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A duplex recombinant viral nucleoprotein microbead immunoassay for simultaneous detection of seroresponses to human respiratory syncytial virus and metapneumovirus infections*

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Abstract

Serologic diagnosis of human respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV) infections has been shown to complement virus detection methods in epidemiologic studies. Enzyme immunoassays (EIAs) using cultured virus lysate antigens are often used to diagnose infection by demonstration of a ≥4-fold rises in antibody titer between acute and convalescent serum pairs. In this study, hRSV and hMPV nucleocapsid (recN) proteins were expressed in a baculovirus system and their performance compared with virus culture lysate antigen in EIAs using paired serum specimens collected from symptomatic children. The recN proteins were also used to develop a duplex assay based on the Luminex microbead-based suspension array technology, where diagnostic rises in antibody levels could be determined simultaneously at a single serum dilution. Antibody levels measured by the recN and viral lysate EIAs correlated moderately (hRSV, $r^2 = 0.72$; hMPV, $r^2 = 0.76$); the recN EIAs identified correctly 35 of 37 (94.6%) and 48 of 50 (96%) serum pairs showing diagnostic antibody rises by viral lysate EIAs. Purified recN proteins were then coupled to microbeads and serum pairs were tested at a single dilution on a Luminex MAGPIX® analyzer. The duplex recN assay identified correctly 33 of 39 (85%) and 41 of 47 (86.7%) serum pairs showing diagnostic rises to hRSV and hMPV, respectively. The recN assay permits simultaneous testing for acute hRSV and hMPV infections and offers a platform for expanded multiplexing of other respiratory virus assays.

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Keywords

Human respiratory syncytial virus (hRSV); Human metapneumovirus (hMPV); Immunoassay; Serology

1. Introduction

Human respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV) are negative-sense single-stranded RNA viruses classified within the family *Paramyxoviridae*, *Pneumovirinae* subfamily (Papenburg and Boivin, 2010; van den Hoogen et al., 2001). Each is comprised of two major antigenic subgroups, A and B, and multiple genotypes (Peret et al., 1998; Biacchesi et al., 2003). hRSV is the leading cause of bronchiolitis and viral pneumonia in infants and young children and accounts for substantial annual morbidity and mortality worldwide (Nair et al., 2010). Severe hRSV infections are also common in persons with underlying chronic disease and the elderly (Hall et al., 1986, 1990; Dias et al., 1988; Falsey et al., 1992; Gilchrist et al., 1994; Han et al., 1999). hMPV infections are clinically indistinguishable from hRSV and the same high risk groups are prone to severe disease (Falsey et al., 2003; Williams, 2005; Kahn, 2006; Williams et al., 2006), although with typically lower incidence rates of infection.

hRSV and hMPV infections are most often diagnosed by virus culture, antigen detection or nucleic acid amplification assays in the clinical setting (Falsey et al., 2002; Beck and Henrickson, 2010). Although impractical for routine diagnostic work, serologic diagnosis based on demonstration of a significant rise in virus-specific antibodies between acute- and convalescent-phase serum specimen pairs has been shown to complement virus detection and increase diagnostic yield in epidemiologic and disease burden studies (Sawatwong et al., 2012; Feikin et al., 2013). Enzyme immunoassays (EIAs) using whole-virus culture lysate for antigen have been used commonly for serodiagnosis of hRSV and hMPV, but cultured viral antigen is difficult to standardize and poses some biosafety risk. As alternatives, recombinant viral proteins expressed in prokaryotic and eukaryotic systems have compared favorably with cultured virus antigen in serological assays for these viruses, including the fusion (Sastre et al., 2012) and matrix proteins (Hamelin and Boivin, 2005). The nucleocapsid (N) protein in particular has been shown to be effective in serologic assays for the paramyxoviruses, being highly immunogenic and inducing an early and long lasting antibody response (Hummel et al., 1992; Buraphacheep et al., 1997; Liu et al., 2007).

As with multiplexed molecular assays that combine multiple individual pathogen assays in a single reaction to reduce reagent and sample consumption and increase testing throughput, the Luminex bead-based suspension array technology has also been used successfully to develop multiplexed serologic assays for human and animal viruses (Anderson et al., 2011; Liao et al., 2011; Hernandez et al., 2012; van der Wal et al., 2012). In this study, the N proteins of hRSV and hMPV were expressed in a baculovirus system and their performance compared against whole virus lysate antigen in in-house serologic enzyme immunoassays (EIAs). These proteins were then used to develop and evaluate a duplex assay for hRSV and hMPV on a Luminex MAGPIX® analyzer.

2. Materials and methods

2.1. Human serum samples

Human serum specimens used for assay development were obtained originally from a fever surveillance study conducted from April, 2004 through February, 2006, in Kamalapur, an urban community in Dhaka, Bangladesh, by the International Center for Diarrheal Diseases (ICDDR,B) (Brooks et al., 2007). Paired acute- and convalescent-phase serum specimens were collected within a median of 6 days (standard deviation, 4 days) of onset of symptoms and 14 days after illness resolution, respectively, from 788 children <5 yrs of age presenting with fever or respiratory syndromes of any severity. The sera were sent to the Centers for Disease Control and Prevention (CDC) where they were tested for diagnostic rises in IgG antibodies to hRSV, hMPV and other respiratory viruses by in-house EIAs (see below). The study was approved by the research and ethical review committees of ICDDR,B and the Institutional Review Board of CDC.

