and analyzed by SDS-PAGE.

3.5. Purification of the ZIKV-E Protein

The bacteria were collected and washed four times by phosphate buffered saline (PBS; KCl 2.7 mmol/L, KH₂PO₄ 2 mmol/L, NaCl 137 mmol/L, Na₂HPO₄ 10 mmol/L, pH 7.4) and subjected to sonication on ice. The supernatant and precipitation were separated after centrifuged at 10,000 \times g, for 20 minutes at 4°C. The precipitations were denatured by 8 M urea (8 mol/L urea, 100 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.9) for 3 hours at 4°C. After that, nickel nitrilotriacetic acid (Ni₂ + -NTA) agarose resin was used to purify ZIKV-E proteins. Then, purified ZIKV-E proteins were dissolved in PBS buffer for refolding, and determined by SDS-PAGE assay.

3.6. Polyclonal Antibody Production Against ZIKV-E Protein

The purified recombinant ZIKV-E was used to produce antibodies in BALB/c mice. Before immunization, blood was collected from the mice. Sera were collected for negative control purposes after centrifugation. Furthermore,

20 μg ZIKV-E proteins mixed with Freund's complete adjuvant were emulsified to immunize mice by multipoint subcutaneous injections. Two weeks later, the injections were given with a mixture that contained 40 μg ZIKV-E proteins in each incomplete Freund's adjuvant. Three days later, after the booster immunization, blood was collected from the mouse. The serum was achieved as described as above.

3.7. Western Blot

The character of anti-ZIKV-E polyclonal antibody was analyzed by Western Blot. The native ZIKV-E proteins from Vero cells infected with ZIKV were used for SDS-PAGE. Then, proteins were transferred to a nitrocellulose (NC) membrane. The NC membrane was blocked with 5% skimmed-milk, which dissolved in the phosphate buffer saline containing 0.05% Tween-20 (PBS-T; KCl 2.7 mmol/L, KH_2PO_4 2 mmol/L, NaCl 137 mmol/L, Na_2HPO_4 10 mmol/L Tween-20 22.5 mmol/L pH 7.4) for 2 hours at 37°C. Following that, anti-E polyclonal (1:1000) was added and the NC membrane was incubated for 2 hours at 37°C. After being washed three times by PBS-T, HRP conjugated secondary antibodies (1:5000) were added and the NC membrane was maintained for 1 hour at 37°C. After that, the NC membrane was washed as described above. Finally, the Western Blot Kit Easy See (TransGen, Beijing, China) was used for the detection of the reactivity of the anti-serum.

3.8. Indirect Immunofluorescence Assay

Vero cells were infected with the ZIKV, cultured 36 hours, and fixed 12 hours in pre-chilled acetone-methanol (1/1) for 20 minutes at 4°C. Then, 5% skimmed milk was used to block cells for 1 hour at 37°C. Next, 100 μL antibodies against ZIKV-E (1:1000) diluted in 5% skimmed milk was added and incubated for 2 hours at 37°C. After washing, 100 μL FITC-conjugated goat anti-mouse antibodies (1:2000) was added and maintained for 1 hour at 37°C. Finally, 200 μL PBS was added into 96-well for fluorescence detection at 488 nm using Leica DMI3000B microscope.

3.9. Primer Design

LAMP primers were designed from the nucleotide sequences of ZIKV (GenBank accession numbers MF574579.1) by Primer Explorer V5 software. Five pairs of primers were designed to the homologous regions of ZIKV, and then selected by its amplification efficiency to the target gene (Figure 1).

3.10. The LAMP and PCR Assay

The LAMP reaction containing a set of four primers (F3, B3, FIP, BIP), 320 U/mL Bst DNA polymerase, 2 μL DNA template, 2.5 μL of $10 \times$ LAMP buffer, dNTP mixture, and add water to 25 μL . The mixture was maintained for 50 minutes at 63°C and terminated for 5 minutes at 85°C. The LAMP products were analyzed by separation on 3% agarose gel. Besides, the primers of F3 and B3 were also used for PCR reaction. The reaction was performed in a 25 μL volume containing 1 μL of F3, B3, 12.5 μL of $2 \times$ TSINGKE Master Mix, and genomic DNA. The conditions of PCR reaction contained initial incubation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C extension for 7 minutes. The products were analyzed in 1% agarose gel by electrophoresis.

