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Neutralization assays for differential henipavirus serology using Bio-Plex Protein Array Systems

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Abstract

Hendra virus (HeV) and Nipah virus (NiV) are related emerging paramyxoviruses classified in the genus *Henipavirus*. Both cause fatal disease in animals and humans and are classified as biosafety level 4 pathogens. Here we detail two new multiplexed microsphere assays, one for antibody detection and differentiation and another designed as a surrogate for virus neutralization. Both assays utilize recombinant soluble attachment glycoproteins (sG) whereas the latter incorporates the cellular receptor, recombinant ephrin-B2. Spectrally distinct sG_{HeV}- and sG_{NiV}-coupled microspheres preferentially bound antibodies from HeV- and NiV-seropositive animals, demonstrating a simple procedure to differentiate antibodies to these closely related viruses. Soluble ephrin-B2 bound sG-coupled microspheres in a dose-dependent fashion. Specificity of binding was further evaluated with henipavirus G-specific sera and MAbs. Sera from henipavirus-seropositive animals differentially blocked ephrin-B2 binding, suggesting that detection and differentiation of HeV and NiV neutralizing antibodies can be done simultaneously in the absence of live virus.

Keywords: Hendra; Nipah; Envelope; Receptor; Multiplexed neutralization

1. Introduction

Nipah virus (NiV) and Hendra virus (HeV) are recently identified paramyxoviruses and are the prototypic members of the new genus *Henipavirus*. Paramyxoviruses are large, enveloped, negative-sense single stranded RNA viruses, and include members such as Measles virus, Mumps virus, and Respiratory syncytial virus (Lamb and Kolakofsky, 2001). It is a diverse virus family, with various members causing common upper and lower respiratory tract diseases and although less common, other members can cause neurological disease. In contrast, although closely related to each other, NiV and HeV are distinguished from all paramyxoviruses most notably by their broad species tropism and ability to cause fatal disease in animals and humans. HeV first appeared in Australia in 1994 and was transmitted from horses to humans (reviewed Murray et al., 1998); NiV emerged in 1998–1999 in peninsular Malaysia and primarily infected pigs and subsequently humans, however, several other animal species became infected (reviewed Chua, 2003). For both viruses amplification and disease in domestic animals commonly occur prior to transmission of the virus to humans, where infection is manifested as a severe respiratory illness and/or febrile encephalitis (Selvey et al., 1995; Tan and Wong, 2003; Wong et al., 2002). The natural reservoir host of HeV and NiV is believed to be fruit bats in the genus *Pteropus* (Chua et al., 2002; Halpin et al., 2000). In recent years, both viruses have continued to re-emerge; HeV reappeared in Australia in 1999, 2004, and 2006 with fatal infections in horses and one non-fatal, but sero-converting, human case (Anon., 2004a; McDonald, 2006; Westbury, 2000). In 2004 and 2005, there were three independent NiV outbreaks in Bangladesh (Anon., 2004b, 2005; Hsu et

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al., 2004). Significant observations in these outbreaks included a higher incidence of acute respiratory distress syndrome in conjunction with encephalitis, person-to-person transmission, and potentially higher case fatality rates (~75%). Furthermore, direct transmission of NiV from flying foxes to humans was suggested (Anon., 2004c).

Paramyxoviruses contain two major membrane-anchored envelope glycoproteins that are required for infection of a receptive host cell. All members contain an F glycoprotein which mediates pH-independent membrane fusion between the virus and its host cell, while the second is the attachment glycoprotein which binds the host cell receptor (reviewed in Lamb and Kolakofsky, 2001). Attachment glycoproteins are oligomeric type II membrane glycoproteins, and both dimeric and/or tetrameric (dimer of dimers) configurations exist (Crennell et al., 2000; Morrison, 1988; Russell et al., 1994; Sheehan et al., 1987). For HeV and NiV, the attachment proteins lack hemagglutinin and neuraminidase activities and are designated G. Recently, recombinant, soluble versions of the henipavirus G glycoproteins (sG) were generated which retained several important structural features, such as oligomerization and the ability to bind henipavirus host cell receptors (Bossart et al., 2005). When used as immunogens, homologous and heterologous anti-sG titers were significantly different (Bossart et al., 2005; Mungall et al., 2006), suggesting G may be an ideal antigen for serological differentiation of these two closely related viruses. Additionally, soluble HeV G elicited higher titers of cross-reactive neutralizing antibodies as determined by heterologous serum neutralization titer and may represent a viable vaccine candidate (Mungall et al., 2006).

Recently, ephrin-B2 and ephrin-B3 were identified as receptors employed by HeV and NiV for infection (Bonaparte et al., 2005; Negrete et al., 2005, 2006). Ephrin-B2 and ephrin-B3 are only the third and fourth host proteins to be identified as paramyxovirus receptors. Both are highly conserved across vertebrate species, expressed in multiple organ systems and are members of a family of receptor tyrosine kinase ligands (Drescher, 2002; Poliakov et al., 2004). Ephrin-B2 and ephrin-B3 have been researched extensively for their role in cancer biology (Castellano et al., 2006; Martiny-Baron et al., 2004; Masood et al., 2005; Nakada et al., 2006) and as a consequence soluble recombinant versions are readily available. When initially discovered as the henipavirus receptor, the affinity of soluble ephrin-B2 for the HeV attachment glycoprotein was demonstrated using Biacore surface plasmon resonance (Bonaparte et al., 2005). More recently, the affinities of ephrin-B2 and ephrin-B3 for the NiV attachment glycoprotein were characterized and when compared, ephrin-B3 had a relatively lower affinity for the attachment glycoprotein but still permitted virus entry (Negrete et al., 2006).

NiV and HeV are classified as biosafety level 4 (BSL4) viruses and as they continue to re-emerge, the ability to diagnose infection becomes critical; however, serological test requiring live virus can be done in only a small number of BSL4 laboratories world wide. Here we combined the use of the soluble attachment glycoproteins, their receptor and a multiplex microsphere platform to develop new assays capable of measuring both virus-specific and neutralizing antibodies. Specifically, we coupled soluble HeV and NiV G (sG_{HeV} and sG_{NiV}) to different microspheres for use on the Bio-Plex Array platform. Henipavirus specific antibodies in sera from seropositive animals were detected and differentiated in a single test. Soluble ephrin-B2 bound both sG_{HeV}- and sG_{NiV}-coupled beads in a dose-dependent fashion. Ephrin-B2 appeared to bind sG_{NiV} more efficiently suggesting that NiV G may have a higher affinity for the host cell receptor. HeV and NiV G-specific sera and monoclonal antibodies (MAbs) were evaluated for their binding to sG and their ability to compete with ephrin-B2 for sG binding. Seropositive sera from different species, including horse and pig field sera, differentially blocked receptor binding to sG_{HeV} and sG_{NiV}, further demonstrating the presence of potentially neutralizing antibodies as well as their specificity. To our knowledge, this is the first report of multiplexed binding and pseudo-neutralization assays that use only recombinant proteins and for HeV and NiV represent a significant advance in serological capability.