2.2. Virus

hRSV laboratory strains *A2* (subgroup A) and *CH-18537* (subgroup B) and hMPV strains *CAN97–83* (subgroup A) and *CAN98–75* (subgroup B) were passaged in VERO E6 cells and stock virus stored at −70 °C until use.

2.3. Cells

VERO E6 cells (ATCC CRL-1587) were grown at 35 °C in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Life Technologies, Grand Island, NY, USA). *Spodoptera frugiperda* (Sf9) cells were grown at 27 °C in Sf-900™ II serum free media supplemented with antibiotics (penicillin, 10,000 U/mL; streptomycin, 10,000/mL) (GIBCO, Life Technologies, Grand Island, NY, USA).

2.4. Cultured virus antigen preparation for EIA

hRSV and hMPV strains were inoculated separately into 150 cm^2 flasks containing nearly confluent monolayers of VERO E6 cells at 10 p.f.u./cell with media change to EMEM with 2% FBS. Cultures were monitored daily for cytopathic effect. When most cells were infected, the cultures were subjected to 3× freeze-thaw cycles and the lysate centrifuged at low speed to remove large cellular debris. The supernatants were then removed and aliquoted in small volumes and stored at −70 °C until use. Uninfected cultures were processed identically to serve as negative antigen control. Optimal concentrations of the whole virus culture lysates used individually or combined as antigen in EIAs (see below) were determined by checker board titrations against human serum pools with and without detectable IgG antibodies to hRSV or hMPV, respectively.

2.5. Expression of hRSV and hMPV N proteins

hRSV (strain *A2*) and hMPV (strain *CAN97–83*) RNA was extracted and the respective nucleocapsid (N) genes amplified by RT-PCR using the following primer pairs: hRSV, forward 5′-CACCATGGCTCTTAGCAAAGTCAA-3′ and reverse, 5′-TCAAAGCTCTACATCATTATCT-3′; hMPV, forward 5′-

CACCATGTCTCTTCAAGGGATTCAC-3′ and reverse, 5′-TTACTCATAATCATTTTGACTGTC-3′. Following manufacturer's instructions, the amplified N genes were cloned respectively into entry vector pENTR/D-TOPO using the Bac-to Bac® Baculovirus Expression System with Gateway® Technology (Invitrogen, Grand Island, NY, USA). The coding regions of the N genes were sequence confirmed and clones showing no amino acid substitutions were selected and transferred into destination vector pDEST™ 10. The resulting expression clones were then transformed into competent DH10 Bac™ *E. coli* cells and the recombinant Bacmid DNA containing hRSV-N or hMPN-N inserts were isolated and transfected into Sf9 cells. The recombinant BAC-hRSV-N and BAC-hMPV-N vectors were harvested when cultures reached 50% nonviability and expression of the recombinant histidine-tagged nucelopcapsid (recN) proteins was confirmed by reaction with mouse anti-histidine-tagged antibodies (Santa Cruz Biotechnology, Dallas, TX) on Western blot (see below).

2.6. Purification of recN proteins for EIA

For EIA antigen, recN proteins were semi-purified using the Ni-NTA Purification System (Life Technologies, Grand Island, NY, USA) following the manufacturer's instructions. Briefly, 500 mL of SF9 cell culture at 2×10^6 cells/mL were infected respectively with BAC-hRSV-N and BAC-hMPV-N at 1 pfu/cell. When cultures reached 50% nonviability (virus titer ~1×10^7 pfu/mL), the cells were collected and resuspended in 8 mL Native Binding Buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 10 mM Imidazole). The cells were then lysed by two freeze-thaws and 4-times passage through an 18-gauge needle and the large cellular debris removed by low speed centrifugation. The supernatant was loaded on a Ni-NTA agarose purification column and the binding reaction carried out at room temperature for 60 min with gentle rotation. The resin was pelleted and washed four times with Washing Buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 20 mM Imidazole) and the bound protein eluted with 8 mL of Elution Buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 250 mM Imidazole). The protein concentration was determined spectrophotometrically and the protein stock diluted to 50% with glycerol and stored at −20 °C.

For coupling to Luminex microbeads, recN proteins required further purification which was performed by a commercial vender (ATCC, Manassas, VA, USA). Briefly, cell pellets from 1000 mL of infected Sf9 cells were lysed by 3× freeze and thaw cycles in 65 mL native binding buffer (50 mM Na_3PO_4, 500 mM NaCl, pH 8.0) and soluble and insoluble fractions were separated by ultracentrifugation. The soluble fraction was filtered through 0.45 μm filter and loaded on 2 × 5 mL HisTrap FF column at 4 °C. The column was washed with 200 mL of native binding buffer followed by 200 mL of wash buffer (50 mM Na_3PO_4, 500 mM NaCl, 100 mM Imidazole, pH 8.0). The target protein was then eluted with 60 mL of elution buffer (50 mM Na_3PO_4, 500 mM NaCl, 250 mM Imidazole, pH 8.0). The eluted protein fraction was concentrated using 30 Kd MWCO membrane followed by filtering with a 0.45 um Acrodisc® 32 mm syringe filter (Pall, Port Washington, NY, USA). Protein purity was confirmed by SDS-PAGE.

