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Short communication

ClearColi BL21(DE3)-based expression of Zika virus antigens illustrates a rapid method of antibody production against emerging pathogens

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ABSTRACT

Available rapid, simple and accurate methods for detection and diagnosis of emerging viral diseases are required. Recently, there was an urgent need for specific antibodies against mosquito-borne Zika virus (ZIKV), which is an emerging zoonotic disease of medical concern in different regions of the world. Here, we showed that overexpression of ZIKV antigens in ClearColi BL21(DE3), a bacteria strain expressing a non-endotoxic form of LPS, is suitable for the production of specific ZIKV antisera. Two major ZIKV antigenic domains, the domain III from envelope E glycoprotein, which brings the virus-specific epitopes, and the N-terminal region of nonstructural NS1 glycoprotein, which is responsible for pathophysiological conditions, were overexpressed in ClearColi BL21(DE3). Immunization of adult rat with insoluble recombinant ZIKV antigens in inclusion bodies resulted in the production of specific antibodies in a few weeks. Anti-E and anti-NS1 antibodies are efficient as biological tools for ZIKV detection by indirect ELISA and immunoblot assay. This method could successfully be applied to any emerging viruses.

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1. Introduction

An availability in antibody is required for *in situ* detection of a newly identified pathogen as well as diagnosis of the pathogen-related disease by screening tests. Neglected or emerging viral diseases are often lacking high quality antibodies. It is usual that available pathogen antibodies show a mediocre specificity and/or sensitivity because the more relevant antigens are poorly characterized or display an unsatisfying immunogenicity in animals. Mosquito-transmitted Zika fever is considered as emerging infectious disease of medical concern worldwide [1]. Phylogenetically, Zika virus (ZIKV) is mainly classified into African and Asian genotypes, the latter being involved in the recent epidemics in South Pacific, Latin America and Caribbean islands [2]. ZIKV infection can cause severe neurological disorders and birth defects (microcephaly) in humans [3]. To face ZIKV emergence, it is urgent to dispose of efficient anti-Zika antibodies as immunological tools for the detection of ZIKV *in vitro* and *in vivo*. Thus, we decided to produce major viral ZIKV antigens in bacteria expression system in order to generate specific anti-ZIKV antibodies in rodent. Like other medically important related mosquito-borne RNA viruses such as dengue (DV), West Nile (WNV), yellow fever (YFV) and Japanese encephalitis, ZIKV contains a positive-stranded genomic RNA which is translated into a large and unique polyprotein, the latter being processed into structural proteins (C, prM/M and E), and nonstructural (NS) proteins, NS1 to NS5, by host and viral proteases [4]. Flavivirus E and NS1 proteins are two major antigenic markers for viral infection. The E protein mediates virus cell entry process (receptor binding and membrane fusion). It has three structural distinct domains: a central ß-barrel shaped domain I (EDI), a finger-like domain II (EDII) and a C-terminal immunoglobulin-like domain III (EDIII) [5]. Several reports have shown that flavivirus type-specific neutralizing antibodies are mainly linked to EDIII which is stabilized by a single disulfide bridge [6]. The flavivirus glycosylated NS1 protein associates with lipids to form a homodimer (46–55 kDa) inside the cells. The intracellular form of NS1 contributes to viral replication, whereas the extracellular NS1 exists as

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a hexameric lipoprotein, which interacts with the host factors leading to pathophysiological conditions [7].

A crucial step in the production of viral antigen antibody is the expression and purification of an immunogenic protein that is suitable for the stimulation of humoral response in animal recipient. It is common that overexpression of recombinant viral antigens in *E. coli* leads to inclusion body formation that can be isolated from crude cell lysates by action of detergents or chaotropic agents [8]. Because the protein refolding is frequently unsuitable, the recovered recombinant antigens are often deficient in their biological properties or antigenicity. As alternative to step purification of inclusion bodies, the crude cell lysate can be directly inoculated in animals but such method often causes acute inflammatory response to endotoxins, more frequently lipopolysaccharides (LPS), and then lethality [9]. In the present study, we showed that immunization of adult rats with non-purified recombinant ZIKV antigens overexpressed in ClearColi BL21(DE3), a bacteria strain expressing a non-endotoxic form of LPS, stimulates the production of specific antibodies. The efficient expression of recombinant antigenic domains from E (EDIII) and NS1 (the N-terminal region) as viral immunogens has allowed the rapid and efficient production of monospecific immune sera that are useful biological tools for ZIKV detection.