2. Materials and methods

2.1. Multiplex microsphere assay equipment, software and calibration

Assays were performed on a Bio-Plex Protein Array System integrated with Bio-Plex Manager Software (v 3.0) (Bio-Rad Laboratories, Inc., CA, USA). The high setting was used for the reporter target channel (RP1) and fluorescent identification of microspheres. Reporter conjugate emission wavelengths were maintained using a Bio-Plex Calibration Kit (Bio-Rad, cat. no. 171-203060). Consistent optical alignment, fluidics performance, doublet discrimination and identification of individual bead signatures were assured using a Bio-Plex Validation Kit (v 3.0) (Bio-Rad, cat no. 171-203000). The coefficient of variation for bead discrimination and reporter channel identification did not exceed 7.0 and 8.0%, respectively.

2.2. Coupling of purified sG_{HeV} and sG_{NiV} glycoproteins to microspheres

Purified sG_{HeV} and sG_{NiV} were prepared as previously described (Bossart et al., 2005; Mungall et al., 2006). Briefly, HeLa-USU cells were infected with recombinant vaccinia viruses that encoded either S-peptide epitope tagged sG_{HeV} (vKB16) or S-peptide epitope tagged sG_{NiV} (vKB22). Culture supernatants were harvested 36 h post-infection and clarified by centrifugation. sG_{HeV} and sG_{NiV} were affinity purified from the culture supernatants by their S-peptide tags and S-protein columns. Purified proteins were further passed over a Superdex 200 gel filtration column and dimer-containing fractions were collected, pooled and protein concentrations were determined (Bossart et al., 2005). 30 μg of purified sG_{HeV} or sG_{NiV} was coupled to 1×10^6 carboxylated (COOH) microspheres, subsets #24 and #42, respectively (Bio-Rad Laboratories, Inc.). For microsphere activation, each subset was added to one well of a pre-wet (Ca^{2+}/Mg^{2+} free PBS (PBSA)) MultiScreen-BV

1.2 μm hydrophilic, low protein binding, 96-well filter plate (Millipore Australia Pty. Ltd., North Ryde, NSW, Australia). Liquid was removed using the vacuum manifold and microspheres were washed twice in activation buffer (Amine Coupling Kit, Bio-Rad Laboratories, Inc.) Subsequently, microspheres were incubated in activation buffer containing 5 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) (Pierce, Rockford, IL, USA) and 5 mg/ml *N*-hydroxysulfosuccinimide (*S*-NHS) (Pierce) for 20 min at room temperature with shaking in the dark. Liquid was removed using the vacuum manifold and 30 μg of sG_{HeV} or sG_{NiV} was added to microspheres, subsets #24 and #42, respectively, in 100 μl of PBSA. Microspheres and antigen were incubated for 2 h at room temperature with shaking in the dark. Microspheres were washed twice with 0.05% Tween-20/PBSA and removed from the filter plate by resuspension in 600 μl of storage buffer (1% BSA/PBSA/0.05% sodium azide containing protease inhibitor cocktail) (Roche Diagnostics Australia Pty. Ltd., Castle Hill, NSW Australia).

2.3. Animal sera

Peptide-specific HeV and NiV G rabbit sera have been described previously (Bossart et al., 2001; Bossart et al., 2002). Rabbit 405 HeV antiserum has been described previously (Bossart et al., 2005). All human, horse, pig and cat sera used in Figs. 1 and 5 were from naturally or experimentally infected individuals and were gamma-irradiated prior to use. All horse sera were from infected animals and were gamma-irradiated prior to use. All other pig and bat sera were diagnostic samples submitted to the Australian Animal Health Laboratory (AAHL) and were heat inactivated at 56 °C for 30 min prior to use. Diagnostic sera samples that contained henipavirus antibodies as measured by SNT were defined as seropositive however; whether these animals were infected remains unknown. For multiplexed differentiation of henipavirus antibodies, human, bat, cat and pig sera were used at a dilution of 1:250. HeV-positive horse and rabbit 405 sera concentrations were adjusted to 1:500 and 1:1000,

