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Research paper

Development and characterization of a Zaire Ebola (ZEBOV) specific IgM ELISA

Tanmaya Atre[a], Revell L. Phillips[b], Kayvon Modjarrad[c], Jason A. Regules[d], Elke S. Bergmann-Leitner[a,*]

[a] Malaria Vaccine Branch, Walter Reed Army Institute of Research, Silver Spring, MD 20910, United States of America
[b] Joint Science and Technology Office, Defense Threat Reduction Agency, Fort Belvoir, VA 22060, United States of America
[c] Emerging Infectious Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, MD 20910, United States of America
[d] Department of Clinical Research, United States Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD 21702, United States of America

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ABSTRACT

Immunoglobulin M (IgM) is the first antibody induced after the onset of an adaptive immune response against a pathogen or vaccine. Serological assays play a central role in evaluating these adaptive immunological responses. Such assays are not only crucial for the assessment of vaccine immunogenicity, but also inform on exposure to pathogens and cross-reactivity with other viruses. To date, there is no ELISA-based assay available that measures IgM responses against Zaire Ebola virus (ZEBOV). To address this critical need, our laboratory has developed a novel immunoassay capable of detecting total IgM against ZEBOV glycoprotein in serum samples from individuals exposed to the antigen through infection or vaccination. Here, we describe a sensitive, high-throughput, and inexpensive assay that can be performed in any laboratory. The performance criteria of the newly developed ZEBOV glycoprotein-based IgM ELISA were assessed using antisera collected from human patients immunized with the rVSVΔG-ZEBOV-GP vaccine being tested in a phase 1 clinical trial. This assay demonstrates high specificity and sensitivity and will also be a valuable tool in the mission to find immune correlates of protection for a successful Ebola vaccine.

1. Introduction

The largest Ebola outbreak was witnessed in West Africa between the years 2014–2016 with over 28,000 cases and 11,000 deaths (*Ebola Virus Disease-Situation Report,* 2016). Ebola outbreaks are associated with a fatality rate between 25 and 90% in humans (Srivastava, 2015). Ebola infection is caused by the Ebola virus, which belongs to the Filoviridae (filovirus) family. These viruses are among the most lethal pathogens known to infect humans and non-human primates. In humans, the virus enters the host by contact with mucous membranes and infects endothelial and liver cells. The virus replicates rapidly in monocytes, macrophages and dendritic cells, subsequently causing sepsis (Ansari, 2014). Five species of EBOV have been reported so far, namely Zaire Ebola virus (ZEBOV), Reston Ebola virus (RESTV), Sudan Ebola virus (SUDV), Tai Forest Ebola virus (TAFV), and Bundibugyo Ebola virus (BDBV). Recent outbreaks of Ebola were reported to largely be caused by ZEBOV, SUDV, and BDBV.

Although Ebola is largely endemic in central and West Africa, it continues to remain a global threat due human-to-human transmission by direct contact with blood or body fluid secretions from infected individuals. Effective vaccines play a vital role in the prevention and control of such infections. As such, the replication-competent recombinant vesicular stomatitis virus (rVSV), in which the VSV glycoprotein (VSV-G) gene was replaced by the ZEBOV glycoprotein (GP), is a leading vaccine candidate for Ebola (Henao-Restrepo et al., 2015, 2017). It is a recombinant, live attenuated virus vaccine that has shown high efficacy with rapid onset of protection in both humans (Henao-Restrepo et al., 2015, 2017) and non-human primates (Jones et al., 2005; Geisbert et al., 2008). However, the immune mechanisms of protection induced by this vaccine are poorly understood. Serological responses measured from a human phase 1 clinical trials using the rVSVΔG-ZEBOV-GP vaccine revealed IgG antibody responses to ZEBOV glycoprotein in almost all vaccinated subjects (Heppner Jr. et al., 2017). The quantification of cellular responses has indicated that ZEBOV-specific circulating follicular T helper cells (cTfh), particularly the cTfh17 subset, correlate with antibody titers (Farooq et al., 2016).

Immunoassays are the predominant tool for evaluating pathogen-induced immune responses. They have been extensively used to assess

* Corresponding author at: 503 Robert Grant Ave, 3W58, Silver Spring, MD 20910, United States of America.
E-mail address: elke.s.bergmann-leitner.civ@mail.mil (E.S. Bergmann-Leitner).