2. Results and discussion

2.1. ZIKV antigens are express as inclusion bodies in E. coli

In order to produce anti-ZIKV antibody, EDIII and the N-terminal region of NS1 (NS1-1 to NS1-151) from Asian ZIKV clinical isolate FP-25013-18 [1] were selected for viral antigen production in *E. coli*. The EDIII contains ZIKV-specific epitopes which have been reported to stimulate the production of anti-ZIKV neutralizing antibody [6]. It is of interest to note that antigenic cross-reactivity has been observed between ZIKV and other medically important flaviviruses such as DV, WNV, and YFV mainly due to ED I/II epitopes [10]. Mounting evidence indicts that ZIKV NS1 antibodies are largely virus-specific compare to E protein [11,12]. It has been reported that the N-terminal region of NS1 is sufficient to stimulate the production of anti-NS1 antibody [13,14].

To produce recombinant EDIII (rEDIII) and fragment $NS1^{1-151}$ in *E. coli*, synthetic genes with optimized codons to improve protein expression in bacteria cells were cloned into pET28a plasmid. An in-frame polyhistidine-tag was added to the C-terminus of viral antigens. The sequences of ZIKV rEDIII and $rNS1^{1-151}$ are listed in supplementary data. The corresponding recombinant plasmids pET28/ZIKV.rEDIII and $pT28/ZIKV.rNS1^{1-151}$ were transformed into ClearColi BL21 (DE3) bacteria, which contain a genetically modified LPS that does not trigger an endotoxic response [15]. The bacterial clones obtained were expanded and induced with 1 mM IPTG at 37 °C during 4 h in order to test the expression of the recombinant viral antigens. Bacterial cultures were lyzed by sonication and centrifuged. The collected protein fractions (total extracts, supernatants and pellets) were analyzed by SDS-PAGE (Fig. 1A). Coomassie Blue staining showed that rEDIII (apparent molecular weight 12 kDa) and $rNS1^{1-151}$ (apparent molecular weight 20 kDa) were essentially recovered as insoluble aggregates, which correspond to inclusion bodies. To circumvent this issue, we tested different strategies. We were not able to obtain soluble rEDIII and $rNS1^{1-151}$ using bacterial strains engineered to favor refolding like Origami2 or shuffleT7. Refolding rates were not either increased by the addition in the culture media of chemical agents like sorbitol, glycerol, high salt, betaine, sucrose or $MgCl_2$ [8,16] (data not shown). The fusion of DV antigens to thioredoxin has previously been reported to increase recombinant protein solubility [6,17,18]. In our hands, thioredoxin fusion showed no effect on the insolubility of ZIKV rEDIII and $rNS1^{1-151}$ (data not shown).

