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Development of a bead-based immunoassay using virus-like particles for detection of alphaviral humoral response

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ABSTRACT

There is a pressing need for sustainable and sensitive immunodiagnostics for use in public health efforts to understand and combat the threat of endemic and emerging infectious diseases. In this proof-of-concept work, we describe an immunodiagnostic approach based on the utilization of virus-like particles (VLPs) in a magnetic bead-based platform for multiplexed detection of antiviral humoral response. A retroviral-based VLP, that presents Venezuelan equine encephalitis virus E1/E2 glycoprotein antigen on its surface, was synthesized and coupled to magnetic beads to create VLP-conjugated microspheres (VCMs). Using these VCMs, IgM and IgG antibodies were detectable in nonhuman primate (NHP) and human clinical serum samples at dilutions of 1×10 Basile et al. [4] and greater. We also extended the VCM methodology to an Old World alphavirus, chikungunya virus, demonstrating the flexibility of this approach toward different VLP architectures. When multiplexed on the MAGPIX® platform, this method provided differential detection between Old World and New World alphaviral IgM. This flexible, immunodiagnostic method, based on the MAGPIX® platform, demonstrates compatibility of particulate antigens with bead-based assays, improves sensitivity by up to 2-logs, and has faster sample-to-answer time over traditional methods.

1. Introduction

Immunoassays are reliable and robust assays used for surveillance and diagnosis of infectious and non-infectious diseases worldwide because they are technologically accessible to many laboratories (Banoo et al., 2019; Spackman, 2012). Traditionally, serosurveillance or clinical diagnosis utilizes inactivated whole virus or lysates from infected cells bound to a solid support to capture virus-specific antibodies in a sample. Use of these inactivated viral reagents as resources for viruses requiring high level biological containment (biosafety level [BSL] 3 or 4) is challenging since their specialized production is laborious and dangerous. To utilize the assays outside of biological containment the virus targets must be inactivated, which requires extensive safety testing and sometimes destroys vital epitopes (Feng et al., 2011). Recombinant proteins are increasingly being used as substitutes for native virus and other native biologically relevant antigens, since they do not require specialized containment facilities for production. Viral surface glycoproteins are frequently immunodominant antigens and therefore immunoassay targets (Beck et al., 2015; Rodriguez-Martinez et al., 2015). Soluble recombinant glycoproteins are not without problems, since they often require truncation to facilitate soluble release of the protein, and epitope display can be flawed due to the lack of a membrane anchor or improper folding (Caliendo et al., 2013; Gaudin et al., 1999; Jose et al., 2009; Nguyen et al., 2017; Wang et al., 2014). For example, problems associated with recombinant expression of alphaviral E1/E2 heterodimeric glycoproteins are well known, and the use of whole virus antigen is recommended for antibody detection (Erasmus et al., 2015). Maintaining alphavirus antigenic integrity in immunodiagnostic assays is critical for accurate detection since glycoproteins play a dominant role in host immune response (Petitdemange et al., 2015; Smith et al., 2018; Weaver et al., 2012). Ideally, the glycoprotein antigenic target would be displayed in an authentic viral envelope structure while still being safe to use at the BSL-2 level. Self-assembling virus-like particles (VLPs) are a sustainable alternative to

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native virus or recombinant antigen. These particles are extremely safe since they contain no viral genome and can be versatile platforms for glycoprotein antigen presentation due to their ability to incorporate both homologous and heterologous glycoproteins during their egress through cellular membranes (Pitoiset et al., 2015; Srinivasan et al., 2014).

Not only is the design and development of reagents critical when designing sustainable immunodiagnostic assays, but equally important is the choice of platform. While traditional serological methods such as plaque reduction neutralization tests (PRNTs) and ELISA have served as a workhorse for diagnostic and serosurveillance efforts for decades, several immunoassay platforms have emerged to make sample analysis faster, more sensitive, and multiplexed at both point-of-care and centralized laboratories. One such system is the xMAP® technology on the MAGPIX® platform developed by Luminex Corporation (Austin, Texas USA) (Basile et al., 2013). It is similar to ELISA in that it detects a typical antigen/antibody interaction, but employs fluorescently labeled magnetic particles as a solid support. Compared to ELISAs, MAGPIX assays are much faster, have increased sensitivity, and better able to be multiplexed (O'Hearn et al., 2016; Satterly et al., 2016).