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the immunogenicity of vaccine formulations in vaccine development, but there continues to be a need for novel and optimized readout methods to better define vaccine or pathogen-induced immune responses. The most commonly used assay to test immunogenicity and potency is the Enzyme Linked Immunosorbent Assay (ELISA). This method is a quick and high-throughput method to accurately measure serum antibodies. Most of the ELISA-based data available are derived from evaluating IgG in sera. Relatively little attention is paid to the presence of IgM antibodies when assessing vaccine immunogenicity. IgM is the first isotype produced after the initial encounter of an antigen by a naïve B cell and generally has lower affinity than subsequently produced IgG. However, IgM may play an important role in rapid protection against lethal pathogens. In addition, evaluating the IgM response to vaccination or after exposure can provide important insights into the onset of an immune response which makes it an important tool in diagnostics. A recent publication characterized the predominant IgM response to the rVSVΔG-ZEBOV-GP vaccine highlighting the need a reliable, sensitive serological assay that assesses the IgM immune response (Khurana et al., 2016). Currently, no standardized, inexpensive method is available to measure serum IgM.

Here, we describe a novel capture ELISA to measure serum IgM antibody responses against Zaire Ebola (ZEBOV) glycoprotein. This assay demonstrates high sensitivity and specificity, and enables rapid, high throughput serological testing of samples. We believe that the implementation of this assay to measure rVSVΔG-ZEBOV-GP vaccine-induced IgM responses will be a valuable tool in the mission to find effective vaccine correlates of protection in the effort to eradicate Ebola.

2. Materials and methods

2.1. Study samples

Samples were obtained from subjects participating in a Phase I clinical trial (NCT02269423) conducted at the Clinical Trials Center at WRAIR. The methods were carried out in accordance with the approved guidelines. All experimental protocols were approved by the WRAIR Human Subject Protection Branch (WRAIR#2163) and all study subjects had provided informed consent for future use of the samples. Sera were collected on Days 0, 7, 28, 56, 84, and 180 after a single vaccination and stored at −20 °C.

2.2. IgM ELISA

Immulon 2HB plates (Thermo Scientific, Waltham, MA) were coated with a mouse monoclonal antibody (mAB) specific to human IgM (clone CM7, BioRad, 0.5 μg/ml, 50 μl/well, Bio-Rad, Hercules, CA) as the capture antibody and incubated overnight at 4 °C. The next day, plates were washed and blocked using Superblock buffer in PBS (Thermo Scientific, Waltham, MA). Primary antibody (human serum from rVSVΔG-ZEBOV-GP vaccinated individuals) were diluted in PBS (pH 7.4), added to respective wells (100 μl/well) and incubated at room temperature (RT) for 2 h. Unbound antibodies were removed by washing plates with PBS + 0.05% Tween 20 (wash buffer). ZEBOV glycoprotein (Sino Biological Inc., Beijing, China) was diluted in PBS at suitable concentration and added to respective wells (100 μl/well). Plates were incubated at RT for 1 h. Following washing, detecting antibody (rabbit polyclonal antibody ZEBOV glycoprotein (Sino Biological Inc., Beijing, China)) was prepared in PBS (pH 7.4), and added to respective wells (100 μl/well). Plates were incubated for 1 h at RT. After washing, plates were incubated with mouse-anti rabbit IgG-Biotin (Southern Biotech, Birmingham, AL, USA) diluted in PBS (pH 7.4) at 1:2000 dilution. Plates were incubated at RT for 1 h. Plates were washed with wash buffer and incubated at RT for 1 h with Streptavidin-AP (Southern Biotech, Birmingham, AL, USA) diluted 1:2000 in PBS (pH 7.4). Plates were then washed and substrate (BluePhos, SeraCare, Gaithersburg, MD) was added. Plates were read on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) at 620 nm.

2.3. Statistical analysis

Statistically significant differences between the various assay conditions were determined by using two-sided *t*-tests (Minitab 17, State College, PA).