2.7. Western blot

The purified recN proteins were resuspended in 2× Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA) and loaded onto a 10% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA). The separated proteins were transferred to an Immobilon®-P Polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and the membrane was blocked with phosphate-buffered saline (PBS, pH 7.4) containing 0.02% Tween® 20 (PBST) and 5% (w/v) powered milk (PBSTM) for 60 min at room temperature. The blotted proteins were reacted against mouse anti-histidine-tagged antibodies (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit hyperimmune antisera against hRSV (Millpore, Billerica, MA, USA) or mouse hyperimmune antisera against hMPV (CDC) diluted 1:500 in PBSTM. The membrane was incubated with primary antibody overnight at 4 °C followed by three washes with PBST and addition of horseradish peroxidase (HRP)-conjugated species-specific secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:20,000 in PBSTM. Following 1 hr incubation at 37 °C, the membrane was washed the proteins visualized with ECL Western Blotting Detection Reagents (GE Healthcare, Fairfield, CT, USA) following manufacturer's instructions.

2.8. Enzyme immunoassays (EIAs)

Indirect EIAs for anti-hRSV and -hMPV IgG antibodies based on viral lysate and recN protein antigens were performed following a standard in-house protocol. For each serum specimen, 75 μL of whole virus lysate or semi-purified recN protein antigen as described above were diluted to optimal concentration in carbonate–bicarbonate buffer (pH 9.6) and added to duplicate wells each of a 96 well Immulon II microtiter plate (Dynex Technologies, Chantilly, VA, USA); duplicate wells each were also coated with uninfected VERO E6 or Sf9 cell lysates processed identically as negative control antigen. After overnight incubation at 4 °C, the plate was washed 3× with PBST and 75 μL of human serum sample diluted 1:200 in PBSTM was added to the 4 antigen coated wells and incubated for 1.5 h at 37 °C. The plate was then washed three times and incubated for 1 h at 37 °C with horseradish peroxidase conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:20,000 in PBSTM. Plates were washed 6× with PBST and 75 μL of 3,3′5.5′-tetramethyl benzidine substrate (Invitrogen, Grand Island, NY, USA) was added to all wells. The reaction was allowed to proceed for 10 min at room temperature and then 75 μL of 2N phosphate acid was added to each well to stop the reaction. The absorbance (A) was then measured at 450/650 nm on an MRX^e Microplate Reader (Dynex Technologies, Chantilly, VA, USA). For each serum specimen, the mean $A_{450/630}$ of the duplicate wells of positive (P) and negative (N) antigen was determined and the net difference (P − N) and ratio (P/N) calculated.

Serum specimens with both P − N values ≥0.1 and P/N values ≥2 by viral lysate EIAs were considered positive for IgG antibodies to the respective viruses. Serum pairs with convalescent (c) to acute (a) ratios ($P - N_c/P - N_a$) ≥ 1.5 were retested by serial 4-fold dilutions and those pairs showing a ≥4-fold rise in IgG antibody titer were considered diagnostic for recent virus infection. IgG positive serum pairs with no or <4-fold titer rises were considered stable and indicative of past infection.

2.9. Coupling of recN proteins to beads

Efforts to couple the Ni-NTA semi-purified recN proteins to MagPlex® beads (Luminex, Austin, TX, USA) were unsuccessful and the proteins were subjected to further purification as noted previously. These highly purified hRSV-recN and hMPV-recN proteins were coupled to beads #20 and #18, respectively, using a *N*-hydroxysulfosuccinimide-enhanced carbodiimide-mediated coupling reaction as described by the manufacturer. Briefly, 2.5×10^6 beads were sonicated for 20 s and then resuspended in 80 μL of 0.1 M sodium phosphate buffer (pH 6.2). Bead carboxylated sites were activated by adding 10 μL each of a 50 mg/mL solution of *N*-hydroxysulfosuccinimide (Sulfo-NHS) and a 50 mg/mL solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and incubating for 20 min at room temperature in the dark. The activated beads were washed twice with 250 μL of PBS (pH 7.4) and 10 μg of each purified recN protein was separately added in 500 μL of PBS and placed on a rotational shaker for 2 h at room temperature in the dark to allow the recN proteins to covalently bind to the beads. The recN protein-coupled beads were then washed three times each with 1 mL of PBS + 1% bovine serum albumin and 0.02% Tween 20 and then stored in 0.5 mL PGT buffer (PBS + 4% normal goat serum, 0.02% Tween 20 and 0.05% azide) at 4 °C in dark.