Herein, we outline the development of a multiplexable immunodiagnostic method, which pairs the antigenic integrity of a VLP with the sensitivity of the MAGPIX® platform, to serve as a tool for detection of anti-viral glycoprotein humoral responses in a serum sample (Fig. 1). As a pilot study, we focused on production, characterization, and optimization of alphavirus reagents, specifically the New World alphavirus Venezuelan equine encephalitis virus (VEEV) and the Old World alphavirus chikungunya (CHIKV). This proof-of-concept work highlights how VLPs coupled with multiplexed immunodiagnostic platforms offer an efficient, sustainable, and flexible immunoassay approach for detection of humoral response to viral infection.

2. Materials and methods

2.1. Antibodies and sera

Positive and negative control sera from NHPs vaccinated with VEEV plasmid DNA were generated as described previously (Dupuy et al., 2011). VEEV and CHIKV IgM and IgG positive human sera originated from either vaccination or natural infection. All normal human sera was obtained from healthy military volunteers or purchased from BioIVT (Hicksville, NY).

2.2. VLP production

Plasmids encoding codon-optimized glycoprotein genes (E3-E2-6K-E1) of VEEV IAB (strain Trinidad donkey) inserted into the mammalian expression vector pWRG7077 was previously described (Dupuy et al., 2018, 2011). For the construction of the Gag-encoding plasmid, the first 538 residues of murine leukemia virus (MLV) Gag-Pol ORF (GenBank: AF033811.1) were codon optimized, synthesized, and cloned into pWRG7077 using flanking 5' *Not*I and 3' *Bgl*II restriction sites relative to the transgene insert (Atum Inc, Menlo Park, CA, USA). HEK293 T cells were seeded in T150 flasks (Corning, Inc., Corning, NY, USA) and incubated at 37 °C with 5% CO_2 until reaching 70–80% confluency prior to transfection with 27 μg of pWRG7077-Gag and 9 μg of pWRG7077-VEEV plasmid DNA using Fugene 6 (Promega, Fitchburg, Wisconsin, USA) according to manufacturer's instructions. Cell supernatants were collected at 24 and 48 h post-transfection, pooled, clarified by centrifugation, and filtered through a 0.45 μm filter. VLPs were concentrated through a Centricon® filter unit with a 100-kDa cutoff (EMD Millipore, Burlington, MA, USA) according to manufacturer's instructions. VLPs were then pelleted through a 20% sucrose cushion in virus resuspension buffer (VRB; 130 mM NaCl, 20 mM HEPES, pH 7.4) by centrifugation for 2 h at 106,750 × g in an SW32 rotor at 4 °C. VLP pellets were resuspended overnight in VRB at 4 °C, pooled, and diluted ten-fold with VRB. The diluted VLPs were re-pelleted without a sucrose cushion as described above. VLPs were resuspended in 1/1,000 vol of VRB relative to starting supernatant and then stored at −80 °C.

CHIKV VLPs were produced in a manner previously described (Akahata et al., 2010). Briefly, a DNA construct encoding the capsid-E3-E2-6K-E1 structural protein genes of CHIKV (strain 0706aTw) was synthesized and cloned into pWRG7077. HEK 293 T cells were then transfected with 25 μg of the VLP construct using Fugene 6 as described earlier. Supernatants were harvested at 24, 48, and 72 h post-transfection and were purified. Protein concentration for all VLPs was determined by BCA assay (ThermoFisher, Waltham, MA, USA).

2.3. Conjugation of VLPs to magnetic microspheres

VEEV and CHIKV VLPs were conjugated to magnetic microspheres using the Luminex xMAP® antibody coupling kit (Luminex Inc., Austin, TX, USA) according to the manufacturer's instructions. Briefly, 100 μL of Magplex microspheres (12.5 × 10 (Caliendo et al., 2013) microspheres/mL) were washed three times using a magnetic microcentrifuge tube holder and resuspended with 480 μL of activation buffer. Then, 10 μL of both sulfo-*N*-hydroxysulfosuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) solutions