3. Results and discussion

3.1. Development of ELISA

Initial experiments clearly demonstrated that a simple ELISA method with antigen-coated plates was not an option as no specific signal could be detected (data not shown). The lack of specificity despite a high signal may have been due to the large concentration of IgG antibodies in the sera that were competing with the lower affinity IgM antibodies. Therefore, a sandwich ELISA using an anti-human IgM monoclonal antibody as the capture antibody was used as a starting point for assay development. Test samples from a recent clinical trial evaluating the rVSVΔG-ZEBOV-GP vaccine (Merck/NewLink) were used for the assay development. The vaccine was tested in a Phase 1 clinical trial with a dose escalation (Regules et al., 2017). Throughout the assay development and characterization, sera from the different dose cohorts were used to evaluate the assay.

Matrix experiments testing (1) the format of the anti-human IgM capture reagents (i.e., polyclonal vs. monoclonal antibodies); (2) the format of the detecting antibody (rabbit anti-ZEBOV polyclonal antibodies): biotinylation vs. alkaline phosphatase (AP) vs. horseradish peroxidase (HRP); (3) the format of the secondary antibody for the detection of bound rabbit anti-ZEBOV (biotinylation vs. enzyme-conjugated anti-rabbit secondary); and (4) the optimal enzyme for the final detection (i.e., AP vs. HRP). The various parameters were chosen based on their superiority regarding signal-to-noise ratio and sensitivity. The optimal experimental setup involves plates coated with a capture mAb specific for human IgM (Fig. 1). Next, test sera (primary antibodies, i.e., sera from rVSVΔG-ZEBOV-GP vaccinated subjects) are added; IgM in the samples binds to the capture mAb regardless of antibody specificity. Recombinant ZEBOV-GP added to the plate is bound by specific human IgM resulting in immune complexes. Visualization of these immune complexes is achieved by adding the polyclonal rabbit-anti-ZEBOV-GP to the plates followed by a biotinylated secondary (mouse anti-rabbit Ig) and Streptavidin-alkaline phosphatase. ZEBOV-GP-specific IgM is quantified by adding BluePhos substrate and measuring optical density in an ELISA plate reader.

Once the basic setup was established, the optimal concentrations of the various components had to be determined (Fig. 2). The optimal concentration of the capture antibody and the range at which the IgM in test samples can be quantified were identified by performing titration experiments. The final concentration for the monoclonal capture antibody was 0.5 μg/ml (Fig. 2A) and the serum dilution with the highest signal-to-noise ratio was 1:25 (Fig. 2B). The optimal concentration for the mouse-anti rabbit IgG-Biotin was determined to be 1:2000 (Fig. 2C). Once these assay conditions were established, various blocking buffers were tested to reduce the background noise to a minimum. 0.5% casein in PBS, 2% non-fat milk in PBS, 1% BSA in PBS, and Superblock buffer in PBS (Thermo Fisher) were tested and Superblock buffer in PBS performed best (Fig. 2D). While the final protocol is complex, it was required to maximize the signal-to-noise-ratio and the sensitivity.

3.2. ELISA specificity

Testing immune serum pools (n = 10 subjects/cohort; study Day 28) from the three cohorts of the clinical study demonstrated high specificity of the IgM ELISA against ZEBOV glycoprotein (Fig. 3A). The

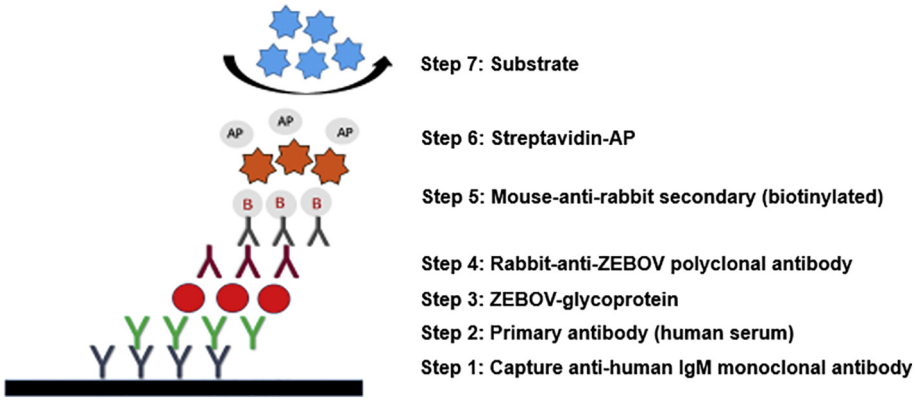

Fig. 1. Schematic overview of the ZEBOV-GP specific IgM ELISA. Numbers indicate the logistical order for adding reagents.