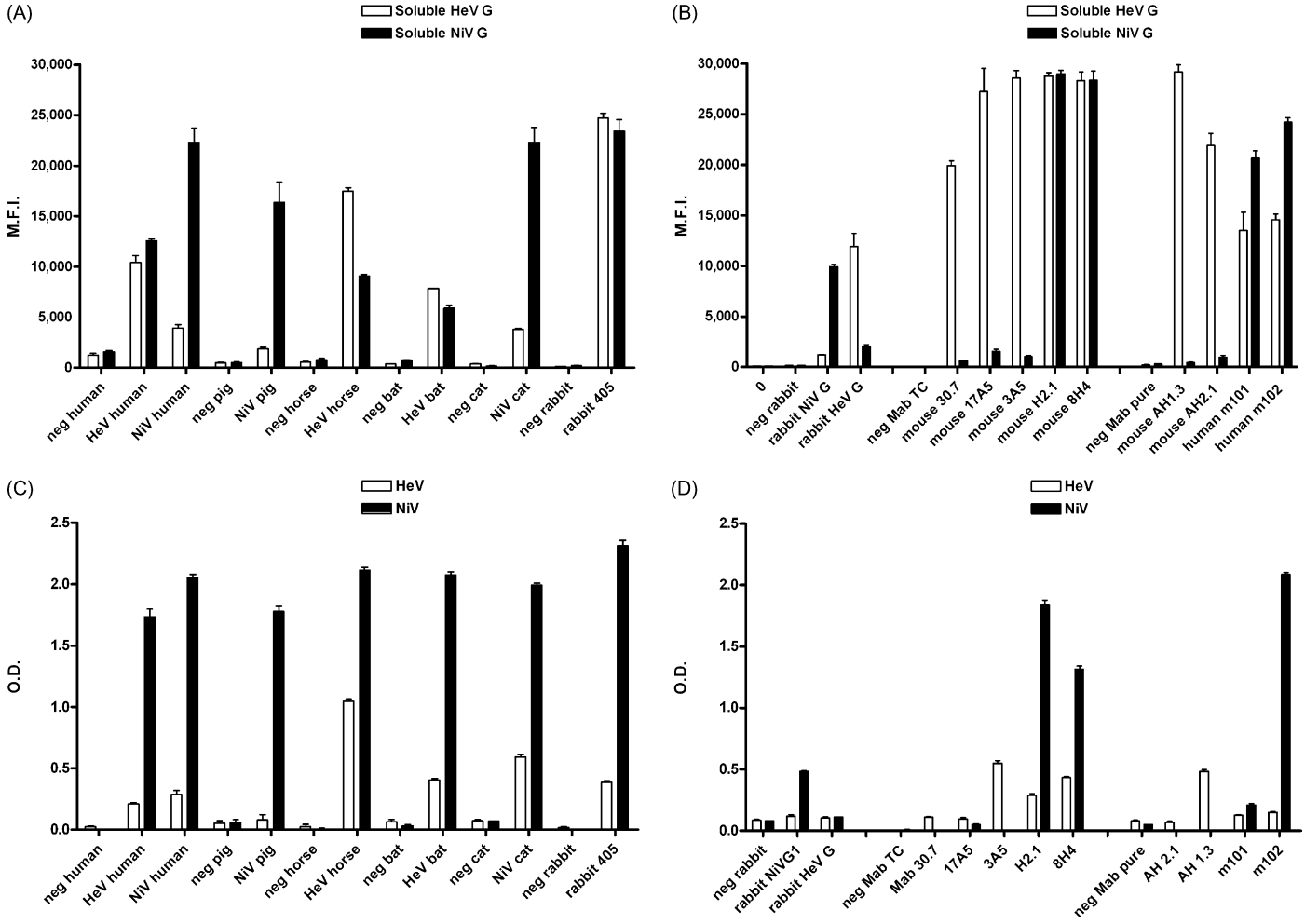

Fig. 1. Differential binding of henipavirus antibodies to sG_{NiV} and sG_{HeV}. Antisera from henipavirus-infected humans and animals (panel A) or peptide-specific sera and MAbs (panel B) were assayed using the antibody detection multiplexed microsphere assay as detailed in Section 2. Median fluorescence intensities (M.F.I.) are shown for each microsphere population: sG_{HeV} (white bars) and sG_{NiV} (black bars). Antisera from henipavirus seropositive humans and animals (panel C) or peptide-specific sera and MAbs (panel D) were evaluated using two ELISAs and optical densities are shown; HeV (white bars) and NiV (black bars). For all panels HeV or NiV infection and species of sera and MAbs are indicated on the *x*-axis labels. Rabbit 405 was a hyper immune serum generated against sG_{HeV} and was used as a positive control. All multiplexed microsphere assays were done in triplicate; the average M.F.I. is shown for each microsphere subset and error bars represent the range of M.F.I. for each microsphere subset. All ELISAs were done in duplicate, average optical densities are shown with standard deviations.

respectively, to achieve optimal differentiation. Peptide-specific rabbit sera concentrations were adjusted to 1:20 to increase detection. Murine MAbs to hog cholera virus (HCV) and anti-HeV MAbs 30.7, 17A5, 3A5, H2.1, and 8H4 concentrated tissue culture (TC) supernatants were used at a dilution of 1:250. Due to their high level of purity, human MAbs and murine MAbs AH1.3 and AH2.1 were used at a dilution of 1:1000. For most ephrin-B2 blocking assays a 10-fold increase of sera or MAbs were used as compared with the antibody detection assay. Human, pig, cat, and bat sera, TC MAbs, purified MAbs, HeV-positive horse sera, rabbit 405 serum and peptide-specific rabbit sera were used at dilutions of 1:25, 1:25, 1:100, 1:50, 1:100 and 1:20, respectively. Negative horse and pig sera were used at dilutions of 1:100 and 1:25, respectively. Tissue culture supernatants for HCV murine MAb and purified SARS S human MAb were used as negative controls at 1:25 and 1:100, respectively. For sensitivity and specificity assays, sera were assayed at dilutions ranging from 1:250 to 1:64,000 in antibody detection assays and from 1:25 to 1:8000 in receptor inhibition assays.

2.4. Multiplex microsphere assays

96-well filter plates were pre-wet with PBSA. Test sera, biotinylated Protein A and Protein G, and streptavidin–phycoerythrin were diluted in PBSA. For ephrin-B2 binding, soluble biotinylated mouse ephrin-B2 (R&D systems, Minneapolis, MN, USA) was diluted in PBSA. For each multiplexed assay, sG-coupled microspheres were vortexed and sonicated for 1 min. sG_{HeV}- and sG_{NiV}-coupled microspheres were mixed after sonication such that all assays were multiplexed. PBSA was removed from 96-well filter plates using the vacuum manifold and 100 μl of PBSA containing 1500 microspheres of each bead set was added per well. Buffer was removed using the vacuum manifold. For detection of henipavirus antibodies, 100 μl of sera was added to appropriate wells and incubated with the microspheres for 30 min at room temperature with shaking in the dark. Liquid was removed using the vacuum manifold and 100 μl of a mixture of biotinylated Protein A (1:500) and biotinylated Protein G (1:500) (Pierce) was added to each well and incubated for 30 min as described above. For murine MAbs, biotinylated goat anti-mouse (1:500) (Pierce) in PBSA was used instead of Protein A and Protein G. Liquid was removed using the vacuum manifold and 100 μl of streptavidin–phycoerythrin (1:1000) (Qiagen, Doncaster, Vic., Australia) was added to each well and incubated for 30 min as described above. 30 μl PBSA was added to each well. Samples were assayed for median fluorescence intensities (M.F.I.) using a protocol template for microsphere sets #24 and #42 on the Bio-Plex Protein Array System. For ephrin-B2 binding, 100 μl of soluble biotinylated ephrin-B2, ranging in concentration from 250 to 1.25 ng/ml, was added to microsphere containing wells and incubated for 30 min at room temperature with shaking in the dark. Liquid was removed using the vacuum manifold and 100 μl of streptavidin–phycoerythrin (1:1000) was added to each well and incubated for 30 min as described above. 30 μl PBSA was added to each well and samples were assayed as described above. For inhibition of ephrin-B2 binding, 100 μl of sera was added to appropriate wells and incubated for 30 min at room temperature with shaking in the dark. Liquid was removed using the vacuum manifold and 100 μl of 125 ng/ml soluble biotinylated ephrin-B2 was added and incubated for 30 min as described above. Liquid was removed using the vacuum manifold and 100 μl of streptavidin–phycoerythrin (1:1000) was added to each well and incubated 30 min as described above. A 30 μl PBSA was added to each well and samples were assayed as described above. For clarity, all inhibition of ephrin-B2 binding results are shown as percent inhibition which were calculated for each microsphere population independently using the following equations: $(1 - \text{M.F.I. serum/M.F.I. negative serum}) \times 100$ or $(1 - \text{M.F.I. MAb/M.F.I. negative MAb}) \times 100$.

2.5. HeV and NiV ELISAs

Current henipavirus diagnostic ELISAs have been previously described (Daniels et al., 2001). Briefly, gamma-irradiated whole cell lysates from HeV-or NiV-infected Vero cells were prepared by the Diagnosis, Surveillance and Response (DSR) group at AAHL and were donated to this study for use as ELISA antigens. All sera were tested at 1:100. All sera and human MAbs were detected with a Protein A/G-HRP conjugate; murine MAbs were detected with an anti-mouse-HRP conjugate. All ELISAs were developed using TMB substrate.

2.6. HeV and NiV serum neutralization test (SNT)

For SNT, all live virus experiments were conducted under strict bio-containment procedures in a BSL-4 laboratory. Sera were diluted by doubling dilution starting at 1:10. Sera were added to wells in quadruplicate in a 96 well plate followed by 50 μl virus containing 200 $TCID_{50}$ of either HeV or NiV and incubated at 37 °C for 30 min. Vero cells were added and plates were incubated at 37 °C for 4 days in a humidified 5% CO_2 atmosphere. Serum neutralization titers were determined by presence of cytopathic effect (CPE) and recorded as the serum dilution where no viral CPE was evident. All MAbs and peptide-specific sera SNTs were done within the same week as multiplexed microsphere assays. The polyclonal sera used in these studies were tested by SNT upon receipt by AAHL and were not retested recently. The HeV-specific human, bat and horse sera were tested only in HeV SNT because their submission was prior to the emergence of NiV.