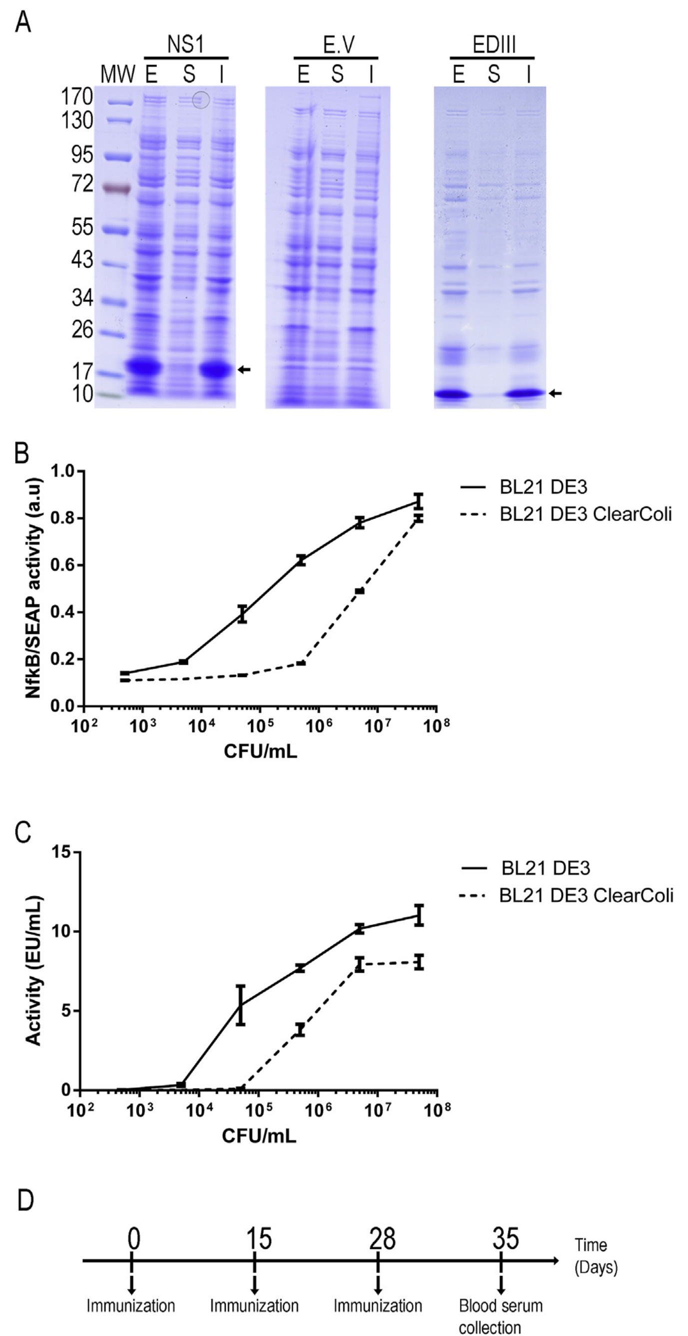

Fig. 1. Expression of recombinant ZIKV antigens in *E. coli* and immunization protocol in rat. **A**. ClearColi BL21(DE3) cells were transformed with pET28/ZIKV.rEDIII (EDIII) or $pT28/ZIKV.rNS1^{1-151}$ (NS1) or empty vector (E.V.). Proteins in crude cell lysates (E) were separated by SDS-PAGE followed by a Coomassie blue staining. The soluble (S) and insoluble (I) fractions of cell lysates were stained. The arrow heads indicate the position of $rNS1^{1-151}$ and rEDIII proteins. **B**. RAW-Blue cells were incubated with increasing doses of sonicated crude cell extracts from ClearColi BL21(DE3) or BL21(DE3). NF-κB activity was measured using QUANTI-blue assay. **C**. Endotoxic activity of sonicated BL21 Clear Coli or BL21DE3 was determined by LAL assay. **D**. Protocol of rat immunization. Crude extract of ClearColi BL21(DE3) cells containing ZIKV antigens were subcutaneously inoculated in adult female Wistar rats. The animals received two booster doses at days 15 and 28 after the first inoculation. Immune sera were collected one week after the last boosting inoculation.