capture mAb has been shown to be highly specific for IgM and does not cross-react with other isotypes (Antibodies, 2018; Pendyala et al., 2009). Test sera were run at sample dilutions of 1:25, 1:50, 1:100 and 1:200. For this assay, two specificity controls were used: (1) omitting the ZEBOV antigen but adding all other reagents; (2) matched pre-vaccination sera. Both negative controls yielded low (background) signals indicating that a positive signal was dependent on the addition of ZEBOV antigen and that the subjects had no cross-reactive antibodies capable of binding ZEBOV-GP prior to vaccination. Immune serum pools showed highest response at a serum dilution of 1:25 (Fig. 3B). The assay also showed a coefficient of variation (% CV) less than 10% (Table 1) summarizing three independent experiments. As expected, the cohort with the lowest titer had the highest % CV.

3.3. ELISA sensitivity

To demonstrate the sensitivity of the assay, serum samples from five time points for the low-dose vs. high-dose cohort were tested (Fig. 4). The results demonstrated that the IgM ELISA described here is capable of detecting trace amounts of ZEBOV-specific IgM antibodies. It also

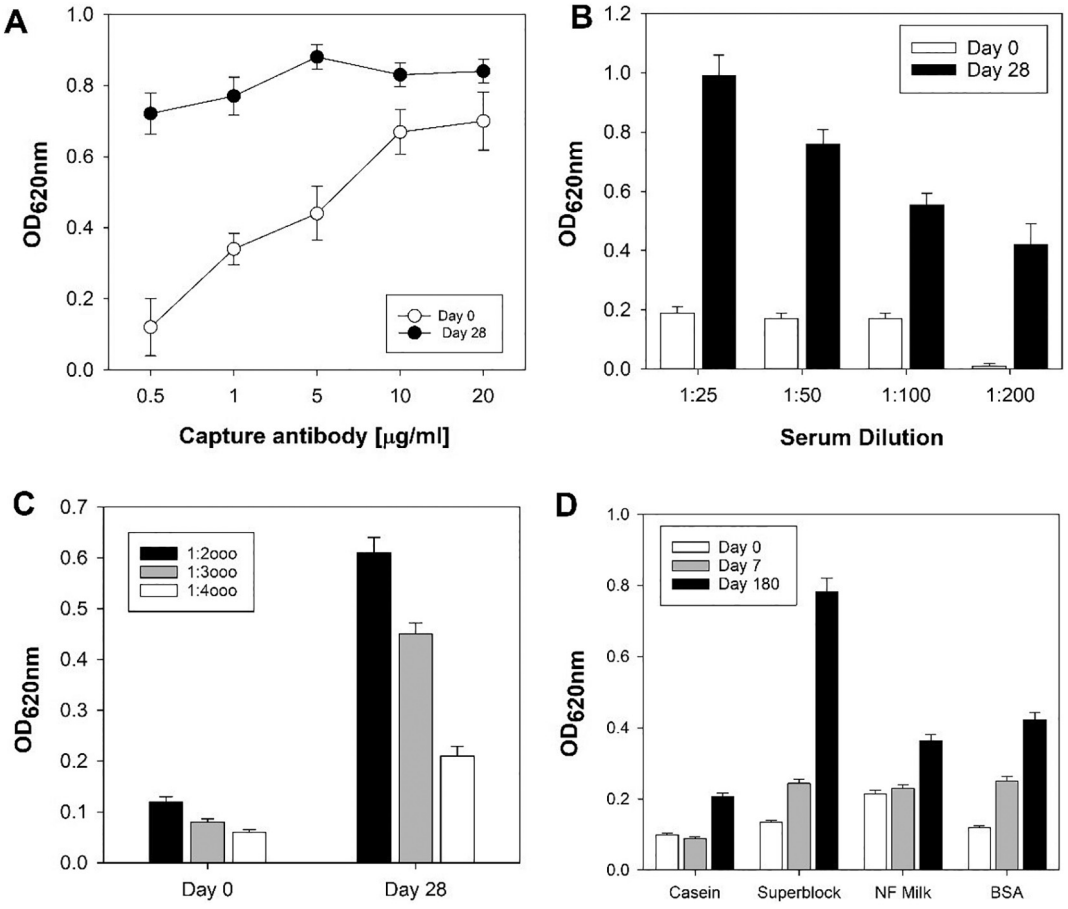

Fig. 2. Development of the ZEBOV-GP-specific human IgM ELISA. (A) Titration of capture mAb (mouse anti-huIgM). ZEBOV-GP immune human sera were tested at 1:25. (B) Titration of ZEBOV-GP immune human sera (n = 3 subjects) from study Day 0 and Day 28 with the optimal capture mAb concentration (0.5 μg/ml). (C) Titration of secondary antibody mouse-anti rabbit IgG-Biotin using ZEBOV-GP immune human sera were tested at 1:25. (D) Testing of different blocking buffers with individual serum samples (n = 3 subjects) for Day 0, Day 7 and Day 180. Data are expressed as mean OD_{620nm} (error bars represent standard deviation) of three rVSVΔG-ZEBOV-GP vaccinated subjects tested in triplicates.