2.10. MAGPIX® recN assay

The recN-coupled beads were vortexed briefly, sonicated for 20 s and adjusted to a working concentration of 50 beads/μL in PGT buffer. A white 96-well round bottom microtiter plate (Fisher Scientific) was prewet with 100 μL/well of PGT buffer and 50 μL of working bead mixture was added to each well. Fifty μL of each serum sample diluted 200× in PGT buffer was then added and the plates were incubated for 90 min at room temperature on a microplate shaker in the dark. After incubation, the plates were washed 2× with 100 μL PGT buffer and the beads resuspended in 100 μL phycoerythrin (PE)-labeled anti-human IgG antibody at concentration of 4 μg/mL in PGT buffer and incubated for 30 min at room temperature as above. The plate was then washed 2× and the beads resuspended in 100 μL of PGT buffer before loading onto the MAGPIX® instrument (Luminex, Austin, TX, USA). Reporter fluorescence was measured and expressed as mean fluorescence intensity (MFI) of at least 50 beads per well.

2.11. Statistical analysis

hRSV and hMPV EIAs using whole virus lysate as antigen were used as the reference standard for assay comparisons. A ≥4-fold rise in IgG antibody titer as defined above was considered definitive for recent acute virus infection. The degree of concordance between two assays was assessed using the unweighted kappa statistic (Fleiss et al., 1969). Kappa values ranging from 0.41 to 0.6, 0.61 to 0.8, and 0.81 to 1 were considered to represent fair, good and very good agreements, respectively.

3. Results

Archived serum pairs from the study described above had been tested previously by the in-house viral lysate EIAs and classified as having (i) no detectable IgG antibodies to hMPV or hRSV (no prior exposure to virus); (ii) stable IgG antibody levels (past exposure) or (iii) ≥4-

fold rises in IgG antibody levels (current or recent infection). Representative serum pairs covering a wide range of antibody levels were selected from this collection and were retested simultaneously by the viral lysate EIAs for study comparisons.

3.1. Comparison of hRSV and hMPV subgroup-specific viral lysate EIAs

hRSV and hMPV each contain two major antigenic subgroups, A & B, distinguishable genetically and immunologically by neutralization with animal hyperimmune antisera and reactivity with monoclonal antibodies (Anderson et al., 1985; van den Hoogen et al., 2004). These antigenic differences are primarily associated with the viral envelope G and F glycoproteins and are less pronounced with the immunodominant nucleocapsid (N) proteins that show >95% amino acid sequence identity between subgroups. To determine if antigenic differences between viral subgroups could influence serologic test results, EIAs using whole virus lysate prepared from representative strains of each virus subgroup were compared with panels of 30 serum pairs selected to represent a broad range of IgG antibody levels. IgG antibody levels determined by individual viral subgroup EIAs for both viruses were highly correlated (hRSV, $r^2 = 0.96$; hMPV, $r^2 = 0.95$) (Fig. 1). The N protein from only one strain of each virus subgroup (hRSV, *A2*; hMPV, *CAN97–83*) was therefore chosen to be expressed for the recN serological assays.

3.2. recN protein expression and comparison of recN and viral lysate EIAs

Protein bands of expected size for each virus were identified on Western blot following reaction with anti-histidine-tagged antibodies confirming successful expression of the recN proteins (data not shown). Following larger scale production and purification, reactivity and purity of the recN proteins were confirmed with hyperimmune animal antisera previously prepared to hRSV and hMPV, respectively

The rec-N proteins were first compared with viral lysate antigens using in-house EIAs. Optimal concentrations of the semi-purified rec-N proteins were determined by checkerboard titrations against pooled IgG positive and negative reference sera. The optimized rec-N and viral lysate EIAs were then compared directly using patient serum pairs selected for high, medium, low and no detectable IgG antibodies to each virus (hRSV, 53 serum pairs; hMPV, 55 pairs). IgG antibody levels measured for both virus assays correlated moderately over the range of concentrations selected (hRSV, $r^2 = 0.72$; hMPV, $r^2 = 0.76$) (Fig. 2).

The ability of the recN EIAs to detect diagnostic antibody rises indicative of acute hRSV or hMPV infection was assessed using serum pairs that showed ≥4-fold rises in IgG antibody titers by the viral lysate EIAs. Serum pairs selected for low, medium and high antibody titer rises were diluted serially and retested by both assays. The recN EIAs detected ≥4-fold antibody rises in 35 of 37 hRSV (94.6%) and 48 of 50 hMPV (96%) positive serum pairs. Two discrepant serum pairs for each virus assay showed no or <4-fold antibody titer rises by recN EIAs on original and repeat testing.

3.3. MAGPIX® recN seroassay

The two purified recN proteins were coupled chemically to separate microbead sets at varied concentrations and reacted with serially diluted positive and negative serum pools to identify recN concentrations giving maximal fluorescence with minimal background. Representative dose response curves obtained with serially diluted pooled sera reacted against optimally coupled individual bead sets are shown in Fig. 3. Reactivity of the hRSV positive serum pool with the hRSV recN-coupled beads plateaued at between 8000 and 10,000 MFI (serum dilutions 1:20–1:80) indicating bead-antibody saturation; the hMPV positive serum plateaued at a lower MFI, between 4000 and 6000 (serum dilutions 1:40–1:160). Serum dilutions between 1:160 and 1:640 gave the highest signal to noise ratios comparing positive and negative serum pools and a single serum dilution of 1:200 was chosen for subsequent studies. To demonstrate the specificity of the duplex MAGPIX hRSV and hMPV recN protein bead sets, we tested 14 hRSV IgG + /hMPV IgG− and 12 hRSV IgG−/hMPV IgG + by the duplex MAGPIX® recN assay. As shown in Fig. 4, hRSV-N or hMPV-N coupled beads bound to serum IgG antibodies in a virus-specific manner.