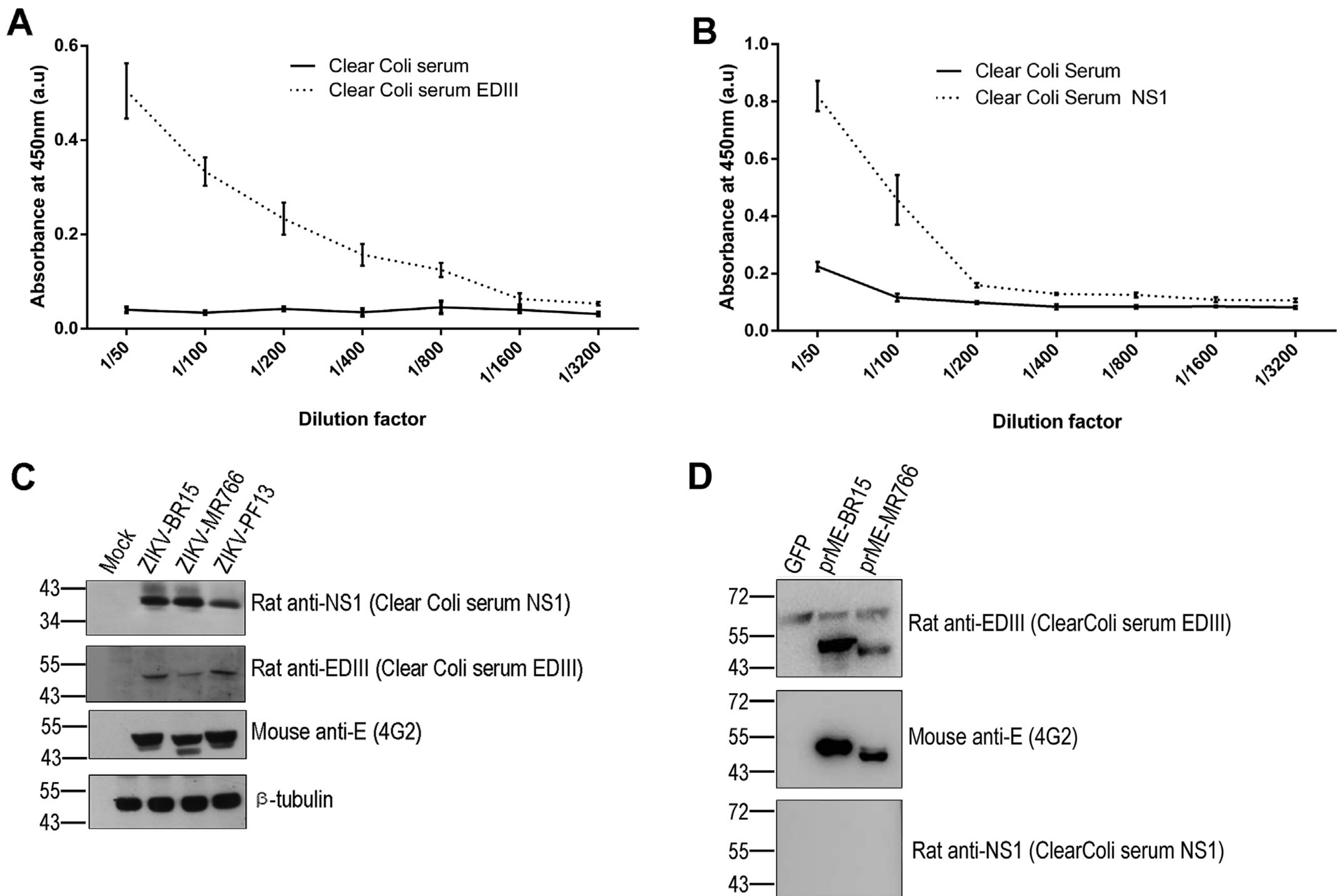

Fig. 2. Reactivity of rat immune sera against ZIKV E and NS1 proteins. **A.** Indirect ELISA was performed to detect anti-ZIKV IgGs in rat immunized with bacterially-ovexpressed rEDIII using inactivated ZIKV strain BR15 (10^6 PFU per well) as viral antigen. **A.** Indirect ELISA was performed to detect anti-NS1 IgGs in rat immunized with bacterially-expressed $rNS1^{1-151}$ using purified recombinant ZIKV.NS1 (100 ng per well) as viral antigen. **C**. RIPA cell lysates obtained from Vero cells infected with ZIKV strains BR15, MR766 or H/PF/2013 were tested with antisera from rat immunized with bacterially-expressed $rNS1^{1-151}$ or rEDIII by immunoblot assay. Anti-flavivirus E mAb 4G2 served as a positive antibody control. **D**. RIPA cell lysates obtained from HEK-293 cells transiently transfected with pcDNA3/MR766.prME (prME-MR766), pcDNA3/BR15.prME (prME-BR15) or pcDNA3/GFP (GFP) were tested with antisera from rat immunized with bacterially-expressed rEDIII by immunoblot assay. Anti-flavivirus E mAb 4G2 served as a positive antibody control. Antisera from rat immunized with bacterially-expressed $rNS1^{1-151}$ served as a negative antibody control.

2.2. ClearColi BL21(DE3) whole cell extracts exhibit low endoxicity activity

Because our attempts to solubilize ZIKV recombinant proteins were unsuccessful, we decided to directly immunize rats with inclusion bodies. LPS is one major endotoxic factor of *E. coli,* which induces hypodynamic state and septic shock in rodent. We decided to use for further investigations the ClearColi BL21(DE3) bacteria as they bear a lipid A displaying a low level of acylation, which is responsible for LPS-induced immune response activation and toxicity. However, we cannot fully rule out that inoculation of a total crude extract of ClearColi BL21(DE3) bacteria triggers adverse events *in vivo*. To verify the safety of the ClearColi BL21(DE3) extracts, we performed a macrophage reporter RAW-Blue cell assay, which allows to test the host innate immune response. In RAW-Blue cells, the presence of LPS induces TLR4/MD-2 receptors-dependent signaling pathway leading to activation of NF-kB and the subsequent production of secreted alkaline phosphatase (SEAP) reporter. As shown in Fig. 1B, there was a 100-fold lower NF-κB activity in response to sonicated ClearColi BL21(DE3) crude extracts when compared to BL21(DE3). The Limulus Amebocyte Lysate assay (LAL assay), another endotoxin reaction test, confirmed that the biological material was poorly endotoxic (Fig. 1C).