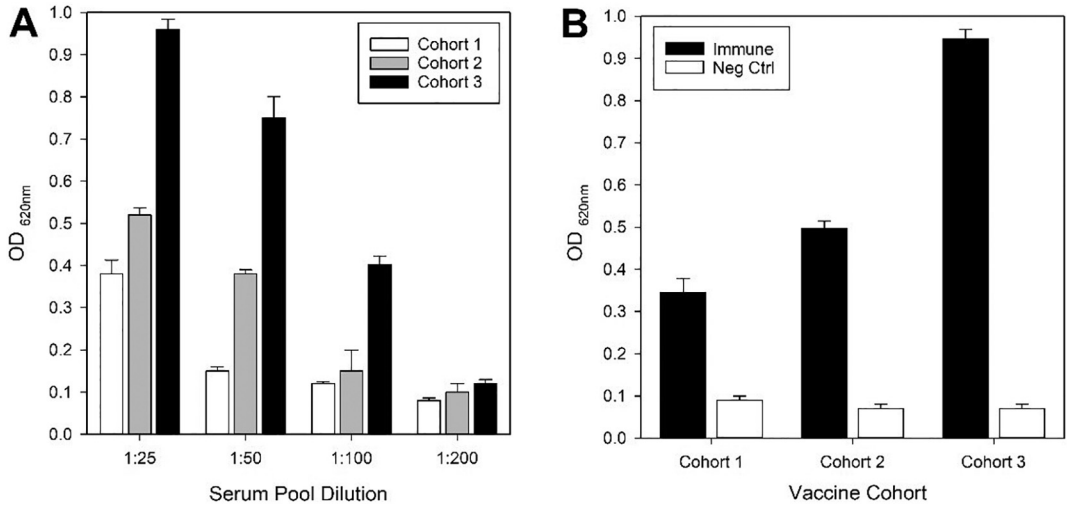

Fig. 3. (A) Different magnitude of responses in the rVSVΔG-ZEBOV-GP vaccine cohorts. Titration of immune serum pools for cohort 1, cohort 2 and cohort 3; (B) assay specificity. Data expressed as mean OD_{620nm} (standard deviation) of three independent experiments using immune pools of cohort 1, cohort 2 and cohort 3 at a 1:25 dilution. Specificity was demonstrated by omitting ZEBOV-GP from the assay (negative control).

Table 1
Intra-assay variability of the ZEBOV-GP specific IgM ELISA.

Samples[a]	Mean (SD)[b]	% CV
Cohort 1	0.35 (0.033)	9%
Cohort 2	0.50 (0.017)	3%
Cohort 3	0.95 (0.024)	3%

[a] Day 28 rVSVΔG-ZEBOV-GP immune serum pools (n = 10 subjects/cohort) were tested in triplicates at a 1:25 dilution.
[b] Values expressed as mean OD_{620nm} (SD).

demonstrates the variability in the serological responses between the vaccinees and the persistence of the response in the different study participants. The persistence of ZEBOV-specific IgM is not surprising as it has been reported that virus-specific IgM^+ plasma cells persist for at least one year after vaccination with a live attenuated influenza vaccine (Bredholt et al., 2014). Another study demonstrated not only persistence, but also protective efficacy mediated by long-lived IgM^+ plasma cells (Bohannon et al., 2016). Such persistence is not restricted to attenuated viral vaccines, but has also been demonstrated in studies with attenuated whole parasite vaccines against malaria where antigen-specific IgM exhibit anti-parasite activity 189 days post vaccination (Daubenberger et al., 2018).