The duplex MAGPIX® recN assay was evaluated further by testing paired serum specimens previously classified as having (i) no detectable IgG antibodies (hRSV, 33; hMPV 30); (ii) stable IgG antibody levels (hRSV, 45; hMPV, 43); and (iii) ≥4-fold rises in IgG antibody titer (hRSV, 39; hMPV, 45) (Table 1). “Cutoff” values to define the MFI boundaries for detection of IgG antibodies to the respective viruses by the MAGPIX® recN assay were chosen to minimize false positive results. These values were set at the mean MFI plus three standard deviations calculated for serum pairs with no detectable IgG antibodies by the viral lysate EIAs (hRSV MFI, 240.2; hMPV MFI, 354.2). Based on these criteria, for each virus, all but one serum pair that was negative by viral lysate EIA was negative by the MAGPIX® assay and all serum pairs with stable detectable IgG antibodies were positive, exceeding the MFI cutoff values.

Convalescent to acute serum MFI ratios for all serum pairs were then determined and cutoff values calculated to define the ratio boundaries for ≥4 fold antibody rises by the MAGPIX® recN assay. Cutoffs were set at the mean MFI ratios plus 3 standard deviations for serum pairs with stable IgG antibodies by viral lysate EIAs (hRSV MFI ratio, 2.3; hMPV MFI ration, 2.0). Based on these criteria, ratios obtained for all serum pairs with stable IgG antibodies were below the cutoff values, and 85% and 86.7% of serum pair ratios showing ≥4 fold antibody rises by cultured virus EIA exceeded the cutoff values for hRSV and hMPV, respectively (Table 1). Because some specimen pairs that showed diagnostic rises by viral lysate EIA, but were below the respective cutoff ratios, had elevated IgG antibodies (high MFI values) in the acute-phase sample at 1:200 dilution, these specimens were subjected to further dilution and reran by the MAGPIX® recN assay. On retesting, 3 of 6 hRSV and 1 of 6 hMPV positive serum pairs showed ≥4 fold titer rises by MAGPIX® assay.

4. Discussion

To overcome the limitations of using whole virus lysate antigen in the in-house serologic EIAs, the N proteins of representative strains of hRSV and hMPV were expressed and their performance was evaluated with paired sera from young children previously classified as

having (i) no detectable IgG antibodies to hMPV or hRSV (no prior exposure to virus); (ii) stable IgG antibody levels (past exposure); and (iii) ≥4-fold rises in IgG antibody levels (current or recent infection). Concern that N proteins alone, derived from single virus strains, might be less effective than antigenically complex whole virus for serodiagnosis proved unfounded. The recN EIAs correctly classified most serum pairs and offered other important benefits; the baculovirus expression system provided large quantities of well-defined recombinant antigen, reducing the need for frequent virus cultures, and was purified more easily from host cellular components, reducing potential for nonspecific antibody reactions. These findings confirmed other studies that found that the N protein could be used successfully as antigen in serological assays to detect antibody responses elicited by hMPV (Liu et al., 2007; Rasa et al., 2011) and hRSV (Buraphacheep et al., 1997) infections. However, two serum pairs for each virus exhibited diagnostic antibody rises by viral lysate EIAs that showed <4-fold on no titer rises on initial and repeat testing by the recN EIAs. Although these antibody rises could potentially be false positives (only one of these 4 patients was confirmed positive for hRSV on RT-PCR testing of concurrently collected respiratory specimens), it is possible that individual variation in the IgG antibody responses of these patients directed predominantly to other viral proteins accounts for this finding.

In an effort to reduce reagent and sample consumption and increase testing throughput and convenience, a pilot study was conducted to evaluate the feasibility of using the recN proteins to develop a duplexed assay for hRSV and hMPV using Luminex fluorescent bead-based suspension array technology. This technology was enhanced recently with the introduction of the MAGPIX® system that uses special magnetic fluorescent microbeads and a less costly and easier-to-use fluorescence analyzer making it more accessible to resources constrained laboratories. Using in-house viral lysate EIAs as the reference “gold-standard” for comparison, the duplex MAGPIX® recN assay was able to clearly discriminate between viral IgG positive and negative sera and correctly identified most serum pairs with ≥4-fold IgG antibody titer rises at a single serum dilution. Several serum pairs that showed diagnostic antibody rises, but were misclassified by the MAGPIX® assay at this dilution, had high acute-phase antibody levels and therefore low convalescent to acute MFI ratios (below the cutoff values). Elevated acute-phase serum antibodies could be due to late acute serum collection or repeat virus infection resulting in an anamnestic antibody response. For these, diagnostic rises could be still be demonstrated by the MAGPIX® assay if higher serum dilutions were included in the run. Serum pairs that could not be corrected by further dilution occurred mostly with hMPV. Lower peak fluorescence and a narrower dynamic range observed with the MAGPIX® hMPV recN assay shown with serially diluted pooled positive sera might reflect insufficient protein purity or loss of immunoreactivity due to conformational changes in hMPV recN proteins on coupling to the microbeads. Further optimization of hMPV recN purification and coupling conditions are being investigated.

