Original Article

Expression and Immunogenicity of VP40 Protein of ZEBOV

Yinbiao Wang[1,#], Xiao Liu[2,#], Ling Tao[1], Pengwei Xu[1], Xia Gao[1], Huijun Li[1], Zhongzhi Yang[1], Weidong Wu•[1]

Abstract

Background: EBOV outbreaks continue to threaten the world due to the absence of effective vaccines and therapeutics. Easy-to-use and rapid diagnostic tests for EBOV are highly desired for prevention and control of the EVD epidemic.

Methods: *Escherichia coli* expression system was used to express VP40 protein of Zaire Ebola virus (ZEBOV) as water-soluble protein upon optimization of temperature, time, and IPTG concentration. VP40 protein was purified through Ni-NTA affinity chromatography and applied to immunize rabbits for immunogenicity analysis. Rabbit polyclonal antibodies against VP40 protein was produced and antibody response was analyzed using Western blot, enzyme-linked immunosorbent assay (ELISA), and immunoperoxidase monolayer assay (IPMA).

Results: Recombinant full-length VP40 protein of ZEBOV was expressed in *E. coli* Rosetta (DE3) cells as water-soluble protein. Analysis of antibody responses showed that rabbit polyclonal antibodies against VP40 protein could react specifically with this *E. coli*-expressed protein in Western blot and ELISA, and antibody titers in ELISA reached 1:25600. Besides, the produced rabbit polyclonal antibodies bound to VP40 proteins eukaryotically expressed by transfecting pcDNA-eGFP-VP40 into BHK-21 cells in IPMA.

Conclusion: These results show that the prokaryotically expressed VP40 protein has high immunogenicity and can be used as diagnostic antigen in ELISA and other immunoassays. The strategy used in this study might be a potential way for preparing diagnostic agents for prevention and control of exotic diseases.

Keywords: Antibody responses, expression and immunogenicity, VP40 protein, ZEBOV

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Introduction

Ebola virus disease (EVD) is a severe viral hemorrhagic fever syndrome caused by Ebola virus (EBOV), affecting humans and non-human primates. An EVD epidemic, which occurred in West Africa in 2014, has been declared by the World Health Organization (WHO) as public health emergency of international concern with severe global economic burden.[1] Five species of EBOV have been identified: Zaire (ZEBOV), Sudan (SEBOV), Bundibugyo (BEBOV), Tai Forest (TEBOV), and Reston (REBOV). ZEBOV is the most lethal species and the causative agent for the 2014 West African outbreak.[2] The genome of EBOV is an envelope, non-segmented, negative single-strand RNA, encoding seven structural proteins: nucleoprotein (NP), virion protein 35 (VP35), VP40, glycoprotein (GP), VP24, VP30, and L protein.[3] The L protein functions as the viral RNA-dependent RNA polymerase. VP35 may be a component of the transcriptase complex. NP is the major component of the viral nucleocapsid and can be divided into a hydrophobic N-terminal half which may play a role in folding and/or RNA binding, and a hydrophilic and acidic C-terminal half which may interact with the matrix protein and/or other viral structural proteins.[3] VP30 represents a minor nucleoprotein which is also an essential activator of viral transcription.[4] VP40 (matrix protein) is the most prominent viral structural protein and plays essential roles in virus assembly and budding together with VP24.[5,6] GP is the only membrane protein exposed on the viral surface and mediates receptor binding and virus entry.[7]

Although great efforts have been made to develop effective vaccines and therapeutics, no licensed vaccine or treatment is currently available.[8,9] Moreover, as international trade and travel still continue to increase, the risk that EBOV would be introduced into virus-free countries is significant.[10] Hence, the development of laboratory diagnostic systems for EVD is an important subject for disease prevention and control even in countries without viral hemorrhagic fevers. Manipulation of EBOV requires biosafety level 4 (BSL-4) laboratories, which are available only in a limited group of countries. Therefore, recombinant viral antigens are being used instead of the virus to develop diagnostics for EBOV. Specifically, recombinant NP, VP40, and GP have been expressed, and serological diagnostic methods for detecting viral antigen and IgM or IgG antibodies have been developed.[11-13] In this study, we aimed to express a recombinant His_6-tagged VP40 protein of ZEBOV and evaluate its immunogenicity by immunization of rabbit to provide a potential diagnostic agent for EBOV antibody detection.