3.4. ELISA repeatability and reproducibility

Assay repeatability was assessed by testing longitudinally collected sera (Day 0, 7, 28, 56, and 180 post vaccination) from three individuals in repeat experiments run over the course of one week (Fig. 5). The results indicated that the assay has a high reproducibility with an intra-assay CV ranging from 4% to 10%.

To evaluate assay reproducibility and robustness, a set of de-identified human sera were tested by two operators at the same time with separate setup and acquisition (Fig. 6). Minor variations in results indicate that this assay is highly reproducible.

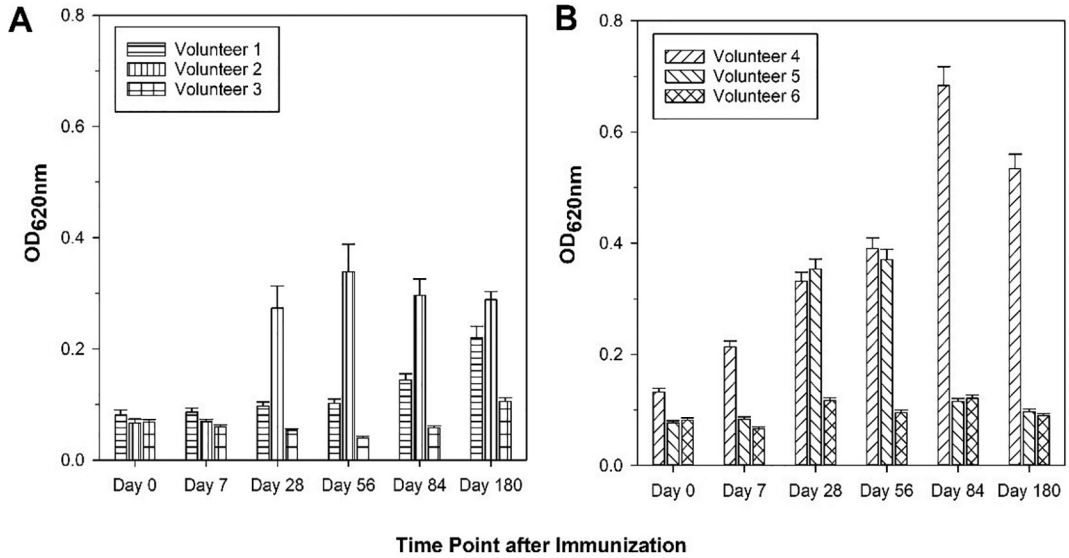

Fig. 4. Total IgM response to ZEBOV-GP for three randomly selected volunteers from the low-dose Cohort 1(A) and high-dose Cohort 3 (B). Data expressed as mean OD_{620nm} (SD) of triplicate wells (three separate experiments).