During the course of this study, several technical factors were identified that affected the performance of the MAGPIX® recN assay. recN purity was essential for efficient bead coupling; incomplete purification resulted in significantly reduced fluorescence intensity and higher non-specific fluorescence levels. Careful cross-titrations were necessary to obtain the optimal concentration of recN proteins for coupling to beads. The choice of serum diluent influenced greatly assay non-specific fluorescence. Normal goat serum was found to block

non-specific fluorescence better in the MAGPIX® assay than bovine serum albumin or dried milk that are often used in conventional EIAs. Once optimally coupled, the recN protein-coupled microbeads were found to be highly stable, and performed equivalently after at least one year storage at 4 °C.

The study had several limitations. First, serum specimens available for assay development were derived from a demographically limited population with sampling restricted to a single community over a fairly narrow time frame. Local differences in host humeral responses and infecting virus strains could potentially bias test results. Additional serum collections from other populations will need to be evaluated to demonstrate consistent assay performance. Also, very limited sample volumes prevented more extensive assay validation studies. Furthermore, the challenge of developing a practical serological test based on demonstration of a diagnostic rise in antibody levels still remains; collection of paired serum specimens is often difficult and delays laboratory diagnosis, limiting assay utility. An alternative approach that warrants further investigation would be to test for hRSV- and hMPV-specific IgM antibodies in single acute serum specimens, where detection has been shown to correlate with recent hRSV infection (Vikerfors et al., 1988).

As proof of concept, these data demonstrate the capacity of baculovirus expressed recN proteins to replace cultured virus antigen in serologic assays for hRSV and hMPV and the feasibility of multiplexing these assays using the Luminex MAGPIX® system for rapid, high throughput testing for acute respiratory virus infections. Future efforts will focus on expanding this system to include assays for other respiratory viral pathogens and use of mixtures of other proteins and peptide antigens to enhance diagnostic yield for these viruses.

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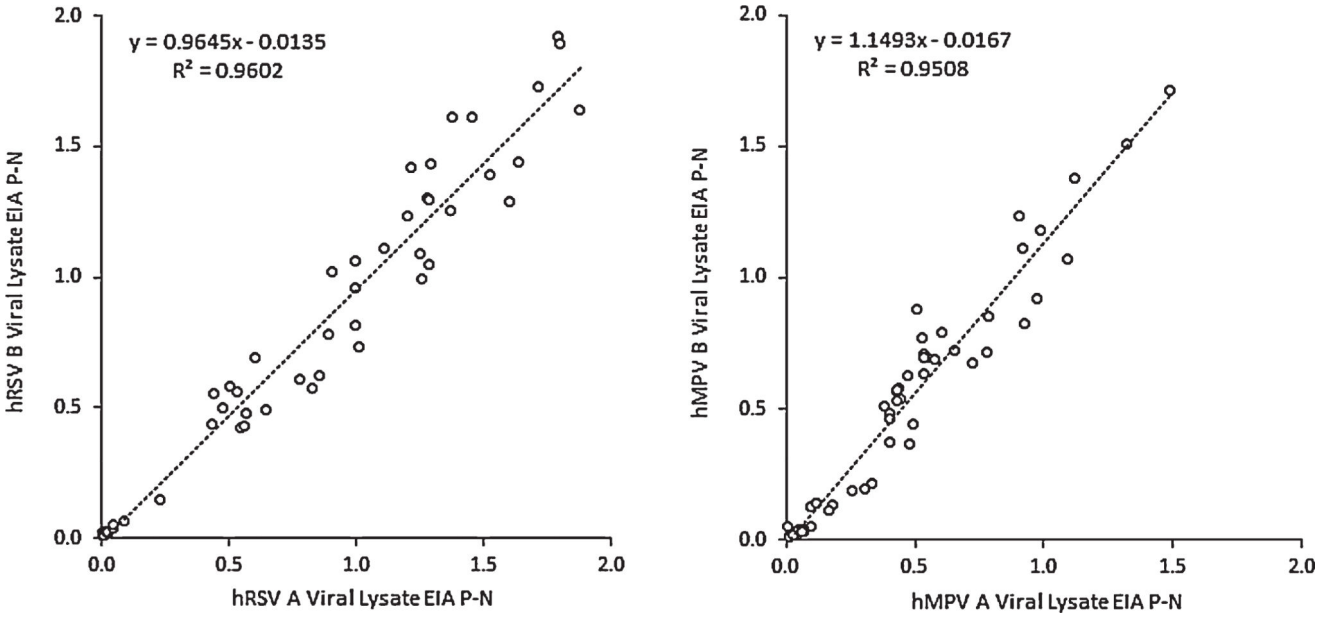

Fig. 1.