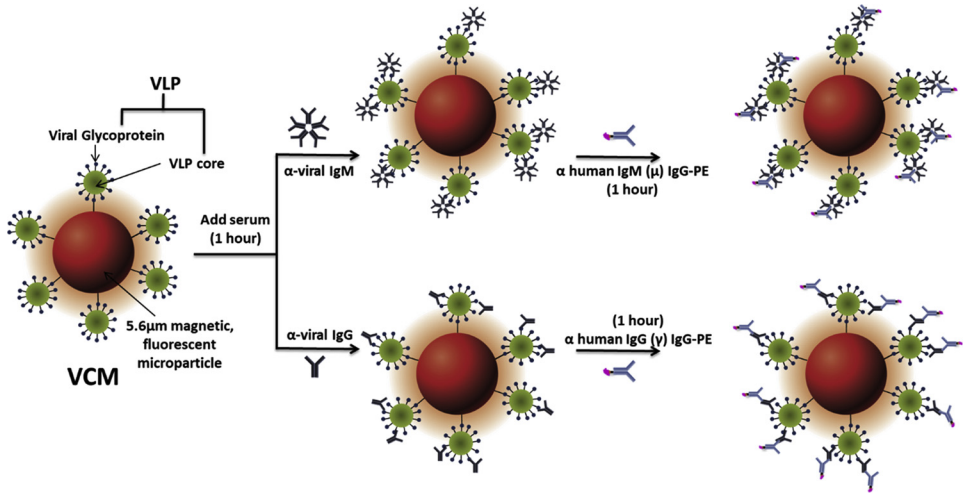

Fig. 1. General virus-like particles (VLP) conjugated microspheres (VCM) assay schematic for detection of both antiviral IgM and IgG responses in a serum sample.

were added to the resuspended microspheres. The tube was covered with aluminum foil and placed on a benchtop rotating mixer for 20 min. After surface activation with EDC, the microspheres were washed three times with activation buffer prior to adding the VLPs at a final concentration of 10 µg VLPs/1 × 10 (Caliendo et al., 2013) microspheres. The tube was again covered with aluminum foil and placed on a benchtop rotating mixer for 2 h. After this coupling step, the microspheres were washed three times with wash buffer and resuspended in wash buffer to the original stock concentration of 12.5 × 10 (Caliendo et al., 2013) microspheres/mL for further use. The VLPs were conjugated to Magplex microsphere bead sets #75 (VEEV) and #25 (CHIKV) (Luminex), in order to facilitate multiplexing experiments.

2.4. Detection of anti-viral IgG or IgM in NHP or human sera using VLP-coupled magplex microspheres

VCMs were diluted 1:250 in phosphate buffer saline (PBS) with 0.02% Tween-20 (PBST) and added to the wells of a Costar polystyrene 96-well plate at 50 µL per well (2500 microspheres/well). The plate was placed on a Luminex plate magnet, covered with foil, and microspheres were allowed to collect for 60 s. While still attached to the magnet, the buffer was removed from the plate by shaking. Then, 50 µL of test serum and appropriate negative NHP or human serum, diluted in PBST with 5% skim milk (PBST-SK), was added to the plate, covered, and incubated with shaking for 1 h at room temperature (RT). The plate was washed three times with 100 µL of PBST using the plate magnet to retain the Magplex microspheres in the wells and then 50 µL of a 1:100 dilution of goat anti-human IgG (H&L) phycoerythrin conjugate (Sigma-Aldrich, St. Louis, MO, USA) or goat anti-human IgM (anti-mu) phycoerythrin conjugate (Abcam, Cambridge, UK) in PBST-SK were added to the wells. These conjugates were used for both NHP and human samples, as the conjugates are known to cross-react between the two species. The plate was covered and incubated with shaking for 1 h at RT. After incubation, the plate was washed three times and the Magplex microspheres were resuspended in 100 µL of PBST for analysis on the MAGPIX®. For multiplexed experiments, the VLP-coupled microsphere sets were added to the plate so that 2500 microspheres of each VCM set were dispensed per well. The remainder of the multiplexed assay was performed as described above. All samples were run in triplicate and repeated on multiple days to ensure reproducibility. For sera from VEEV-infected NHPs, analysis was conducted in a BSL-3 suite with infected material handled in a Class II Biological Safety Cabinet.