Materials and Methods

Ethical statement

Two young female rabbits were purchased from the Animal Center of Henan Academy of Agricultural Sciences and reared with humane care. Animal experiment was allowed by and strictly carried out according to the practices of the Ethic Committee of Xinxiang Medical University.

Authors' affiliations: [1]School of Public Health, Xinxiang Medical University, Xinxiang 453003, P.R. China. [2]Colleges of Veterinary Medicine and Animal Science, Henan Agricultural University, Zhengzhou 450002, P.R. China.
•**Corresponding author and reprint:** Weidong Wu; E-mail: wdwu2013@126.com
[#] These authors contributed equally to this study.
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Cells and reagents

Baby hamster kidney cells (BHK-21) were cultured in DMEM media containing 10% fetal bovine serum (FBS). Plasmids pET28a and pcDNA3.1-eGFP were propagated in *E. coli* DH5α cells. *E. coli* competent cells Rosetta (DE3) was prepared and maintained at -80°C in our laboratory. Lipofectamine 2000 was purchased from Thermo Fisher Scientific and used as the transfection reagent. High affinity Ni-NTA resin was purchased from GE Healthcare. Anti-His_6 tag monoclonal antibody was obtained from Abcam. HRP-conjugated goat-anti-mouse and goat-anti-rabbit antibodies were from Jackson ImmunoResearch.

Plasmid construction and transfection

After codon optimization for expression in *E. coli* and eukaryotic cells, VP40 gene of ZEBOV (GenBank No. NC_002549) was synthesized by Sangon, China. The gene was doubly digested from pUC19-VP40 plasmid using restriction enzymes *Bam*H I and *Xho* I, and then respectively ligated with pET28a vector or pcDNA3.1-eGFP vector digested with the same enzymes. The construct pET28a-VP40 was transformed into *E. coli* Rosetta (DE3) competent cells for protein expression. pcDNA3.1-eGFP-VP40 was transformed into *E. coli* DH5α cells for plasmid proliferation. All plasmids were identified by PCR and restriction enzyme digestion, and sequenced by Sangon, China.

For expression of VP40 protein in eukaryotic cells, pcDNA3.1-eGFP-VP40 plasmid transfection was performed using lipofectamine 2000 reagent according to the manufacturer's instruction when BHK-21 cell density reached 80-90% in 96 microtiter plates. The amount of 0.2 µg of pcDNA3.1(+), pcDNA3.1-eGFP, and pcDNA3.1-eGFP-VP40 was respectively mixed with DMEM media in 25 µL for 5 min. Also, 0.5 µL lipofectamine 2000 was added into each DMEM media in 25 µL and kept for 5 min. Then, the plasmid and the transfection reagent were mixed and incubated at room temperature for 20 min. The cells were washed twice with DMEM media before the addition of the transfection complex into each well slowly while shaking the plate. After 6 hours, the culture media were changed to DMEM containing 10% FBS. After 48 hours of transfection, the transfected cells were observed under a fluorescent microscope for expression of green fluorescent protein (GFP).

Protein expression and purification

E. coli Rosetta (DE3) cells bearing pET28a-VP40 were cultured in LB liquid media at 37 °C for 2-3 h until OD_{600} value of the cell culture reached 0.4-0.6. Then, the cells were induced with 0.1-1.0 mM isopropyl-β-D-thiogalactoside (IPTG) for 8 h at 25 °C, 30°C and 37°C, respectively. Next, the cells were pelleted by centrifugation at 6000 g for 20 min at 4 °C, and resuspended in Tris buffered saline (pH 8.0) containing 20 mM Tris and 500 mM NaCl (binding buffer). After ultra-sonication on ice for 20 min, the supernatant was harvested by centrifugation at 12,000 g for 20 min at 4 °C, filtered through a 0.45 µm nitrocellulose filter (Millipore, Billerica, MA, USA), and purified through Ni-NTA affinity chromatography. Firstly, the affinity column was equilibrated with binding buffer before loading the supernatant. Secondly, the column was washed with binding buffer containing 40 mM imidazole (wash buffer). Thirdly, VP40 protein was eluted with binding buffer containing 500 mM imidazole (elution buffer). Purified protein was dialyzed against 20 mM Tris (pH 8.0) and then stored at -20 °C. Protein concentration was determined using a Bradford Protein Assay Kit (Sangon Biotech, Shanghai).