3.11. Immunoprecipitation of ZIKV Virion in Cell Culture Medium with Polyclonal Antibody

The immuno-magnetic beads were conducted as described by Zhang et al. (19). A total of 400 μL of mouse anti-ZIKV-E serum (1:100) was transferred into 1.5 mL tubes coated for 0.5 hour at 37°C. The anti-ZIKV-E serum in tubes was removed and washed by PBS-T, and the immuno-magnetic beads were used for capturing ZIKV virion. After that, the mixture containing magnetic beads, antibodies, viruses were used for Western Blot assay and the extraction of cDNA, respectively, under the action of an outside magnetic field. Then, 4 μL of the cDNA was used for PCR, LAMP to verify polyclonal antibody capturing ZIKV. At the same time, the polyclonal antibody of ZIKV-NS1, *Shigella*, and *Salmonella* were also used for capturing ZIKV and the cDNA were used as a negative control, respectively.

4. Results

4.1. Generation of the ZIKV-E Protein Prokaryotic Expression Plasmid

After extract the RNA of the ZIKV SZ-WIV01 strain and PCR reaction, the E protein gene of was amplified successfully (Figure 2, Lane 1). The EcoRI and XhoI were used to digest the PCR product and the fragment was inserted into fraction pET-32a (+) digested with the same enzymes. After that, the recombinant plasmid pET-32a-E was generated successfully. The restriction enzyme digestion and DNA sequencing were used to confirm the plasmid of pET-32a-E (Figure 2, Lane 3). The pET-32a (+) was also by restriction enzyme digestion (Figure 2, Lane 2).

A

AATGAGTGAC CTGGCTAAGC TTGCAATTTT GATGGGTGCC ACCTTCGCGG 50

AAATGAACAC TGGAGGAGAT GTAGCTCATC TGGCGTGAT AGCGGCATTC 100

AAAGTCAGAC CAGCGTTGCT GGTATCTTTC ATCTCAGAG CTAATTGGAC 150

ACCCCGTGAA AGCATGCTGC TGGCCTTGGC CTCGTGICTT TTGCAAACCTG 200

CGATCTCCGC CTTGGAAGGC GACCTGATGG TTCTCATCAA TGGTTTTGCT 250

TTGGCCTGGT TGGCAATACG AGCGATGGTT GTTCC 285

B

FIP: 5'-ATGCTTTCACGGGGTGTCCAATTTTATGTCAGACCAGCGTTGCT-3' F1c+F2

BIP: 5'-GCAAACCTGCGATCTCCGCTTTTTTTTGGCCAACCAGGCCAAAG-3' B1c+B2

F3: 5'-CGCTGATAGCGGCATTCA-3'

B3: 5'-GGAACAACCATCGCTCGTA-3'

Figure 1. LAMP and PCR primers used to detect ZIKV. A, specific sequence of ZIKV (GenBank accession number MF574579.1) used for the design of LAMP primers. The arrows were used for indicating the sequences used for LAMP primers; B, sequences of the four primers used in the LAMP and PCR reaction.

4.2. Expression of the ZIKV-E Protein

After being induced with IPTG, the ZIKV-E protein was expressed at a relatively high level (Figure 3, Lane 2). In addition, the ZIKV-E protein was only expressed after induction (Figure 3, Lanes 2 and 4), while there was no expression of the E protein without IPTG induction (Figure 3, Lane 1). The result of SDS-PAGE showed that the majority of the ZIKV-E proteins were expressed in the cell debris pellets (Figure 3, Lane 4), suggesting that the ZIKV-E protein was insoluble as the form of inclusion bodies.

4.3. The Purification of the ZIKV-E Protein

The Ni²⁺-NTA resin column was used to purify the ZIKV-E protein (14). The result of SDS-PAGE showed that the recombinant ZIKV-E protein could conjugate with Ni²⁺-NTA resin column, and a single target band about 67.1 kDa was

purified (Figure 3, Lane 6). Approximately 1.4 mg ZIKV-E protein was obtained after being determined by the BCA assay.

4.4. Characterization of the Polyclonal Antisera Against ZIKV-E Protein

The reactivity and specificity of the polyclonal antibody against ZIKV-E was evaluated by Western Blot and immunofluorescence assays. The Figure 5 showed that anti-ZIKV-E antibody could recognize the native E protein in ZIKV infected cells, and the negative control had no fluorescence. The results of Western Blot showed that the recombinant ZIKV-E protein and native E protein in ZIKV infected cells were recognized by mouse polyclonal antisera (Figure 4, Lane 2, 3 and Figure 6, Lane 2).