2.5. Ethics statement

All human and NHP sera utilized in this study was acquired from existing collections at USAMRIID. Research on NHPs was conducted under an IACUC approved protocol, V05-17, in compliance with the Animal Welfare Act, PHS Policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Research on human subjects was conducted in compliance with DoD, Federal, and State statutes and regulations relating to the protection of human subjects, and adheres to principles identified in the Belmont Report (1979). All data and human subjects research were previously de-identified and given a “research not involving human subjects” determination by the USAMRIID Office of Human Use and Ethics, OHU&E Log Number FY17-26.

3. Results

3.1. Synthesis and characterization of VLPs

MLV-based VLPs were chosen for VEEV glycoprotein presentation because they are high yielding, homogenous, and can accommodate a wide range of glycoprotein antigens (Datta et al., 2011; Soares et al., 2016). Transient expression of two DNA plasmids encoding both VEEV E1/E2 and the first 538 amino acids of MLV Gag in mammalian cells generated highly homogenous particles presenting both the E1 and E2 VEEV glycoproteins on their surface as determined by electron microscopy (SI Fig. 1A). A molar ratio of 3:1 Gag plasmid to VEEV E1/E2 plasmid yielded the highest incorporation of the glycoproteins into the particles (SI Fig. 1B). As further proof that the MLV-based VEEV VLP glycoproteins were present in a native, functional conformation, we demonstrated successful entry of the VLPs into target cells. This entry was blocked by neutralizing polyclonal sera from VEEV E1/E2 vaccinated NHPs, further supporting the functional conformation of the VLP-embedded glycoproteins (SI Fig. 1C). CHIKV VLPs were developed using an alternate VLP architecture that is dependent on the ability of CHIKV capsid and envelope proteins to spontaneously drive VLP formation (Akahata and Nabel, 2012). Relying on this homogeneous approach, CHIKV VLPs were generated and characterized by both western blot and ELISA (data not shown).

3.2. Conjugation, characterization, and comparison to traditional ELISA of VEEV VCMs

VEEV VLPs were conjugated to Magplex microspheres using carbodimide coupling chemistry to covalently link the amine groups from the surface glycoproteins of the VLP to the carboxylate surface of the microparticle. Saturation of the particle surface with VLPs was observed at a concentration of 10 µg/million microspheres and was chosen as the standard loading concentration for the VCMs (SI Fig. 1D). Upon screening known anti-VEEV IgG positive NHP serum with the VCMs on the MAGPIX®, the limit of detection (LoD) was at a 1×10^5 dilution of serum in assay buffer (Fig. 2A). The change in signal was statistically significant relative to the same dilution of negative NHP sera (t-test; $p < 0.0001$). The VEEV VCM MAGPIX® assay was two orders of magnitude more sensitive toward IgG detection than traditional 96-well ELISA assays using inactivated TC-83 cell lysate or VEEV VLP direct capture antigens (Fig. 2B).

3.3. Characterization of alphavirus VCMs in Singleplex and multiplex formats

CHIKV VLPs were coupled to Magplex beads at a loading concentration of 10 µg/million beads and screened against known IgG positive NHP sera in a singleplex format (Fig. 2A). The limit of detection of the singleplex CHIKV VCM assay was similar to that observed in the pilot test with the VEEV VCMs at a 1×10^5 serum dilution. LoD was not affected by multiplexing the alphavirus VCMs and interrogating individual NHP sera (SI Fig. 2). At a 1:100 dilution of each convalescent NHP sera, some IgG crossreactivity was observed as signal on non-correlative bead sets was significantly above normal NHP serum (2-way ANOVA, 95% confidence interval [CI]). Much of this crossreactivity diminished at a 1:1000 dilution of sera in assay buffer (2-way ANOVA, 95% CI), while still maintaining significant signal for the VEEV and CHIKV specific bead sets. (SI Fig. 3).