Production of rabbit polyclonal antibodies

Two young female rabbits were immunized with purified VP40 protein four times at intervals of three weeks. For the prime immunization, the rabbits received 200 µg protein emulsified in Freud's complete adjuvant subcutaneously. For other immunizations, the same amount of protein emulsified in Freud's incomplete adjuvant was used. Rabbit sera were collected 7 days after the final immunization and serum IgG was extracted using saturated ammonium sulfate precipitation.

SDS-PAGE and Western blot

The expression and purity of VP40 protein was examined in 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and confirmed by Western blot. VP40 protein resuspended in SDS-PAGE reduced buffer was subjected to 12% SDS-PAGE, and stained with 0.25% Coomassie brilliant blue R250 or transferred to a PVDF membrane. After blocking with 5% skimmed milk, the transferred membrane was incubated with 1:5000 diluted anti-His_6 tag MAb or 1:1000 diluted rabbit polyclonal antibodies against VP40 for 1 h at 37 °C. HRP-conjugated goat-anti-mouse IgG or goat-anti-rabbit IgG were used respectively as secondary antibodies, and AEC buffer was used as staining substrate.

Indirect enzyme-linked immunosorbent assay (ELISA)

Serum antibody titer was measured using indirect ELISA. Briefly, 96-well polyvinyl chloride microtiter plates were coated with 50 µL VP40 (5 µg/mL) in carbonate buffered saline (CBS, pH 9.6) and incubated overnight at 4°C. Following three washes in PBS containing 0.05% Tween 20 (PBST), the plates were incubated with blocking buffer (5% skimmed milk) for 1 h at 37°C and with 50 µL rabbit polyclonal antibodies diluted in *E. coli* lysate for 30 min at 37°C. Normal rabbit polyclonal antibodies were used as control and each sample was added in duplicate. After washing six times with PBST, the plates were incubated with 50 µL HRP-conjugated goat-anti-rabbit IgG for 30 min at 37°C. Then, the plates were washed again and finally incubated with 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) for color development. After 10 min of incubation at room temperature, the enzymatic reaction was stopped with 2 M H_2SO_4 and the horseradish peroxidase product was quantified at A450 (OD_{450}) with an automatic plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). All data were measured in triplicate, and the mean OD_{450} value (X) and standard deviation (SD) for the rabbit polyclonal antibodies were calculated. The highest dilution of antibodies that gave a positive/negative value over 2.2 was determined to be the titer of serum antibodies.

Immunoperoxidase monolayer assay (IPMA)

After 48 hours of transfection, when expression of GFP reached the highest level as observed under a fluorescent microscope, the transfected cells were washed three times with PBS and then processed with cold methanol containing 2% H_2O_2 for 15 min. Then, 5% skimmed milk was used as blocking buffer to block available sites on the plate at 37°C for 1 h. A volume of 50 µL of 1:1000 diluted rabbit polyclonal antibodies against VP40 protein was added and incubated with the plate at 37°C for 1 h. A volume of 50 µL of 1:500 diluted goat-anti-rabbit IgG-HRP was used as