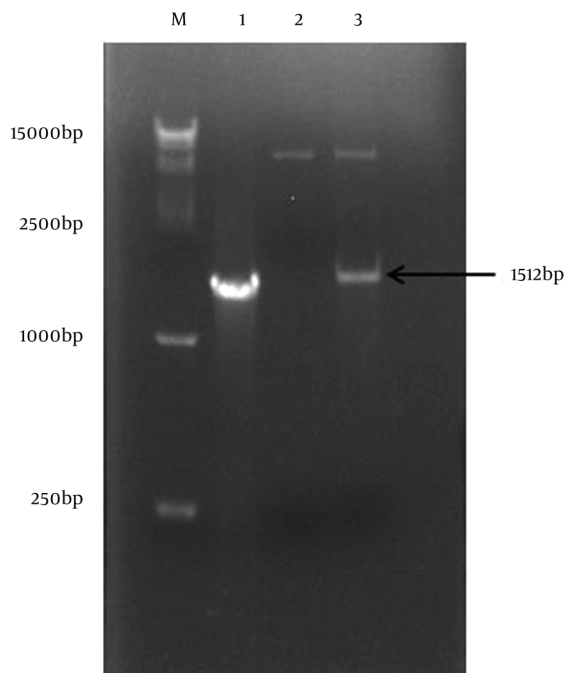


Figure 2. Amplification of ZIKV E protein gene. M: DL15000 DNA marker; lane 1: E protein fragment; lane 2: The pET-32a digested by EcoR I and Xho I Enzymes; lane 3: The pET-32a-ZIKV-E digested by EcoR I and Xho I Enzymes.

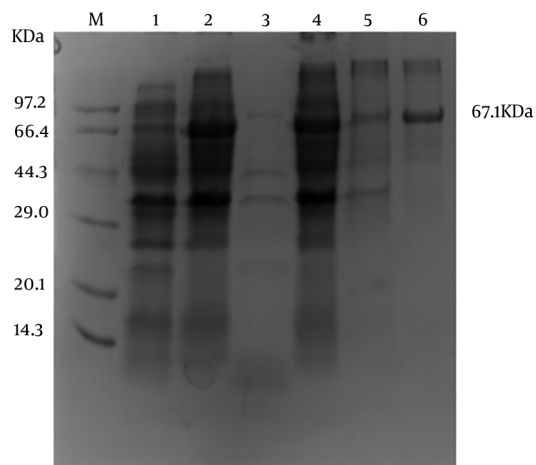


Figure 3. SDS-PAGE analysis of expressed and purified ZIKV-E protein. M: protein marker; lane 1: *E. coli* without IPTG; lane 2: *E. coli* induced with IPTG; lane 3: soluble fractions; lane 4: insoluble fractions; lane 5: the purified ZIKV-E protein; lane 6: the purified ZIKV-E protein after refolding.

4.5. Capture of ZIKV Virion in Cell Culture Medium by Polyclonal Antibody

Protein A/G coated magnetic beads (Biotool) were coated with the polyclonal antibody of ZIKV-E, ZIKV-NS1,

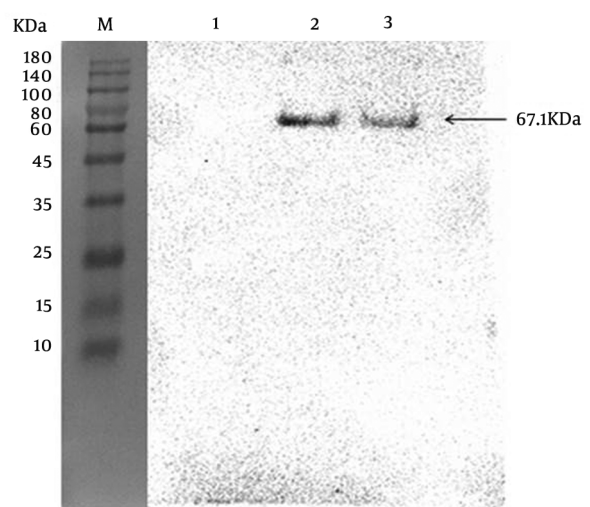


Figure 4. Western Blot analysis of ZIKV-E protein. Lane M: marker; lane 1: *E. coli* before induced; lane 2: *E. coli* was induced with IPTG; lane 3: the purified ZIKV-E protein after dialysis.

Shigella, and *Salmonella* to generate the immuno-magnetic beads. Then, the immuno-magnetic beads were used for capturing the supernatant of ZIKV infected, and mock-infected Vero cells. The mixtures were confirmed by Western Blot (Figure 7A). According to the manufacturer's instructions, the mixture containing magnetic beads, antibodies, viruses were used for the extraction of cDNA. As shown in Figure 7B, a 200 bp fragment (A) and trapezoidal strip (B) were obviously observed in the polyclonal antibody of ZIKV-E and used for capturing ZIKV, however, there was no amplified in other polyclonal antibodies, which were used for capturing ZIKV.