2.3. Direct injection of antigens from ClearColi BL21(DE3) whole cell extracts elicit anti-ZIKV antibodies in hyperimmunized rate

We assessed whether the ClearColi BL21(DE3) bacteria were suitable for animal immunogenicity testing. Adult Wistar rats were primed with 100 μL of ClearColi BL21(DE3) crude extract (2.10^8 bacteria.mL^{-1}) which overexpress ZIKV rEDIII or $rNS1^{1-151}$ in presence of incomplete Freund adjuvant and twice boosted at days 15 and 28 with the same dose of recombinant viral antigens. At day 35, whole blood was collected by cardiac puncture after rat anesthesia (Fig. 1D). To measure the productions of anti-ZIKV IgGs by immunized rats, indirect enzyme-linked immunosorbent assay (ELISA) was performed on rat sera. Viral antigens used for the assay were inactivated ZIKV particles (Fig. 2A) or ZIKV rNS1 purified from supernatants of HEK-293 cells stably transfected with a plasmid pcDNA3.1 containing the complete ZIKV NS1 gene from a contemporary epidemic strain (Fig. 2B). Serum from an adult rat inoculated with bacterially-expressed GFP served as a negative control. Inoculation of crude *E. coli* extract which overexpresses ZIKV rEDIII in rats induced a significant production of anti-ZIKV specific antibodies with a titer reaching up to 1:200. ZIKV $rNS1^{1-151}$ also led to an antibody response against ZIKV NS1 in immunized rats. The titer of anti-NS1 antibody reached up to 1:100.

Reactivity of rat immune sera was further evaluated by immunoblotting using RIPA cell lysates of Vero cells infected by ZIKV (Fig. 2C). Immunoblotting analysis showed that $rNS1^{1-151}$ antibodies reacted with the heat-denatured NS1 protein (apparent molecular weight 42 kDa) regardless of the tested ZIKV strains. A very weak reactivity was observed with the dimeric form of NS1 (apparent molecular weight 72 kDa) indicating that the rat immune serum is preferentially directed against the monomeric form (data not shown). Rats that received ZIKV rEDIII displayed antibodies against ZIKV E proteins (apparent molecular weight 52 or 54 kDa) (Fig. 2C). Anti-rEDIII antibodies recognize the E protein from African prototype strain MR766 (Uganda 1947) as well as Asian epidemic strains FP-25013-18 from French Polynesia (2013) and BeH819015 from Brazil (2015). The specificity of rat anti-ZIKV.EDIII antibody against ZIKV E protein was further evaluated by immunoblotting using RIPA lysates of transiently transfected HEK-293 cells with recombinant plasmid pcDNA3/MR766.prME or pcDNA3/BR15.prME (Fig. 2D). Transfected cells accumulated recombinant E protein as observed by Western blotting using mAb 4G2. The BR15 E protein migrated slower than did MR766 E protein. This slower migration may be due to the N-glycosylated status of the E protein from ZIKV BR15 strain. Rat immunization with rEDIII elicited antibodies that were reactive with the E protein from Asian (BR15) and African (MR766) strains of ZIKV (Fig. 2D). There was no reactivity of rat anti-rNS1 immune serum with cell lysates containing recombinant prM and E from ZIKV. Immunization of rats with rEDIII and $rNS1^{1-151}$ stimulated the production of specific anti-E or anti-NS1 antibodies.

3. Conclusion

In the present study, we showed that injection of whole cell extracts of endotoxin-free ClearColi BL21(DE3) containing viral antigens made available the production of specific antibodies in immunized animal. As a proof of concept, rat immunization was performed with the antigenic domain III of the E protein or the N-terminal region of NS1 from ZIKV. Immune sera from rats immunized with bacterially expressed-recombinant viral antigens were positive for anti-E and NS1 antibodies that recognize similarly ZIKV strains of African and Asian genotypes by indirect ELISA and immunoblot assays. The remarkable efficacy of the ClearColi BL21(DE3)-based expression system at eliciting the rapid production of specific anti-ZIKV antibodies endorses an innovative method, which can be extended to emerging viruses for which development of immunological tools is an urgent prerequisite.

Author agreement

All the co-authors have contributed collectively to this study in order named as full participants. All the co-authors approved the final version of the manuscript and its submission to *BIOCHIMIE*. This work has not previously been published and is not being considered for publication elsewhere.

Author contributions

W.V., P.D designed research; W.V., B.N., W.H., P.K.T., P.D. performed research; W.V., B.N., W.H., P.K.T., S.B., C.E.K., G.G., P.D. contributed new reagents/analytic tools; all authors analyzed data; W.V., G.G., P.D wrote the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biochi.2017.09.011.

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