2.7. Real-time measurement of G-receptor interaction by surface plasmon resonance (SPR)

Kinetic analysis of sG_{NiV} and sG_{HeV} binding to ephrin-B2 was done using a Biacore 1000 instrument (Biacore, Pharmacia, Piscataway, NJ). Soluble mouse ephrin-B2 (R&D systems) was immobilized covalently on a CM5 sensor chip using carbodiimide coupling chemistry with optimized surface density to minimize mass transfer. A reference control surface was prepared for non-specific binding and changes in buffer refractive index. Varying concentrations of affinity purified sG_{NiV} and sG_{HeV} (6–0.75 nM) were injected over each flow cell at a flow rate of 30 μl/min using running buffer containing 150 mM

NaCl, 3 mM EDTA, and 0.005% P-20 (pH 7.4). After completion of each association and dissociation cycle, the surface of each flow cell was pulsed with regeneration solution (0.1 M HCl). All the experiments were performed at 25 °C. The association and dissociation phase data were fitted simultaneously to calculate association rates (k_a), dissociation rates (k_d) and affinity constants (K_D) using the non-linear data analysis program BIAevaluation 3.2 (Biacore AB, Uppsala, Sweden).

3. Results

3.1. Multiplexed antibody detection using sG_{NiV} and sG_{HeV}

Previous studies have demonstrated that HeV and NiV antisera cross neutralize, with each serum being slightly less effective against the heterotypic virus (Berhane et al., 2006; Crameri et al., 2002; Tamin et al., 2002). Additionally, we have demonstrated that antibodies raised against sG_{HeV} were more effective in neutralizing HeV than NiV (Bossart et al., 2005). In more recent immunization studies, sG_{NiV}- and sG_{HeV}-specific cat sera were less effective in SNT against heterotypic virus (Mungall et al., 2006). To develop a test that differentiated antibodies to HeV and NiV and incorporated both sG_{NiV} and sG_{HeV} we sought a technology that could be multiplexed and subsequently chose a microsphere assay based on Luminex® technology.

Luminex® technology utilizes spectrally distinct microsphere subsets that excite at one wavelength but emit at slightly different wavelengths such that individual microsphere subsets can be identified, gated and quantified. The incorporation of different microsphere subsets within an individual test gives rise to a multiplexed assay. Purified sG_{NiV} and sG_{HeV} were coupled to two different microsphere subsets under identical conditions as described in Section 2. For all test samples, sG_{NiV}- and sG_{HeV}-coupled microsphere subsets were pre-mixed and incubated with sera or MAbs, biotinylated Protein A/G and streptavidin–phycoerythrin. Samples were measured and analyzed using the Bio-Plex Protein Array System as described in Section 2. Median fluorescence intensities (M.F.I.) for mixed microsphere subsets were calculated independently from 100 detected events per microsphere subset and multiplexed results are shown in Fig. 1. Because henipaviruses exhibit a broad species tropism we selected sera from a range of infected species as well as the natural reservoir, and evaluated their preferential binding to either HeV or NiV G (Fig. 1A). Additionally, to examine the specificity of the multiplexed microsphere platform, we assayed peptide-specific sera and MAbs with known G-binding preferences (Fig. 1B). All negative sera and MAbs had very low M.F.I. values; whereas, all HeV and NiV-specific sera and MAbs had high M.F.I. values. Importantly, sera bound differentially to sG_{HeV} or sG_{NiV} in accordance with the virus which elicited the antibody response, with one exception, the HeV-specific human serum, which bound both antigens similarly. All MAbs bound in accordance with previously published specificity (Bossart et al., 2001; Bossart et al., 2002; White et al., 2005; Zhu et al., 2006). In parallel we tested all sera and MAbs in HeV- and NiV-specific ELISAs in order to compare the multiplexed microsphere assay with current antibody detection diagnostic tests. The HeV and NiV ELISA antigens were prepared by the DSR team at AAHL; however, when assayed side-by-side, the ELISAs were incapable of antibody differentiation; all sera, regardless of origin, had higher optical densities in the NiV-specific ELISA (Fig. 1C). The ELISA antigens are routinely prepared from gamma-irradiated crude lysates of HeV- or NiV-infected cells and because the amount of virus can vary, direct comparisons of HeV and NiV-specific ELISA results are difficult. With few exceptions, the peptide-specific sera and MAbs bound poorly in both ELISAs (Fig. 1D). Due to the nature of the ELISA antigens, the relevant MAb epitopes were most likely masked.

Next we examined different field sera known to be positive for the presence of henipavirus antibodies by SNT; specifically, we tested sera from infected horses from the 1994 HeV outbreak in Australia and pig sera from the 1998 to 1999 NiV outbreaks in Malaysia and Singapore. All sera were assayed in the new multiplexed microsphere assay and the HeV- and NiV-specific ELISAs and results are shown in Figs. 2 and 3. Using the multiplexed microsphere assay, all of the HeV-infected horses had antibodies that preferentially bound sG_{HeV} with exception of horse 94-4 whose antibody levels were only slightly higher than the negative horse serum control (Fig. 2A). Interestingly, this particular horse serum had been shown previously to have very low levels of antibodies as determined by ELISA and SNT (Selleck, personal communication). All of the HeV-specific horse sera preferentially bound NiV in the ELISAs (Fig. 2B) again demonstrating the problems with the current diagnostic ELISAs. All pig sera, from peninsular Malaysia and Singapore, preferentially bound sG_{NiV} with limited cross reactivity to sG_{HeV} (Fig. 3A). Similar observations were made in ELISA (Fig. 3B).

Collectively, these data demonstrated the use of sG as suitable antigens for henipavirus differential serology and established the multiplex microsphere assay as a viable platform for detecting antibodies to either HeV or NiV G simultaneously.

3.2. Receptor binding to sG_{NiV} and sG_{HeV}

Recently, we demonstrated that ephrin-B2 bound sG_{NiV} or sG_{HeV} in an ELISA format (Bonaparte et al., 2005). We further investigated this interaction using the multiplexed microsphere assay which compared binding of ephrin-B2 to sG_{NiV} and sG_{HeV} simultaneously. The binding of soluble biotinylated ephrin-B2 to sG_{NiV}- and sG_{HeV}-coated microspheres was detected using streptavidin–phycoerythrin. As demonstrated in Fig. 4, soluble ephrin-B2 bound both sG_{NiV} and sG_{HeV} in a dose-dependent fashion with an apparent detection limit of 5 ng/ml. At concentrations higher than 125 ng/ml, there were no significant increases in M.F.I. for sG_{NiV} or sG_{HeV} (data not shown), thus 125 ng/ml was chosen as the optimal concentration for receptor-sG based multiplexed assays. Regardless of the ephrin-B2 concentration, ephrin-B2 bound sG_{NiV} better than sG_{HeV}. To quantify the affinity and the kinetics of the ephrin-B2-G glycoprotein interaction, soluble proteins were analyzed by a Biacore surface plasmon resonance assay over a similar range of concentrations. sG_{NiV} had about two-fold higher affinity for ephrin-B2 (equilibrium dissociation constant $K_D = 0.3$ nM, $k_a = 1.4 \times 10^6\ M^{-1}\ s^{-1}$ and $k_d = 4.1 \times 10^{-4}\ s^{-1}$), while sG_{HeV}