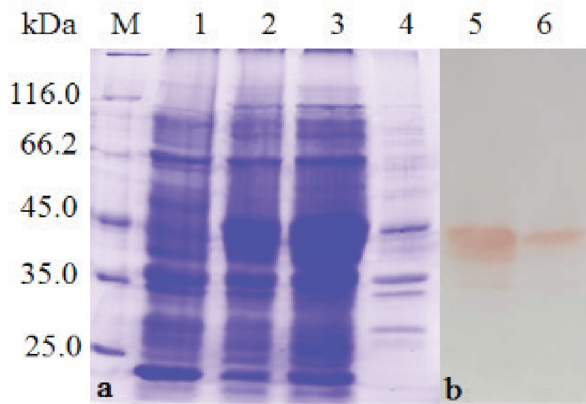

Figure 1. SDS-PAGE (a) and Western blot analysis (b). Lane M: protein marker; Lane 1: non-induced *E. coli* Rosetta (DE3) cells bearing pET28a-VP40; Lane 2: induced *E. coli* Rosetta (DE3) cells bearing pET28a-VP40; Lane 3: supernatant of induced *E. coli* Rosetta (DE3) cells bearing pET28a-VP40 after ultrasonication; Lane 4: precipitant of induced *E. coli* Rosetta (DE3) cells bearing pET28a-VP40 after ultrasonication; Lane 5: reaction between rabbit polyclonal antibodies to VP40 protein and the supernatant; Lane 6: reaction between rabbit polyclonal antibodies to VP40 protein and the precipitant.

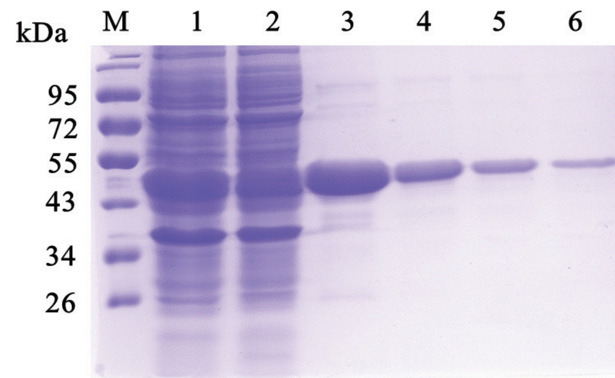

Figure 2. Purification of VP40 protein using Ni-NTA affinity chromatography. Lane M: protein marker; Lane 1: supernatant of induced E. coli Rosetta (DE3) cells bearing pET28a-VP40 after ultrasonication; Lane 2: flow through of the supernatant; Lane 3-6: 1-4 eluent of VP40 protein collected 1 ml per tube.

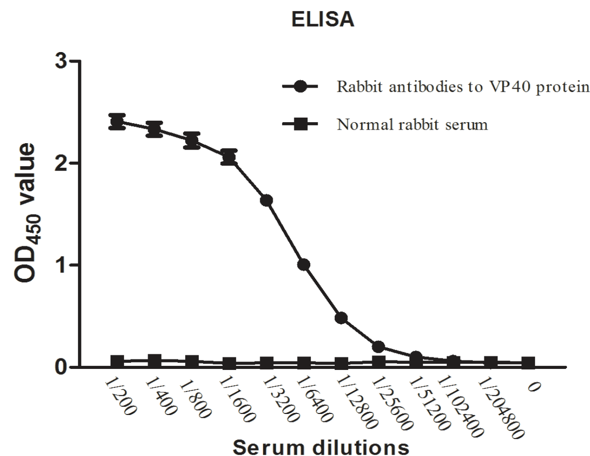

Figure 3. Titers of rabbit polyclonal antibodies tested by ELISA.

secondary antibodies and incubated with the plate at 37°C for 1 h. After each step, the plate was washed three times with PBST. Finally, AEC buffer was added for color development before stopping the color reaction with DDW. The wells were observed under a light microscope.

Results

Expression and purification of soluble VP40 protein

The time, temperature, and concentration of IPTG were optimized for VP40 protein expression. A large amount of soluble VP40 protein was produced by inducing *E. coli* Rosetta (DE3) cells bearing pET28a-VP40 in LB liquid media with 0.2 mM IPTG for 8 h at 25°C (Figure 1a). The expressed protein could be recognized by rabbit polyclonal antibodies against VP40 protein in Western blot (Figure 1b). After ultrasonication on ice and centrifugation, the supernatant was purified through Ni-NTA affinity chromatography. SDS-PAGE showed that VP40 protein bound with Ni^{2+} ions with high affinity, indicating the His_6-tag exposed on the outer aspect of the fusion protein (Figure 2). The overall yield of purified VP40 was over 25 mg/L cell culture.