Comparison of EIAs using viral lysate antigens prepared from representative strains of hRSV and hMPV subgroups A and B with panels of 30 serum pairs with varied levels of IgG antibodies to the respective viruses at a single serum dilution (1:200). Least squares linear regression lines and analyses are shown. P–N, net difference of mean $A_{450/630}$ of duplicate wells of positive (P) and negative (N) antigen.

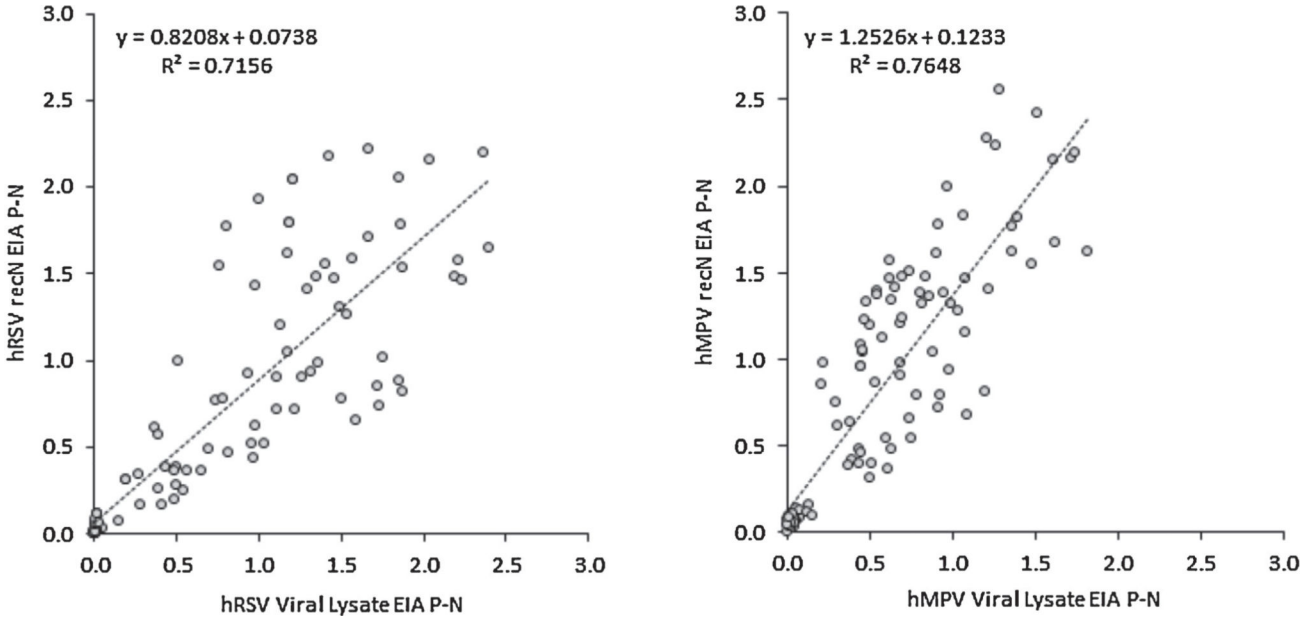

Fig. 2.

Comparison of recN and viral lysate EIAs with panels of serum pairs containing varied levels of hRSV (n = 53 pairs) and hMPV (n = 55 pairs) IgG antibodies to the respective viruses at a single serum dilution (1:200). Least squares linear regression lines and analyses are shown. P–N, net difference of mean $A_{450/630}$ of duplicate wells of positive (P) and negative (N) antigen.