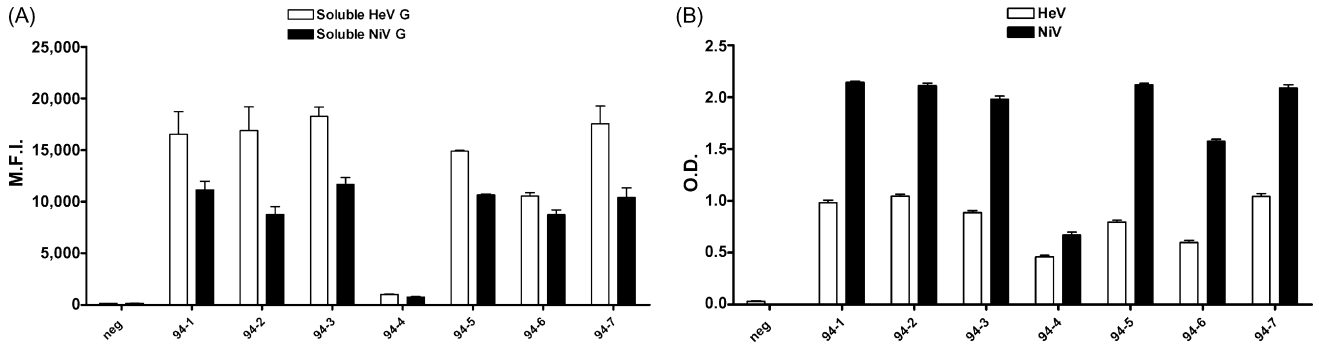

Fig. 2. Detection of HeV-specific antibodies in horse field sera. Horse sera were assayed using the antibody detection multiplexed microsphere assay (panel A) or the HeV and NiV ELISAs (panel B) as described in Section 2. For panel A, median fluorescence intensities (M.F.I.) are shown for each microsphere population; sG_{HeV} (white bars) and sG_{NiV} (black bars); for panel B, optical densities are shown; HeV (white bars) and NiV (black bars). All multiplexed microsphere assays were done in triplicate; the average M.F.I. is shown for each microsphere subset and error bars represent the range of M.F.I. for each microsphere subset. All ELISAs were done in duplicate, average optical densities are shown with standard deviations.

bound to ephrin-B2 with $K_D = 0.5$ nM, $k_a = 6.4 \times 10^5\ M^{-1}\ s^{-1}$ and $k_d = 3.5 \times 10^{-4}\ s^{-1}$ (Supplementary Fig. 1). These results were consistent with the multiplexed assay data and verified that the increased binding of sG_{NiV} to soluble ephrin-B2 was not an artifact of the assay system.

3.3. Establishment of a surrogate neutralization assay based on sG-receptor interactions

In most instances, it is the envelope glycoproteins of viruses to which virtually all neutralizing antibodies are directed (Quinnan,

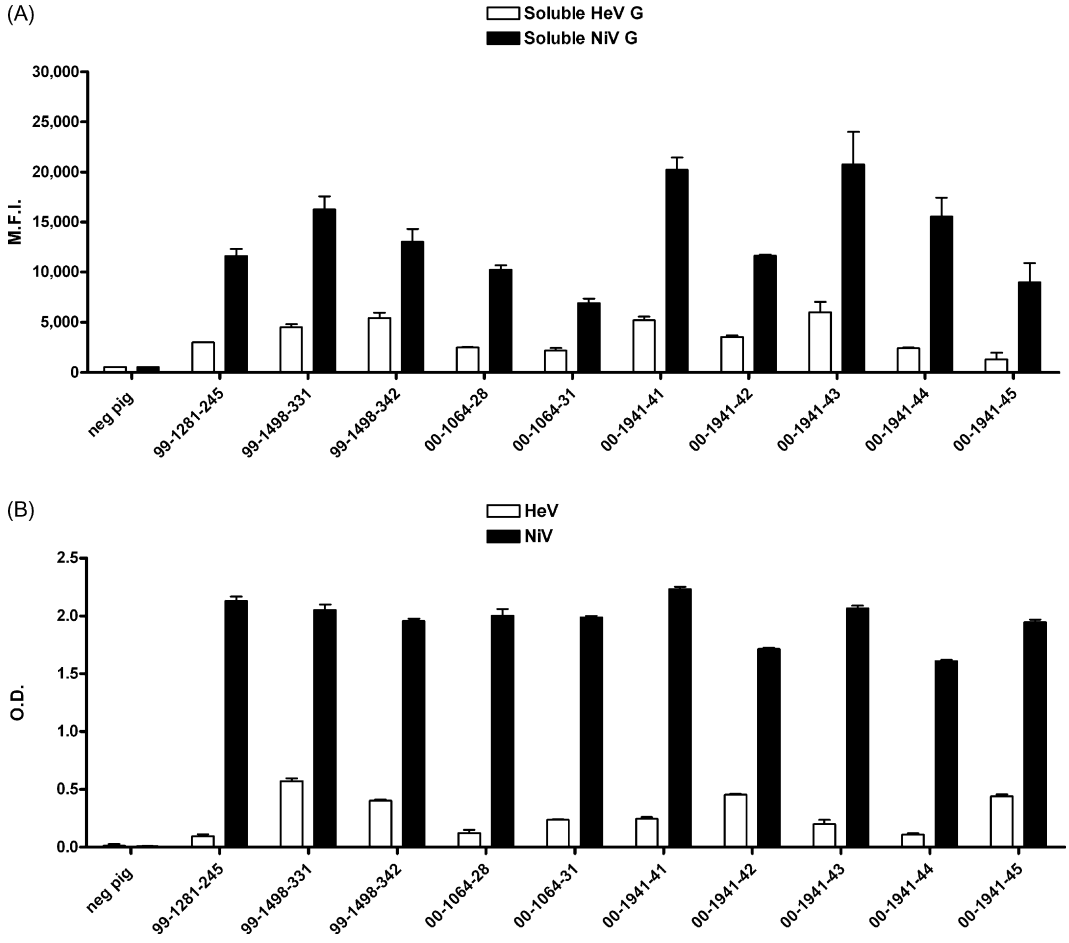

Fig. 3. Detection of NiV-specific antibodies in pig field sera. Pig sera were assayed using the antibody detection multiplexed microsphere assay (panel A) or the HeV and NiV ELISAs (panel B) as described in Section 2. For panel A, median fluorescence intensities (M.F.I.) are shown for each microsphere population; sG_{HeV} (white bars) and sG_{NiV} (black bars); for panel B, optical densities are shown; HeV (white bars) and NiV (black bars). All multiplexed microsphere assays were done in triplicate; the average M.F.I. is shown for each microsphere subset and error bars represent the range of M.F.I. for each microsphere subset. All ELISAs were done in duplicate, average optical densities are shown with standard deviations.