ELISA

Rabbit sera were collected 7 days after the final immunization. Indirect ELISA was performed to measure antibody titers using purified VP40 as the coating antigen and *E. coli* lysate as the dilution buffer for rabbit polyclonal antibodies to avoid non-specific reactions. Normal rabbit serum showed no detectable antibody level and the titer of the rabbit polyclonal antibodies to VP40 protein reached 1:25600 in ELISA (Figure 3).

IPMA

GFP was used to monitor transfection efficiency and expression of VP40 protein in transfected BHK-21 cells. The expression of

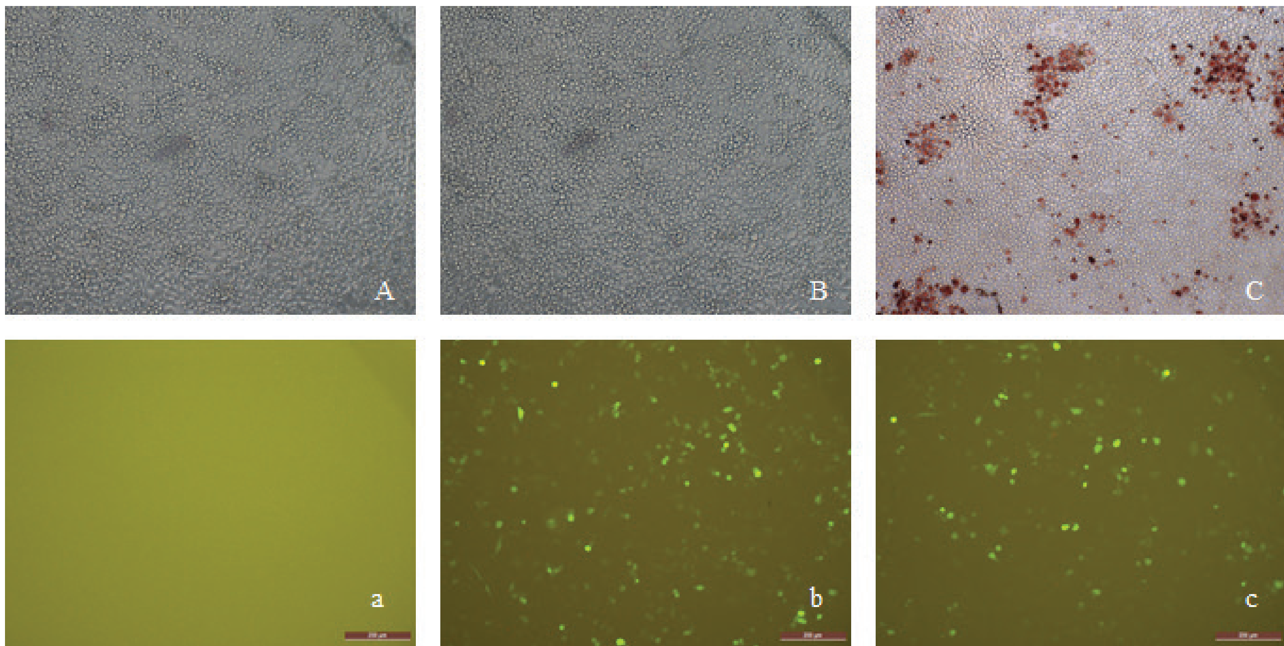

Figure 4. Immunoperoxidase monolayer assay (IPMA). BHK-21 cells transfected with pcDNA3.1, pcDNA3.1-eGFP and pcDNA3.1-eGFP-VP40 were respectively reacted with rabbit polyclonal antibodies to VP40 48 h post-transfection in IPMA. **A)** pcDNA3.1-transfected cells under a light microscope; **a)** pcDNA3.1-transfected cells under a fluorescent microscope. **B)** pcDNA3.1-eGFP-transfected cells under a light microscope; **b)** pcDNA3.1-eGFP-transfected cells under a fluorescent microscope. **C)** pcDNA3.1-eGFP-VP40-transfected cells under a light microscope **c)** pcDNA3.1-eGFP-VP40-transfected cells under a fluorescent microscope.