5. Discussion

Much attention was focused on the ZIKV epidemic in the Americas. There is the possibility of large-scale outbreaks in the equatorial and northern hemisphere, where ~80% of the human population live (21, 22). The bacterial expression has been used widely for its rapid growth rate, relative inexpensive cost, and ease of manipulation (23). In this study, pET-32a-E prokaryotic expression vector was constructed successfully. After induction and purification, the E protein was obtained and then used as an immunogen to inoculate BALB/c mice. After immunization, the serum were collected and used for Western Blot and indirect immunofluorescence analysis. The results showed that the mouse polyclonal antibody could react with the native E protein in Vero cells infected with ZIKV specifically

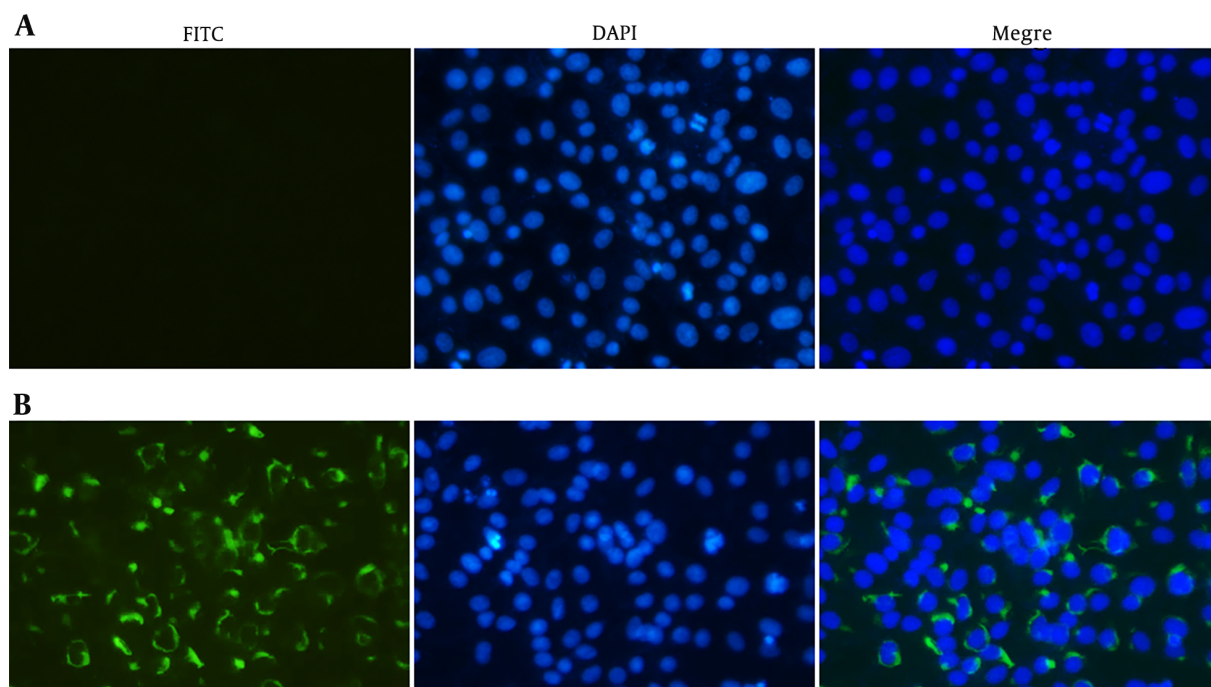


Figure 5. Indirect immunofluorescent assay of the E protein in Vero cells infected with ZIKV. ZIKV infected (B) and mock-infected (A) Vero cells were probed with the E antiserum and observed under fluorescent light. ZIKV infected and mock-infected Vero cells were counterstained with DAPI to visualize the nuclei (40×).



Figure 6. Western Blot analysis of the polyclonal antibody induced by recombinant ZIKV-E protein. ZIKV infected and mock-infected Vero cells were used for Western Blot analysis using GAPDH antibody and anti-ZIKV-E antiserum.