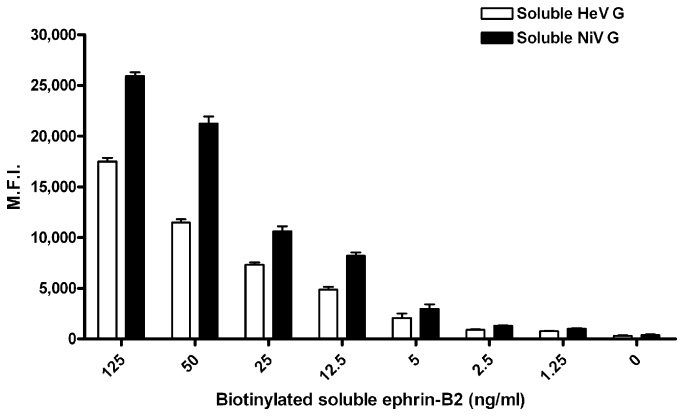

Fig. 4. Multiplexed detection of soluble ephrin-B2 binding. Biotinylated soluble ephrin-B2 was incubated with sG_{HeV} and sG_{NiV}-coated microspheres over a range of concentrations as detailed in Section 2. Median fluorescence intensities (M.F.I.) are shown for each microsphere population; sG_{HeV} (white bars) and sG_{NiV} (black bars). All assays were done in triplicate; the average M.F.I. is shown for each microsphere subset and error bars represent the range of M.F.I. for each microsphere subset.

1997). Because receptor engagement is a crucial step in henipavirus entry, antibodies that target the receptor binding domain of the G glycoprotein, or bind elsewhere on the protein in such a manner as to interfere with the G glycoprotein-receptor interaction, are most likely potent neutralizers of virus. In support of this notion, two human recombinant MAbs, m101 and m102, which were potent neutralizers of HeV and NiV in the traditional SNT (Zhu et al., 2006), were mapped to the receptor binding domain of the G glycoprotein (Bonaparte et al., 2005).

Since sG-ephrin-B2 interactions were readily detected using the multiplex microsphere assay, we hypothesized that sera that blocked these interactions were neutralizing and that our sG-ephrin-B2 assay could be modified to act as a surrogate neutralization assay. To investigate the specificity of such an assay, particular sera and MAbs were selected for analysis based on their known abilities to bind HeV and NiV G, as determined by the antibody detection microsphere assay, their varied neutralization profiles, and their mechanism of neutralization. Specifically, sera generated against HeV and NiV G peptides were chosen as both bound either HeV or NiV G, respectively, and neither were capable of neutralization in SNT (Bossart et al., 2001; Bossart et al., 2002). Murine MAbs 30.7 and AH2.1 bound HeV but did not neutralize; MAbs 17A5, 3A5, and AH1.3 bound HeV and neutralized HeV; and MAbs H2.1 and 8H4 bound HeV and NiV but only neutralized HeV. None of the murine MAb epitopes mapped to the speculated receptor binding domain of HeV G (White et al., 2005) and therefore would not be expected to interfere directly with receptor binding. Human MAbs, m101 and m102, were evaluated because m101 bound HeV and NiV G but only neutralized HeV, whereas m102 bound and neutralized both HeV and NiV. Binding of all sera and MAbs to sG_{NiV} and sG_{HeV} was demonstrated by the antibody detection microsphere assay (Fig. 1B). The ability of the same sera and MAbs to interfere with ephrin-B2 binding is shown in Fig. 5. Collectively, although all peptide-specific sera and MAbs bound sG_{NiV} or sG_{HeV}, only MAbs m101 and m102 significantly inhibited receptor binding (Fig. 5A). Importantly, antibody binding did not non-specifically interfere with ephrin-B2 binding. MAb m101 inhibited 69% of the ephrin-B2-sG_{HeV} interaction, and had only a negligible effect on the ephrin-B2-sG_{NiV} interaction. MAb m102 was capable of inhibiting 89% of ephrin-B2 binding to both sG_{HeV} and sG_{NiV}. The ability of m101 to block ephrin-B2-sG_{HeV} but not ephrin-B2-sG_{NiV} provided further evidence of the high specificity of the G-ephrin-B2 interaction.

We next examined sera from different species to determine if the subpopulation of antibodies that blocked receptor binding were also present in infected hosts and the natural reservoir.

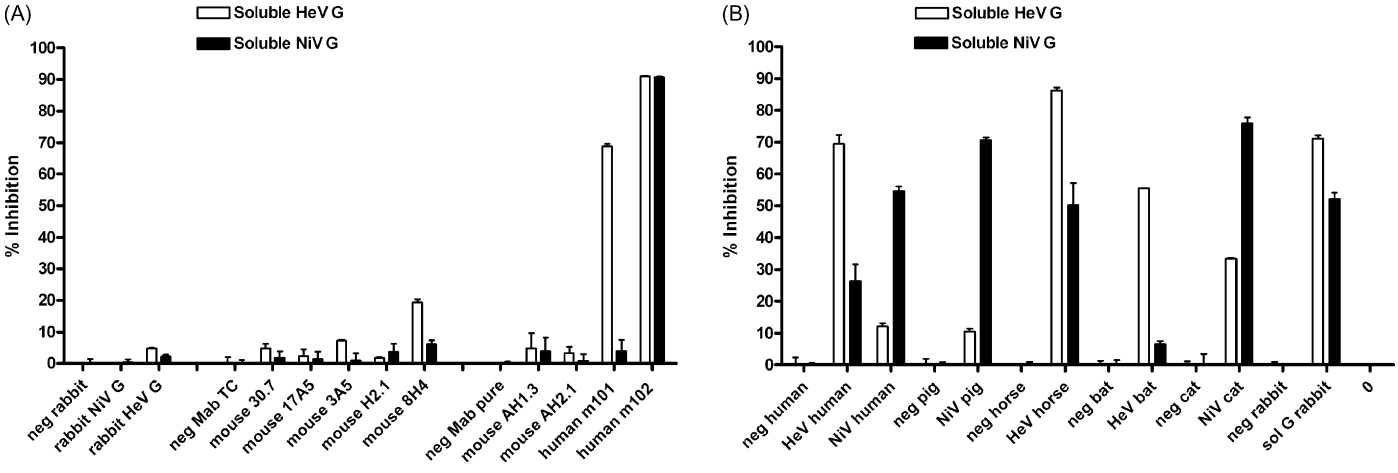

Fig. 5. Specificity of ephrin-B2 binding. Peptide-specific sera and MAbs (panel A) or antisera from henipavirus-infected humans and animals (panel B) were assayed using the inhibition of ephrin-B2 binding multiplexed microsphere assay as detailed in Section 2. The inhibition of ephrin-B2 binding was calculated as a percent inhibition for each microsphere population independently using the equations detailed in Section 2; sG_{HeV} (white bars) and sG_{NiV} (black bars). All assays were done in triplicate; the average percent inhibition is shown for each microsphere subset and error bars represent the range of percent inhibition for each microsphere subset. HeV or NiV infection and species of sera and MAbs are indicated on the *x*-axis labels. Rabbit 405 was a hyper immune serum generated against sG_{HeV} and was used as a positive control. 0 indicates no serum was added.

As demonstrated in Fig. 5B, all infected individuals contained antibodies that interfered with receptor binding. Equally important, specificity of inhibition of receptor-G interaction correlated with the virus which elicited the antibody response. Like the antibody detection multiplexed microsphere assay, the receptor inhibition multiplexed assay had the capability to differentiate HeV and NiV specific antibodies. Of interest, the receptor inhibition assay appeared to have a higher specificity than the antibody detection assay (Fig. 1A) as illustrated by the HeV-infected human and bat sera.