GFP was detected both in pcDNA3.1-eGFP- and pcDNA3.1-eGFP-VP40-transfected cells as early as 18 h post-transfection. After 48 hours of transfection, IPMA was performed to test the reactivity between rabbit polyclonal antibodies produced by immunizing with *E. coli*-expressed VP40 and eukaryotically expressed VP40 in BHK-21 cells. As shown in Figure 4, rabbit polyclonal antibodies to VP40 protein reacted specifically with pcDNA3.1-eGFP-VP40-transfected cells and possessed no reaction with pcDNA3.1- or pcDNA3.1-eGFP-transfected cells.

Discussion

EBOV outbreak continues to threaten the world due to the absence of effective vaccines and therapeutics. Contact with wild animals serves as the main conduit for the initial zoonotic transmission of EBOV into the human population.[14] Fruit bats and great apes have been recognized as the major source of EBOV transmission.[14-16] Although EVD is regarded as an exotic disease for China, great efforts have been made for prevention of EBOV introduction. Wang *et al.* expressed a full-length NP protein of ZEBOV, produced monoclonal antibodies against the protein, and identified an epitope motif PPLESD in the C-terminal which was well conserved among four EBOV species: ZEBOV, BEBOV, TEBOV, and REBOV.[17] Codon bias for EBOV GP protein has been analyzed, and the protein has been expressed in *E. coli*, and in mice by immunization with plasmid DNA encoding GP for the induction of neutralizing antibodies.[18-20]

VP40 is the major structural component and the most abundant protein of the EBOV virions. In this study, after codon optimization for expression in *E. coli* and eukaryotic cells, plasmids pET28a-VP40 and pcDNA-eGFP-VP40 were constructed, respectively. The expression of GFP was utilized for determining transfection efficiency and the expression of VP40 protein in BHK-21 cells. Through induction with 0.2 mM IPTG at 25 °C for 8 h, VP40 protein was present mainly in the supernatant after ultrasonication on ice. More water-soluble VP40 protein might be obtained if protein expression were induced at lower temperatures (15 °C or 20 °C). The His_6-tagged VP40 protein showed strong binding with Ni-NTA resin, indicating that the His_6-tag exposed on the external region of the recombinant protein is accessible to be recognized by nickel ions. Following purification through Ni-NTA affinity chromatography, the overall yield of purified VP40 protein was over 25 mg/L cell culture. The immunogenicity of VP40 protein was evaluated by immunization of rabbit with purified VP40 protein. It was shown that rabbit polyclonal antibodies specifically reacted with VP40 protein in Western blot and ELISA. Antibody titers tested by ELISA reached 1:25600. Besides, rabbit polyclonal antibodies against VP40 protein reacted specifically with pcDNA3.1-eGFP-VP40-transfected BHK-21 cells and showed no reaction with pcDNA3.1- or pcDNA3.1-eGFP-transfected cells. Hence, rabbit polyclonal antibodies produced by immunization with the prokaryotically expressed VP40 protein specifically reacted with the eukaryotically expressed VP40 protein in IPMA.

In conclusion, VP40 protein produced in *E. coli* Rosetta (DE3) cells has high immunogenicity and can be further used as diagnostic antigens in ELISA and other immunoassays. The strategy used in this study of expressing target protein from exotic diseases might be helpful in preparing a specific region for developing diagnostic methods against pathogens not existing in the region. Future study based on this work will focus on the production of monoclonal antibodies (MAbs) against VP40 protein, and development of indirect or direct competitive ELISA using purified VP40 protein and horseradish peroxidase conjugated MAb (HRP-MAb).

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