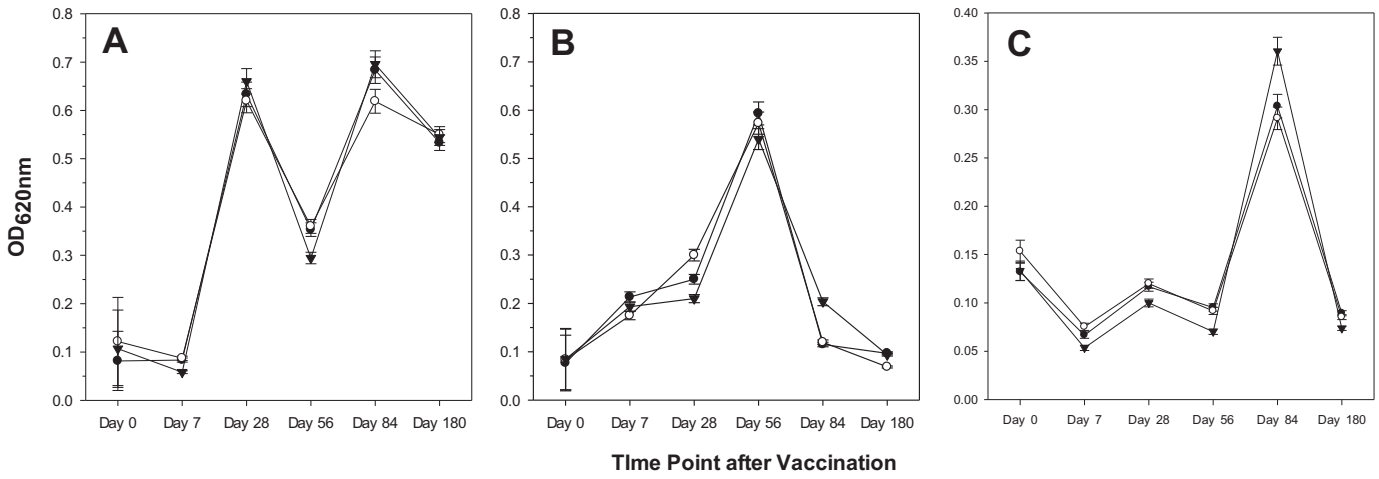

Fig. 5. Assay repeatability of the ZEBOV-GP specific IgM sandwich ELISA. Individual rVSVΔG-ZEBOV-GP immune serum samples (three donors A, B, C) from Day 0, 7, 28, 56, 84, and 180 were tested on three separate assay days. Data is represented as the mean OD_{620nm} ($n = 3$ separate experiments). Lines represent the mean of the individual experiments, error bars represent the standard deviation of the individual experiments.

4. Conclusion

A novel capture ELISA to measure IgM antibodies in serum from rVSVΔG-ZEBOV-GP vaccine recipients has been successfully developed and optimized. The assay has demonstrated high specificity and sensitivity and can be performed by different operators with highly consistent results.

Authors' contributions

TA performed the experiments and compiled the manuscript. EB-L designed the experiments. RLP, KM, JAR, EB-L reviewed and edited the manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data and detailed protocol can be made available by the corresponding author upon request.

Consent for publication

Not applicable.

Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting the views of the Department of the Army or the Department of Defense. This paper has been approved for public release with unlimited distribution. The investigators have adhered to the policies for protection

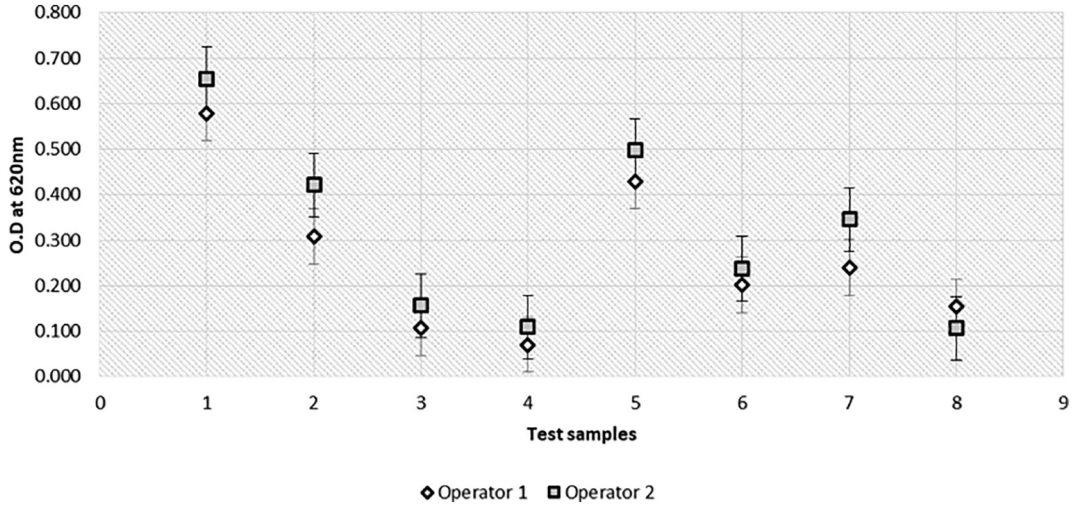

Fig. 6. Inter-operator variability of the ZEBOV-GP specific IgM sandwich ELISA. Eight individual rVSVΔG-ZEBOV-GP immune serum samples (Day 56) were tested for total IgM response by two different operators. Data is represented as the mean OD_{620nm} (SD) (n = 3 separate experiments) for each operator.

of human subjects as prescribed in AR 70-25.

Ethics approval and consent to participate

Not applicable.

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