3.4. Utility of alphavirus VCMs for detection of humoral IgM response in animal models

While the VCMs proved useful for detection of IgG in convalescent NHP sera, pathogen-based biomarker tracking and humoral response requires detection of antiviral IgM antibodies as well. The presence of

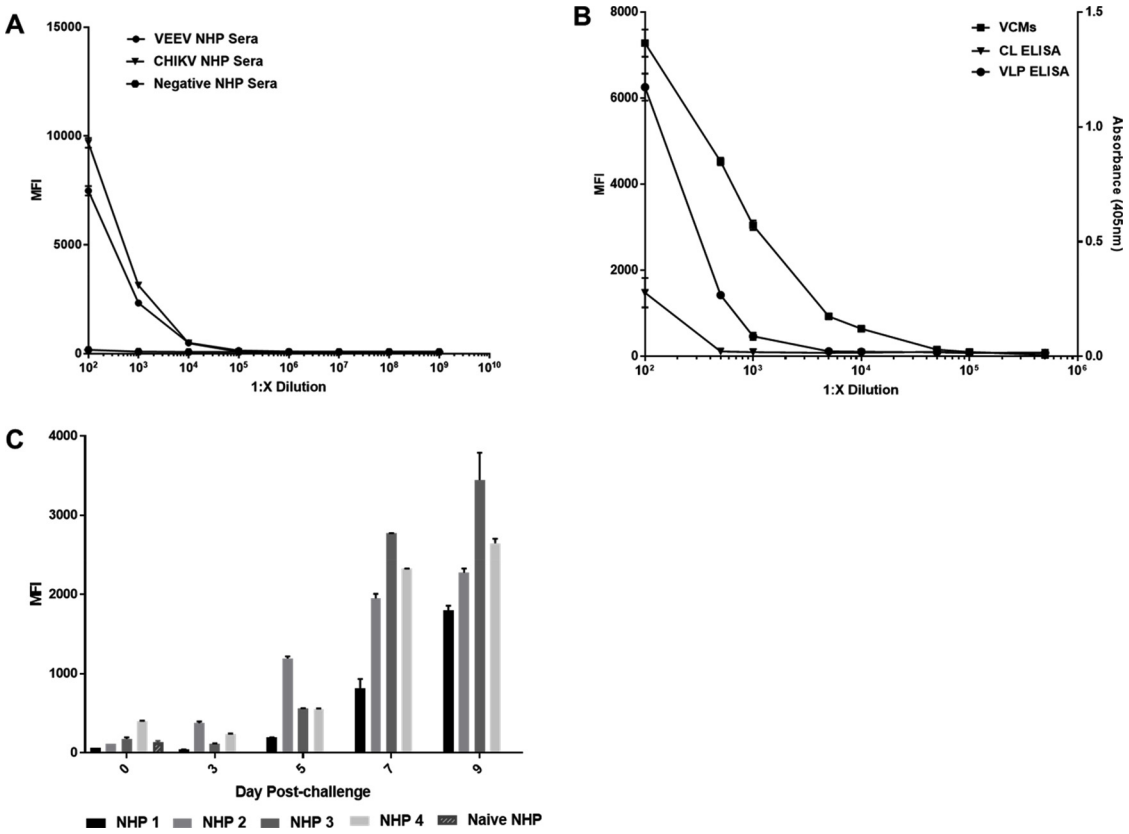

Fig. 2. (A) Limit of detection (LoD) of anti-VEEV IgG and CHIKV IgG in nonhuman primate (NHP) sera using the VEEV/CHIKV VCM MAGPIX assay. Signal was statistically significant ($p = 0.01$; unpaired t-test) over baseline to a dilution of 1×10 (Beck et al., 2015); (B) Comparison of VEEV VCM assay to traditional direct ELISA (based on infected cell lysate, CL, antigen) for detection of anti-VEEV IgG in NHP sera; (C) Detection of anti-VEEV IgM in positive NHP sera at several days post challenge. Error bars represent standard deviation.

pathogen-specific IgM represents the earliest antibody response of an organism to infection. As the course of infection progresses toward convalescence, the presence of IgM generally decreases as IgG rises to dominate the humoral response (Fields et al., 2007). VEEV NHP sera ($n = 4$) collected at multiple time points post-challenge were screened using the VEEV VCM assays. Anti-VEEV IgM response was observed at days 5, 7 and 9 post-infection (2-way ANOVA, 95% CI) (Fig. 2C) as compared to the day 0 time point.