(24). Next, polyclonal antibody against the ZIKV-E protein was used for capturing ZIKV in the cell culture medium. In addition, the polyclonal antibody of ZIKV-NS1, *Shigella*, and *Salmonella* were used for capturing ZIKV. The Western Blot, IC-LAMP, and IC-PCR results showed that only the polyclonal antibodies of ZIKV-E have the ability to capture virion. That means the prokaryotic expression and purification of Zika virus E protein and preparation of its polyclonal antibody was able to identify some epitope on the virions. However, ZIKV-E was expressed in *E. coli*, which did not support the proper folding of protein; the protein refolding is required to attain its native conformation (25).

It is well known that the prokaryotic expression system

lacks the functional glycosylation modification on recombinant protein, thus, the structure of recombinant E and natural E protein expression were different in a certain extent. Denaturing purification strategy was used for the formation of inclusion body, which markedly affects the conformation of the recombinant glycoprotein epitope. Various recombinant protein renature methods had been reported such as refolding in the refolding buffer (100 mM Tris-HCl, 0.5 M L-arginine, 0.2 mM EDTA, pH 7.5) (26), using ion-exchange chromatography (27), and biochemical and biophysical methods (28). Given this reason, the protein was renatured only in PBS and then the mice were immunized. We found that the fluorescence of anti-ZIKV-E polyclonal antibody reacted with Vero cells infected with the ZIKV was stronger than the recombinant E was not renature (data not shown). The reason may be that the E protein's structure was recovered partly after renaturation. In this experiment, the E protein was only renatured in PBS, however, the results of Western Blot and immunofluorescence assays showed that the recombinant proteins conformation was similar to its native protein in a certain extent.

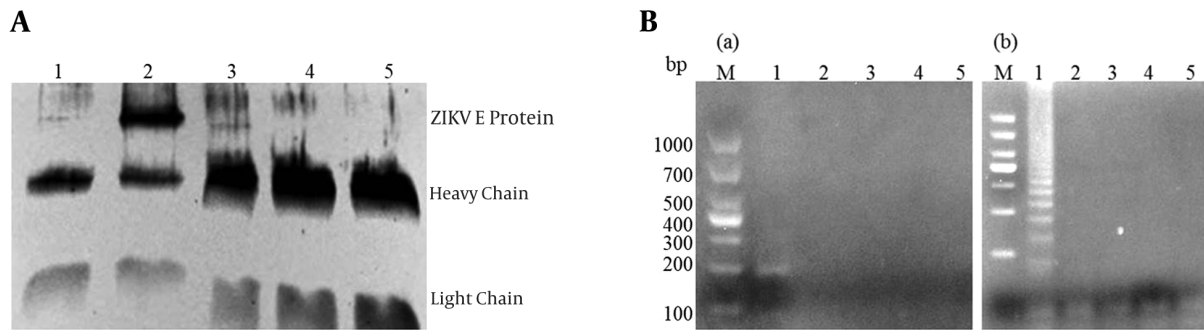


Figure 7. Evaluation of the ZIKV virion capture ability of the ZIKV-E polyclonal antibody by Western Blot, IC-PCR, IC-LAMP assay. A, the supernatant of ZIKV infected Vero cells (lane 2), and mock-infected Vero cells (lane 1) were captured by the polyclonal antibody of ZIKV-E using the immuno-magnetic beads. Besides the Vero cells supernatant of ZIKV infected also captured by the polyclonal antibody of ZIKV-NSI, *Shigella*, and *Salmonella* (lane 3, 4, 5), then the mixture were used for Western Blot analysis using anti-ZIKV-E antiserum; B, (a) the IC-PCR Products were analyzed by gel electrophoresis; lane 1 positive IC-PCR products; lane 2 to lane 4 represent the cDNA were captured by the polyclonal antibody of ZIKV-NSI, *Shigella*, and *Salmonella*, lane 5 negative control; (b) The IC-LAMP Products were analyzed by gel electrophoresis; lane 1, positive IC-LAMP products; lane 2 to lane 4 represent the cDNA were captured by the polyclonal antibody of ZIKV-NSI, *Shigella*, and *Salmonella*, lane 5 negative control.

5.1. Conclusions

Our findings proved that the polyclonal antibodies elicited by recombinant ZIKV-E are able to recognize native virions. In addition, it also provides the basis for further preparation of neutralizing antibodies against ZIKV and the development of the related detection kits such as IC-PCR, IC-LAMP, and sandwich ELISA. This approach could effectively reduce times for the detection of ZIKV. Furthermore, it has important implications for the multivalent vaccine development.

Footnotes

Authors' Contribution: Zhang Liding designed and drafted the work. Zhang Li, Chen Congjie, Dai Li, Zhang Liding, Xu Keqing, Chen Qiang, and Han Qinqin performed the experiments, analyzed the data, and interpreted the results. Zhang Jinyang, Song Yuzhu, and Xia Xueshan designed the work and revised it critically.