Lastly, we examined a panel of HeV-specific horse and NiV-specific pig sera using the receptor inhibition assay. As demonstrated in Fig. 6, all seropositive horses and pigs had antibodies that blocked ephrin-B2 binding. The specificity of ephrin-B2-G glycoprotein interactions were highlighted further by the ability of HeV-infected horse sera to block preferentially the sG_{HeV}-ephrin-B2 interactions whereas NiV-infected pig sera preferentially blocked sG_{NiV}-ephrin-B2 interactions.

3.4. Sensitivity and specificity of microsphere based assays

The new multiplexed microsphere assays provided the ability to differentiate henipavirus-specific antibodies in several hours. To examine the specificity and sensitivity of these new assays in more detail, all sera and MAbs were tested over a range of concentrations and results are shown in Table 1. For comparison, SNT titers were included. Murine and human MAbs were included for reference and endpoint titrations were not obtained. The sensitivity of the antibody detection microsphere assay ranged from 1:500 to 1:64,000. Differences in titer were apparent depending on the species tested; pig field samples had the lowest titers whereas horse sera consistently demonstrated very high titers. Interestingly, although specificity mirrored the virus that elicited the host antibody response, HeV-infected individuals had high levels of sG_{NiV} cross-reactive antibodies; whereas NiV-infected animals had limited sG_{HeV} cross-reactive antibodies. Jointly, these data suggested that the native HeV attachment protein stimulated a more robust and cross-reactive immune response. The receptor inhibition microsphere assay had a decreased sensitivity by comparison, 1:25 to 1:4000, as would be expected when measuring a subset of host antibodies. Like the antibody detection assay, specificity paralleled the virus that induced the immune response. Additionally, HeV-infected individuals possessed higher titers of receptor blocking antibodies providing further evidence of a more robust immune response. Importantly, the differential receptor inhibition titers from infected individuals corresponded with results determined by SNT.

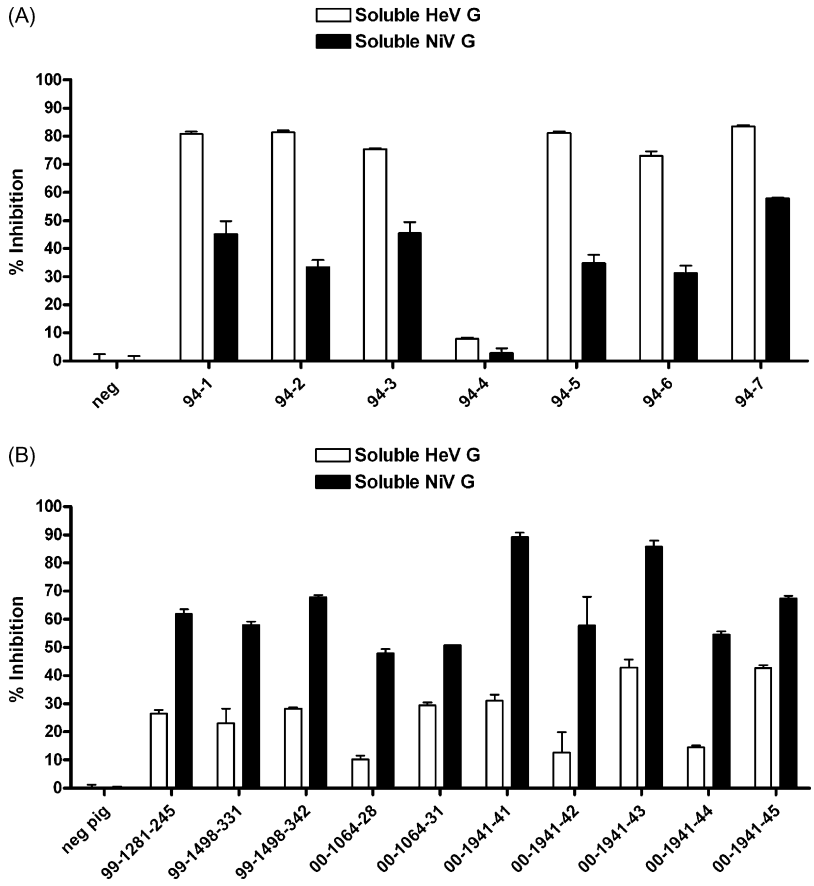

Fig. 6. Blocking of ephrin-B2 binding by henipavirus field sera. Horse (panel A) and pig (panel B) field sera were assayed using the inhibition of ephrin-B2 binding multiplexed microsphere assay as detailed in Section 2. The inhibition of ephrin-B2 binding was calculated as a percent inhibition for each microsphere population independently using the equations detailed in Section 2; sG_{HeV} (white bars) and sG_{NiV} (black bars). All assays were done in triplicate; the average percent inhibition is shown for each microsphere subset and error bars represent the range of percent inhibition for each microsphere subset.

Table 1
Sera and MAb endpoint titers in multiplexed microsphere assays and SNTs

Sera or MAbs	Details	Luminex-antibody detection sG NiV	Luminex-antibody detection sG HeV	NiV SNT	HeV SNT	Luminex-receptor inhibition sG NiV	Luminex-receptor Inhibition sG HeV
Polyclonal sera from different species	Control human	neg	neg	neg	neg	neg	neg
	HeV human	1:16,000	1:8,000	[b]	1:128	1:100	1:250
	NiV Human	1:32,000	1:2,000	1:100	>1:50	1:250	1:100
	Control pig	neg	neg	neg	neg	neg	neg
	NiV pig	1:8,000	neg	1:160	1:2	1:500	neg
	Control horse	neg	neg	neg	neg	neg	neg
	HeV horse	1:8,000	1:8,000	[b]	1:640	1:1,000	1:4,000
	Control cat	neg	neg	neg	neg	neg	neg
	NiV cat	1:64,000	1:1,000	1:160	1:20	1:4,000	1:100
	Control bat	neg	neg	neg	neg	neg	neg
	HeV bat	1:1,000	1:1,000	[b]	1: 640	1:250	1:500
	Control rabbit	neg	neg	neg	neg	neg	neg
	Rabbit 405	1:16,000	1:16,000	1:640	1:1,280	1:250	1:1,000
Peptide-specific sera	Control rabbit	neg	neg	neg	neg	neg	neg
	NiV G1	1:20	neg	neg	neg	neg	neg
	HeV G1	neg	1:20	neg	neg	neg	neg
Murine MAbs from Tissue culture supernatants	Control MAb	neg	neg	neg	neg	neg	neg
	30.7	neg	1:250[a]	neg	neg	neg	neg
	17A5	neg	1:250[a]	neg	1:10	neg	neg
	3A5	neg	1:250[a]	neg	1:256	neg	neg
	H2.1	1:250[a]	1:250[a]	neg	1:16	neg	neg
	8H4	1:250[a]	1:250[a]	neg	1:16	neg	1:25
Purified murine and human MAbs	Control MAb	neg	neg	[b]	[b]	neg	neg
	AH1.3	neg	<1:16,000	neg	1:5,000	neg	neg
	AH2.1	neg	1:16,000	neg	neg	neg	neg
	m101	<1:16,000	<1:16,000	1:160	1:1,280	neg	<1:8,000
	m102	<1:16,000	<1:16,000	1:10,240	1:10,240	1:8,000	<1:8,000
Field pig sera	Control pig	neg	neg	neg	[b]	neg	neg
	99-1281-245	1:1,000	1:50	1:8	[b]	1:500	1:50
	99-1498-331	1:1,000	1:50	>1:16	[b]	1:500	1:100
	99-1498-342	1:1,000	1:50	>1:16	[b]	1:500	1:25
	00-1064-28	1:1,000	neg	1:128	[b]	1:500	neg
	00-1064-31	1:1,000	1:50	1:128	[b]	1:500	1:50
	00-1941-41	1:4,000	1:50	>1:16	[b]	1:1,000	1:50
	00-1941-42	1:500	1:50	>1:16	[b]	1:500	1:25
	00-1941-43	1:8,000	1:50	>1:16	[b]	1:1,000	1:50
	00-1941-44	1:8,000	1:50	>1:16	[b]	1:1,000	1:50
	00-1941-45	1:1,000	1:50	>1:16	[b]	1:1,000	1:250
Field horse sera	Control horse	neg	neg	[b]	neg	neg	neg
	94-1	1:16,000	1:32,000	[b]	1: 640	1:500	1:4,000
	94-2	1:8,000	1:16,000	[b]	1: 640	1:500	1:4,000
	94-3	1:16,000	1:32,000	[b]	1: 1,280	1:500	1:4,000
	94-4	1:500	1:1,000	[b]	1: 20	1:50	1:100
	94-5	1:4,000	1:8,000	[b]	1: 640	1:500	1:4,000
	94-6	1:4,000	1:8,000	[b]	1: 640	1:500	1:4,000
	94-7	1:32,000	1:64,000	[b]	1: 640	1:500	1:4,000