3.5. Differential detection of old world versus new world alphaviruses in human sera

Human sera of known etiology (VEEV sera from vaccination studies, CHIKV sera from natural infections, and normal human sera from healthy donors confirmed previously by ELISA) were screened using VEEV and CHIKV VCM assays for the presence of anti-viral IgG and IgM antibodies. The VEEV and CHIKV singleplex assays were highly sensitive toward IgG and IgM detection in correlating human sera (Fig. 3). LoDs at a dilution of 1×10^5 and 1×10^4 were observed for VEEV IgG and IgM, respectively (t-test; $p < 0.0001$; 95% CI). Likewise, LoDs for CHIKV IgG and IgM fell at dilutions of 1×10^6 and 1×10^4, respectively (t-test; $p < 0.0001$; 95% CI). When these same IgG and IgM positive sera were screened in a duplex VEEV/CHIKV assay at a 1:100 dilution, there was clear preference of the IgG response from the CHIKV sera toward the corresponding VCM, with minimal crossreactivity with the VEEV VCM (2-way ANOVA, 95% CI) (Fig. 4A). No crossreactivity of the IgG response from the VEEV sera was observed with the CHIKV VCM. When a set of healthy human donor samples were tested with the duplex assay, no false positive results were observed (SI Fig. 4). When IgM positive sera was screened in the duplex assay, no crossreactivity was observed (2-way ANOVA, 95% CI) (Fig. 4B).

4. Discussion

In this proof of concept work, we developed a multiplexed immunodiagnostic method that demonstrates how particulate antigens can be integrated onto bead-based assays for detecting host antibody response to both vaccination and natural infection. We developed this methodology for detecting and discriminating between two arthropod-borne pathogens, VEEV and CHIKV, of the RNA-virus family *Togaviridae*. VEEV, a NIAID priority B pathogen with biodefense concerns, is a New World alphavirus that causes disease in equines and humans, whereas the Old World alphavirus CHIKV is a major global health concern due to its widespread dissemination and long-lasting disease burden (Weaver et al., 2012). While RT-PCR assays are useful for detection of acute alphavirus infection, viremia can be transient. In cases of CHIKV infection, PCR-detection reliability rapidly decreases 5 days after onset of symptoms and is supplanted by IgM detection (Marcus et al., 2006).

When incorporated into the MAGPIX® immunoassay platform, alphaviral VCMs were useful for detection of humoral responses in acute and convalescent serum samples. VEEV and CHIKV NHP sera, screened with VEEV or CHIKV VCMs, respectively yielded IgG LoDs at serum dilutions of 1×10^5 (Fig. 2A). This was a marked improvement over traditional ELISA, with a two-order of magnitude increase in IgG sensitivity (Fig. 2B), and the additional benefit of a reduction in time-to-answer (2 h by VCM versus 15+ h by ELISA). When assays were multiplexed for VEEV and CHIKV antibody detection, there was no observed loss in sensitivity with the combined assay components (SI Fig. 2). While some anti-glycoprotein IgG crossreactivity was observed when sera were screened with the duplex assay; this was to be expected since some alphavirus family members share regions of homology and possess conserved epitopes, the E1 glycoproteins in particular (Trent