Funding/Support: This work was supported by the 2017 Year Undergraduate Student Innovation & Carve Out Training Program Granting Project of Kunming University of Science and Technology, National Natural Science Foundation of China (Grant No. 81860625), and Applied Basic Research Projects of Yunnan Province (2018FB128)

References

- Petersen E, Wilson ME, Touch S, McCloskey B, Mwaba P, Bates M, et al. Rapid spread of Zika virus in the Americas—implications for public health preparedness for mass gatherings at the 2016 Brazil olympic games. *Int J Infect Dis.* 2016;**44**:11–5. doi: [10.1016/j.ijid.2016.02.001](https://doi.org/10.1016/j.ijid.2016.02.001). [PubMed: [26854199](https://pubmed.ncbi.nlm.nih.gov/26854199/)].
- Haddow AD, Schuh AJ, Yasuda CY, Kasper MR, Heang V, Huy R, et al. Genetic characterization of Zika virus strains: Geographic expansion of the Asian lineage. *PLoS Negl Trop Dis.* 2012;**6**(2): e1477. doi: [10.1371/journal.pntd.0001477](https://doi.org/10.1371/journal.pntd.0001477). [PubMed: [22389730](https://pubmed.ncbi.nlm.nih.gov/22389730/)]. [PubMed Central: [PMC3289602](https://pubmed.ncbi.nlm.nih.gov/PMC3289602/)].
- Fleming AM, Ding Y, Alenko A, Burrows CJ. Zika virus genomic RNA possesses conserved G-quadruplexes characteristic of the flaviviridae family. *ACS Infect Dis.* 2016;**2**(10):674–81. doi: [10.1021/acscinfecdis.6b00109](https://doi.org/10.1021/acscinfecdis.6b00109). [PubMed: [27737553](https://pubmed.ncbi.nlm.nih.gov/27737553/)]. [PubMed Central: [PMC5067700](https://pubmed.ncbi.nlm.nih.gov/PMC5067700/)].
- Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med.* 2009;**360**(24):2536–43. doi: [10.1056/NEJMoa0805715](https://doi.org/10.1056/NEJMoa0805715). [PubMed: [19516034](https://pubmed.ncbi.nlm.nih.gov/19516034/)].
- Coyne CB, Lazear HM. Zika virus - reigniting the TORCH. *Nat Rev Microbiol.* 2016;**14**(11):707–15. doi: [10.1038/nrmicro.2016.125](https://doi.org/10.1038/nrmicro.2016.125). [PubMed: [27573577](https://pubmed.ncbi.nlm.nih.gov/27573577/)].
- Ibrahim NK. Zika virus: Epidemiology, current phobia and preparedness for upcoming mass gatherings, with examples from World Olympics and Pilgrimage. *Pak J Med Sci.* 2016;**32**(4):1038–43. doi: [10.12669/pjms.324.10038](https://doi.org/10.12669/pjms.324.10038). [PubMed: [27648063](https://pubmed.ncbi.nlm.nih.gov/27648063/)]. [PubMed Central: [PMC5017074](https://pubmed.ncbi.nlm.nih.gov/PMC5017074/)].
- Johansson MA, Mier-y-Teran-Romero L, Reefhuis J, Gilboa SM, Hills SL. Zika and the risk of microcephaly. *N Engl J Med.* 2016;**375**(1):1–4. doi: [10.1056/NEJMp1605367](https://doi.org/10.1056/NEJMp1605367). [PubMed: [27222919](https://pubmed.ncbi.nlm.nih.gov/27222919/)]. [PubMed Central: [PMC4945401](https://pubmed.ncbi.nlm.nih.gov/PMC4945401/)].
- Cao L, Zhang XG, Wang JG, Wang HB, Chen YB, Zhao DH, et al. Pulmonary function test findings in patients with acute inhalation injury caused by smoke bombs. *J Thorac Dis.* 2016;**8**(11):3160–7. doi: [10.21037/jtd.2016.11.94](https://doi.org/10.21037/jtd.2016.11.94). [PubMed: [28066595](https://pubmed.ncbi.nlm.nih.gov/28066595/)]. [PubMed Central: [PMC5179430](https://pubmed.ncbi.nlm.nih.gov/PMC5179430/)].
- Heymann DL, Hodgson A, Sall AA, Freedman DO, Staples JE, Althabe F, et al. Zika virus and microcephaly: Why is this situation a PHEIC? *Lancet.* 2016;**387**(10020):719–21. doi: [10.1016/S0140-6736\(16\)00320-2](https://doi.org/10.1016/S0140-6736(16)00320-2). [PubMed: [26876373](https://pubmed.ncbi.nlm.nih.gov/26876373/)].
- Pardee K, Green AA, Takahashi MK, Braff D, Lambert G, Lee JW, et al. Rapid, low-cost detection of zika virus using programmable biomolecular components. *Cell.* 2016;**165**(5):1255–66. doi: [10.1016/j.cell.2016.04.059](https://doi.org/10.1016/j.cell.2016.04.059). [PubMed: [27160350](https://pubmed.ncbi.nlm.nih.gov/27160350/)].
- K GB, K BMK, N S, D R, N V, B HB. Determination of genotoxic impurity in atazanavir sulphate drug substance by LC-MS. *J Pharm Biomed Anal.* 2017;**132**:156–8. doi: [10.1016/j.jpba.2016.09.025](https://doi.org/10.1016/j.jpba.2016.09.025). [PubMed: [27723524](https://pubmed.ncbi.nlm.nih.gov/27723524/)].