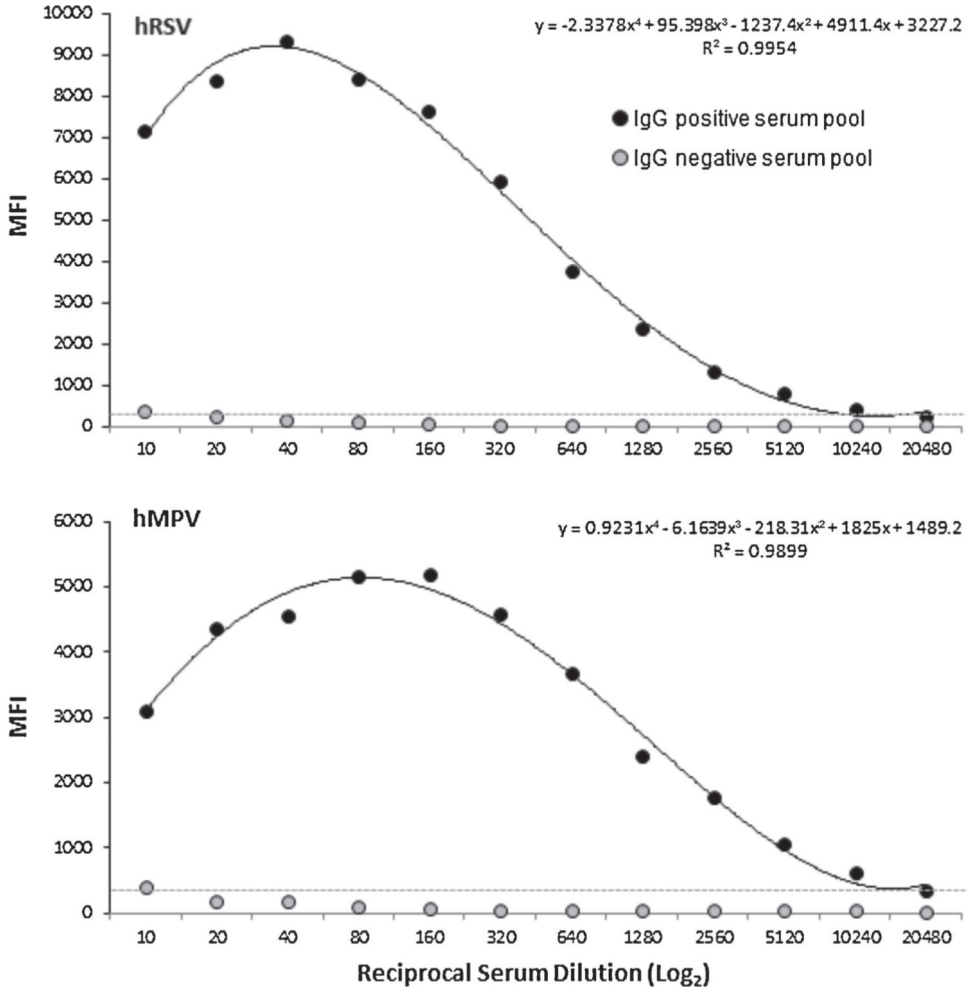

Fig. 3.

recN MAGPIX® assay reactivity with serially diluted serum pools positive and negative for hRSV and hMPV IgG antibodies by viral lysate EIAs. Each data point is averaged from two independent assay measurements. Fitted 4th order polynomial regression lines and analyses shown for positive serum pool dilutions. MFI: median fluorescent intensity.

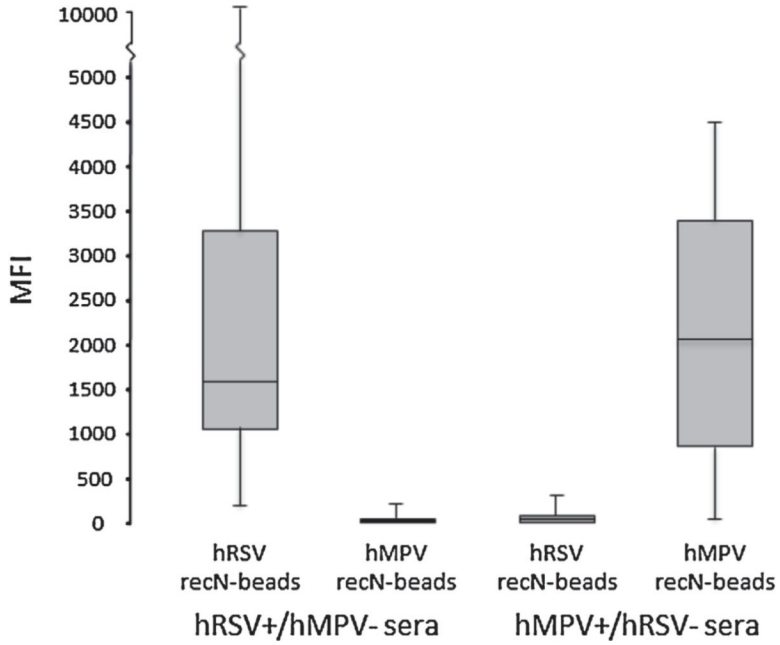

Fig. 4.

recN MAGPIX® assay reactivity with paired sera positive (+) and negative (−) for IgG ntibodies to hRSV (n = 14 pairs) and hMPV (n = 12 pairs). Box and whisker plots showing quartile distribution of median fluorescent intensity (MFI) values.

Table 1

Comparison of duplex MAGPIX® recN seroassay with reference cultured virus EIAs.

Virus	Serum pair categories[a]	No.	Duplex MAGPIX® recN assay No. (% agreement)	Kappa statistic (95% CI)[b]
hRSV	Neg, no detectable IgG antibodies	33	32(97)	0.909 (0.844–0.974)[c]
	Pos, stable IgG antibodies	45	45(100)	0.961 (0.918–1)[d]
	Pos, ≥4-fold rise in IgG antibodies	39	33[c]/37[d] (84.6/94.9)	
hMPV	Neg, no detectable IgG antibodies	30	29 (96.7)	0.911 (0.847–0.975)[c]
	Pos, stable IgG antibodies	43	43 (100)	0.924 (0.864–0.983)[d]
	Pos, ≥4-fold rise in IgG antibodies	47	41[c]/42[d] (87.2/89.4)	

[a] Paired acute and convalescent-phase sera categories determined by cultured virus EIAs. Kappa statistic (95% CI).

[b] Kappa statistic for all categories. CI: confidence interval.

[c] First number, serum pairs with convalescent/acute serum MFI ratios ≥2.3 (hRSV) or ≥2.0 (hMPV) at a single serum dilution of 1:200.

[d] Second number, serum pairs showing ≥4-fold antibody rises by serial 2-fold dilution and tested by the duplex MAGPIX recN seroassay.