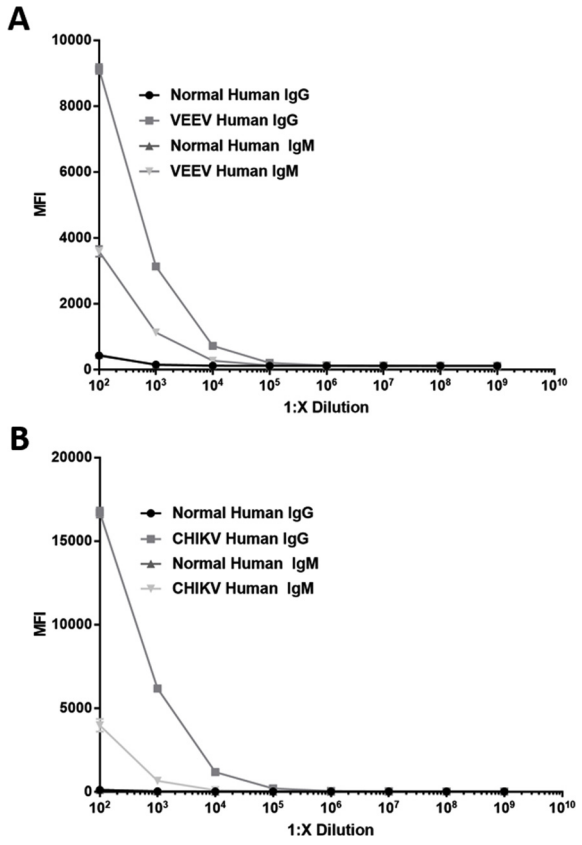

Fig. 3. Dynamic range for IgG and IgM detection in (A) VEEV and (B) CHIKV human clinical samples as compared to normal human samples. LoD for IgG was at a sample dilution of 1×10^5 for both VEEV and CHIKV. LoD for IgM was at a sample dilution of 1×10 (Banoo et al., 2019) for both. All assays were performed in triplicate. Error bars represent standard deviation.

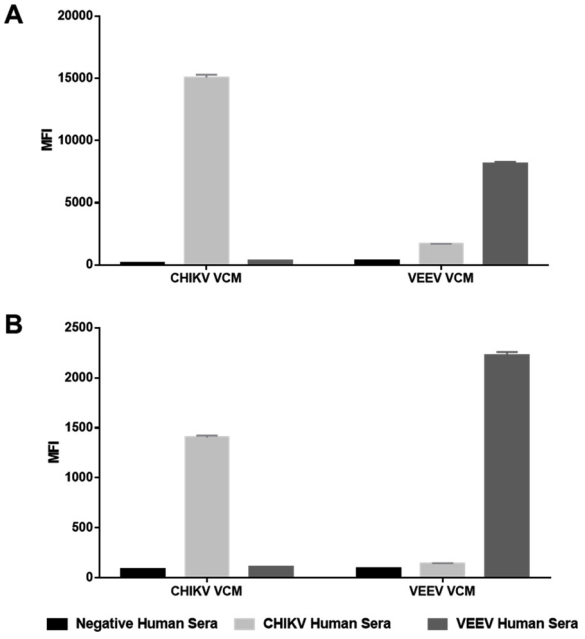

Fig. 4. Discrimination between Old World and New World alphaviruses for (A) IgG and (B) IgM detection from human clinical sera at a 1:100 dilution. All assays were performed in triplicate. Error bars represent standard deviation.

et al., 1984). IgM responses in acute NHP sera to VEEV challenge was observed starting at day 5 post-infection (Fig. 2C). Taken together, these results demonstrate the utility of this method for use in animal models to understand host response to a pathogen exposure or vaccination.

Acute and convalescent human sera, of known VEEV and CHIKV etiology, were screened with VEEV and CHIKV VCMs to demonstrate IgM and IgG detection in human clinical samples (Fig. 3). When utilizing the VEEV/CHIKV duplex, no crossreactivity was observed for IgM detection and only minimal crossreactivity for IgG detection (Fig. 4). This would suggest that differential IgM detection is achievable for identifying Old World versus New World alphaviruses, which would be significant in regions where both the equine encephalitic viruses and CHIKV are endemic. Pairing this multiplexed VCM immunoassay with molecular diagnostic methods in an integrated diagnostic approach, would lead to the highest confidence in specific pathogen identification in acute and early convalescent human clinical samples. While we do not currently have access to large cohorts of CHIKV and VEEV human serum samples from natural infection, we are working with overseas partners to gather both acute and convalescent samples and to further validate these assays for clinical diagnostic utility in the future. Part of this validation will include a larger comparison of matched serum samples using the discussed bead-based method to more traditional serologic approaches such as PRNTs and ELISA. Beyond its diagnostic utility, this assay approach would also be particularly desirable in scenarios in which the antibody response to a particulate vaccine (e.g. VLP or nanoparticle) is being evaluated. Numerous VLP-based vaccines have been introduced in recent years against a wide range of infectious viruses (Mohsen et al., 2017). Since the method described here detects host humoral response, whether that response originated from natural infection or vaccination, the particulate vaccine itself could be conjugated to magnetic beads for serum evaluation in a similar manner. Integration of VLPs into magnetic-bead based immunodiagnostic platforms provides a useful alternative to current 96-well plate ELISA methods used in both human and animal studies to provide a higher throughput, more sustainable, an potentially more sensitive route for detection of humoral response.

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Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.04.013.

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