12. Rabe IB, Staples JE, Villanueva J, Hummel KB, Johnson JA, Rose L, et al. Interim guidance for interpretation of Zika virus antibody test results. *MMWR Morb Mortal Wkly Rep.* 2016;**65**(21):543-6. doi: [10.15585/mmwr.mm6521e1](https://doi.org/10.15585/mmwr.mm6521e1). [PubMed: [27254248](https://pubmed.ncbi.nlm.nih.gov/27254248/)].
13. Ma T, Liu Y, Cheng J, Liu Y, Fan W, Cheng Z, et al. Liposomes containing recombinant E protein vaccine against duck Tembusu virus in ducks. *Vaccine.* 2016;**34**(19):2157-63. doi: [10.1016/j.vaccine.2016.03.030](https://doi.org/10.1016/j.vaccine.2016.03.030). [PubMed: [27016654](https://pubmed.ncbi.nlm.nih.gov/27016654/)].
14. Yavropoulou MP, Kollia P, Chatzidimitriou D, Samara S, Skoura L, Yovos JG. Severe osteoporosis with multiple spontaneous vertebral fractures in a young male carrying triple polymorphisms in the vitamin D receptor, collagen type 1, and low-density lipoprotein receptor-related peptide 5 genes. *Hormones (Athens).* 2016;**15**(4):551-6. doi: [10.14310/horm.2002.1710](https://doi.org/10.14310/horm.2002.1710). [PubMed: [28222408](https://pubmed.ncbi.nlm.nih.gov/28222408/)].
15. Ramakrishnan C, Kutumbarao NHV, Suhitha S, Velmurugan D. Structure-function relationship of Chikungunya nsP2 protease: A comparative study with papain. *Chem Biol Drug Des.* 2017;**89**(5):772-82. doi: [10.1111/cbdd.12901](https://doi.org/10.1111/cbdd.12901). [PubMed: [28054451](https://pubmed.ncbi.nlm.nih.gov/28054451/)].
16. Ye Q, Liu ZY, Han JF, Jiang T, Li XF, Qin CF. Genomic characterization and phylogenetic analysis of Zika virus circulating in the Americas. *Infect Genet Evol.* 2016;**43**:43-9. doi: [10.1016/j.meegid.2016.05.004](https://doi.org/10.1016/j.meegid.2016.05.004). [PubMed: [27156653](https://pubmed.ncbi.nlm.nih.gov/27156653/)].
17. Annamalai AS, Pattnaik A, Sahoo BR, Muthukrishnan E, Natarajan SK, Steffen D, et al. Zika virus encoding non-glycosylated envelope protein is attenuated and defective in neuroinvasion. *J Virol.* 2017. doi: [10.1128/JVI.01348-17](https://doi.org/10.1128/JVI.01348-17). [PubMed: [28931684](https://pubmed.ncbi.nlm.nih.gov/28931684/)]. [PubMed Central: [PMC5686755](https://pubmed.ncbi.nlm.nih.gov/PMC5686755/)].
18. Mossenta M, Marchese S, Poggiana M, Slon Campos JL, Burrone OR. Role of N-glycosylation on Zika virus E protein secretion, viral assembly and infectivity. *Biochem Biophys Res Commun.* 2017;**492**(4):579-86. doi: [10.1016/j.bbrc.2017.01.022](https://doi.org/10.1016/j.bbrc.2017.01.022). [PubMed: [28069378](https://pubmed.ncbi.nlm.nih.gov/28069378/)].
19. Zhang L, Wei Q, Han Q, Chen Q, Tai W, Zhang J, et al. Detection of Shigella in milk and clinical samples by magnetic immunocaptured-loop-mediated isothermal amplification assay. *Front Microbiol.* 2018;**9**:94. doi: [10.3389/fmicb.2018.00094](https://doi.org/10.3389/fmicb.2018.00094). [PubMed: [29467730](https://pubmed.ncbi.nlm.nih.gov/29467730/)].