neg = sera or MAb had no activity at highest concentration.
[a] Only dilution tested.
[b] SNT not done.

4. Discussion

The high level of similarity between HeV and NiV combined with their restriction to high bio-containment laboratories has severely limited the availability of reliable differential serological assays. Although ELISAs have been developed for HeV and NiV, there are technical difficulties in maintaining their reproducibility (Daniels et al., 2001). The antigens are prepared from HeV- or NiV-infected cell cultures and gamma-irradiated for transfer to non-containment laboratories. Consequently, the amount and quality of antigen per batch can vary widely. Currently, diagnostic samples are tested on one ELISA, either HeV- or NiV-specific, depending on the nature and origin of the sample received. As these assays were not developed in concert, have significantly different protocols and are not conducted concurrently, their ability to differentiate henipavirus antibodies is unknown. Regardless, if a test sample is positive by ELISA, SNTs are conducted against both HeV and NiV. SNT is the gold standard currently, however, it is a laborious assay which can only be done in a handful of laboratories. As the test endpoint is dependent on the inhibition of the viral cytopathic effect in cell culture, results are not known for 3–4 days. Furthermore, the ability of a particular serum to neutralize HeV or NiV preferentially can be ambiguous and this precludes a conclusive identification of the henipavirus responsible for eliciting the antibodies.

For all of these reasons we sought to devise new diagnostic tools applicable to the henipaviruses. Specifically, we wanted a single test capable of differentially detecting HeV- and NiV-specific antibodies. Here, we have described two new multiplexed microsphere assays that use sG_{HeV}- and sG_{NiV}-coupled beads. The first assay we developed was capable of detecting and differentiating between anti-HeV and anti-NiV antibodies. Although our results were consistent with the virus eliciting the host antibody response, our results were very different from those obtained using ELISA where all sera bound NiV preferentially. As previously mentioned, HeV and NiV-specific ELISAs are not routinely conducted concurrently; therefore, the relative activity of each antigen is not known. The skewed binding to NiV demonstrated here was most likely due to high quantities of NiV and low quantities of HeV antigen present. The multiplexed antibody detection assay uses microspheres coupled to known equal amounts of recombinant antigens creating less variation and enabling more accurate results. Interestingly, sera from HeV-infected individuals demonstrated significant heterotypic binding to sG_{NiV} whereas sera from NiV-infected individuals bound sG_{HeV} poorly. These data were analogous to our previous findings which demonstrated that sG_{HeV} immunized animals had greater heterotypic SNT titers than animals immunized with sG_{NiV} (Mungall et al., 2006). Together, both results demonstrate that HeV infection or sG_{HeV} immunization can elicit high levels of G-specific cross-reactive antibodies.

The second multiplexed assay was developed to increase specificity by targeting the domain of sG_{HeV} and sG_{NiV} that interacts with the host cell receptor, ephrin-B2. We found that sG_{NiV} appeared to have a slightly higher affinity for ephrin-B2 as measured in both the multiplex bead-based and surface plasmon resonance assays. In both assays the increased binding of sG_{NiV} was approximately two-fold and such differences may be attributed to discrepancies in the oligomeric state of sG_{NiV} and sG_{HeV}. Nevertheless, ephrin-B2 bound to both sG_{HeV} and sG_{NiV} in a dose-dependent fashion, demonstrating the specificity of these interactions. We speculated that specific subpopulations of neutralizing antibodies existed within infected hosts that targeted the receptor binding domain of G. When we examined sera and MAbs with known G binding specificity and SNT profiles we found that inhibition of ephrin-B2 binding had an excellent specificity. When assayed with animal or field sera, blocking of ephrin-B2 binding to G was more efficient in homotypic combinations, with sera from HeV and NiV-infected animals preferentially blocking the binding of ephrin-B2 to sG_{HeV} and sG_{NiV}, respectively. Clearly, infected individuals harbor this potentially neutralizing subset of antibodies. The ephrin-B2-based multiplexed assay has unique advantages over both the antibody detection multiplexed assay and SNT. Here, we can detect and clearly differentiate HeV and NiV neutralizing antibodies in a quantitative manner. Furthermore, due to the multiplex platform and its high sensitivity, only 1–5 μl of serum was required to detect specific neutralizing antibodies, a substantial advantage compared to traditional SNT where 20 μl of serum is commonly used for each assay. This is the first example of a surrogate neutralization assay that employs solely recombinant proteins and produces results that are completely in-step with results obtained by conventional SNT methods. Although we recognize that there will be other neutralizing antibodies in sera from infected hosts such as neutralizing antibodies to F (Guillaume et al., 2004), the preponderance of evidence in the literature indicates that attachment protein-specific antibodies appear to comprise the bulk of paramyxovirus neutralizing antibody (Lamb and Kolakofsky, 2001).

These unique multiplexed assays offer rapid and safe assessment of henipavirus antibody activity and specificity with significantly reduced time and expense. Both assays have considerable sensitivity and represent important new tools for henipavirus serosurveillance. These assays can be done without high biocontainment, require very small amounts of sera and provide valuable data quickly, within 2–3 h. Importantly, we believe such assays will significantly enhance our capability and capacity for serosurveillance in the event of a henipavirus outbreak. Estimation of seroprevalence within an affected area becomes achievable and an accurate account of asymptomatic infections will be possible, which in turn will provide a more accurate assessment of morbidity and mortality.

In recent years Luminex® technology has been primarily used for molecular, cytokine, auto-immune and serum drug detection. Although some multiplexed microsphere assays have been developed to detect pathogen-specific antibodies, very few of these assays differentiate closely related serotypes or closely related viruses and none are capable of selectively detecting neutralizing antibodies. By combining soluble envelope and receptor glycoproteins with the multiplexed microsphere platform we believe we have brought together several concepts that will give rise to new types of diagnostic assays.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2007.01.003.

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