20. Cao XL, Zhang X, Zhang YF, Zhang YZ, Song CG, Liu F, et al. Expression and purification of mouse Ttyh1 fragments as antigens to generate Ttyh1-specific monoclonal antibodies. *Protein Expr Purif.* 2017;**130**:81-9. doi: [10.1016/j.pep.2016.09.013](https://doi.org/10.1016/j.pep.2016.09.013). [PubMed: [27678288](https://pubmed.ncbi.nlm.nih.gov/27678288/)].
21. Shi W, Zhang Z, Ling C, Carr MJ, Tong Y, Gao GF. Increasing genetic diversity of Zika virus in the Latin American outbreak. *Emerg Microbes Infect.* 2016;**5**:e68. doi: [10.1038/emi.2016.68](https://doi.org/10.1038/emi.2016.68). [PubMed: [27381219](https://pubmed.ncbi.nlm.nih.gov/27381219/)]. [PubMed Central: [PMC4972906](https://pubmed.ncbi.nlm.nih.gov/PMC4972906/)].
22. Wang X, Yin F, Bi Y, Cheng G, Li J, Hou L, et al. Rapid and sensitive detection of Zika virus by reverse transcription loop-mediated isothermal amplification. *J Virol Methods.* 2016;**238**:86-93. doi: [10.1016/j.jviromet.2016.10.010](https://doi.org/10.1016/j.jviromet.2016.10.010). [PubMed: [27793644](https://pubmed.ncbi.nlm.nih.gov/27793644/)].
23. Mayer M, Buchner J. Refolding of inclusion body proteins. *Methods Mol Med.* 2004;**94**:239-54. [PubMed: [14959834](https://pubmed.ncbi.nlm.nih.gov/14959834/)].
24. Rawal G, Yadav S, Kumar R. Zika virus: An overview. *J Family Med Prim Care.* 2016;**5**(3):523-7. doi: [10.4103/2249-4863.197256](https://doi.org/10.4103/2249-4863.197256). [PubMed: [28217576](https://pubmed.ncbi.nlm.nih.gov/28217576/)]. [PubMed Central: [PMC5290753](https://pubmed.ncbi.nlm.nih.gov/PMC5290753/)].
25. Tripathi NK, Babu JP, Shrivastava A, Parida M, Jana AM, Rao PV. Production and characterization of recombinant dengue virus type 4 envelope domain III protein. *J Biotechnol.* 2008;**134**(3-4):278-86. doi: [10.1016/j.jbiotec.2008.02.001](https://doi.org/10.1016/j.jbiotec.2008.02.001). [PubMed: [18342971](https://pubmed.ncbi.nlm.nih.gov/18342971/)].
26. Jaiswal S, Khanna N, Swaminathan S. High-level expression and one-step purification of recombinant dengue virus type 2 envelope domain III protein in Escherichia coli. *Protein Expr Purif.* 2004;**33**(1):80-91. doi: [10.1016/j.pep.2003.09.009](https://doi.org/10.1016/j.pep.2003.09.009). [PubMed: [14680965](https://pubmed.ncbi.nlm.nih.gov/14680965/)].
27. Babu JP, Pattnaik P, Gupta N, Shrivastava A, Khan M, Rao PV. Immunogenicity of a recombinant envelope domain III protein of dengue virus type-4 with various adjuvants in mice. *Vaccine.* 2008;**26**(36):4655-63. doi: [10.1016/j.vaccine.2008.07.006](https://doi.org/10.1016/j.vaccine.2008.07.006). [PubMed: [18640168](https://pubmed.ncbi.nlm.nih.gov/18640168/)].
28. Raviprakash K, Kochel TJ, Ewing D, Simmons M, Phillips I, Hayes CG, et al. Immunogenicity of dengue virus type 1 DNA vaccines expressing truncated and full length envelope protein. *Vaccine.* 2000;**18**(22):2426-34. [PubMed: [10738100](https://pubmed.ncbi.nlm.nih.gov/10738100/)].