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ABSTRACT

Characterization of the Fusion and Attachment Glycoproteins of Human Metapneumovirus and Human Serosurvey to Determine Reinfection Rates

Julie A. Pavlin, MD, PhD, MPH, 2007

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Human metapneumovirus (hMPV) is a newly discovered virus that causes acute respiratory illness. It is found worldwide and most people are exposed before 5 years of age. It can cause severe illness and even death at extremes of age, and in those with immunocompromising states and underlying illnesses. Immunity after natural or experimental infection with hMPV is not lasting, although neutralizing antibodies develop to the virus. We have prepared and characterized soluble versions of the fusion (F) and attachment (G) surface glycoproteins of hMPV to further assess the ability of these viral components to induce and detect antibodies. Size exclusion chromatography and sucrose gradient analysis revealed that the sG glycoprotein is a dimer, and the sF glycoprotein a monomer, although other reported sF glycoproteins are trimers. Hamsters, rabbits and non-human primates immunized with hMPV develop antibodies reactive to both the monomeric and trimeric sF glycoproteins, and animals immunized with the same genotype as the sG glycoprotein also demonstrate antibodies to sG, although at much lower levels than sF. Non-human primates immunized with hMPV do not develop cross-

reactive antibodies to respiratory syncytial virus (RSV), the most closely related human viral pathogen. Mice immunized with hMPV sF and sG developed antibodies to each, although at significantly lower levels to G than to F. An indirect screening enzyme-linked immunosorbent assay (ELISA) using sF was used to test for anti-F IgG in 1,380 acute and convalescent sera collected from school age children (7-16 years) who had an acute febrile illness in Kamphaeng Phet, Thailand from 1998-2002. 1,376 (99.7%) showed evidence of prior infection with hMPV. 66 children demonstrated a four-fold or greater rise in titer for an overall re-infection rate of 4.9%. Two children demonstrated evidence of an initial infection. 49 of the 68 new or re-infections occurred in 2000, accounting for 13.2% of all febrile illnesses in the study population in that year. 32 of these cases occurred in a discrete 2 week time period. All positive specimens were also tested for an increase in titer to RSV F and 27% exhibited a four-fold or greater rise in titer, representing cross-reactivity, non-specific clonal expansion of antibody or dual-infections. hMPV may represent a more significant impact in older children than previously realized and may be the cause of significant outbreaks. Taken together, the character of the sF glycoprotein developed here suggests it may be an attractive subunit vaccine candidate. Development of the surface glycoproteins as subunit vaccines may be an effective way to prevent severe illness in high-risk populations.

CHARACTERIZATION OF THE FUSION AND ATTACHMENT GLYCOPROTEINS OF HUMAN METAPNEUMOVIRUS AND HUMAN SEROSURVEY TO DETERMINE REINFECTION RATES

By

Julie A. Pavlin

Dissertation submitted to the

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Chapter 1 – Introduction

Acute respiratory tract infections (ARI) cause significant morbidity and mortality worldwide. Lower respiratory tract infection is the leading cause of mortality in children under the age of 5 (96). In 2000, analyses estimated that 1.9 million children died from ARI, with 70% of them in Africa or southeast Asia (116). Lower respiratory infections take a toll in all age distributions and are the fourth leading cause of death in high- and middle-income countries and the second leading cause of death in low-income countries (5).

Studies in the United States demonstrate that ARIs are the most common illnesses regardless of gender or age (78). In one study, viral agents caused 69% of ARIs, with rhinoviruses as the most common etiologic agent, followed by coronaviruses, influenza, parainfluenza, respiratory syncytial virus and adenoviruses (79). However, in 23% of cases, the etiology could not be determined. Other studies have been unable to find the etiology of lower respiratory tract infection in a third of cases and in nearly half of upper respiratory infections in children (61).

In recent years, many new causes of respiratory illness have been discovered. In 2001, van den Hoogen, et al. discovered a viral agent that caused respiratory illness in children in the Netherlands (112). They isolated a paramyxovirus from nasopharyngeal samples taken over a 20-year period from 28 patients suffering a respiratory tract illness that could not be identified through immunological assays and polymerase chain reaction (PCR)-based methods using virus-specific antibodies or primers. Using random-primed PCR, they obtained genome sequence data that demonstrated a relationship to the avian

metapneumovirus and named it human metapneumovirus (hMPV). Intentional inoculation of birds and non-human primates with viral isolates provided evidence that hMPV is a primate pathogen and not an avian pathogen that can also infect humans.

Testing of retrospective samples using indirect immunofluorescence demonstrated that by the age of 5, all 60 samples tested had evidence of exposure to hMPV (112). An additional 72 sera taken in 1958 from humans aged 8 to 99 also showed 100% seroprevalence, indicating that the virus has been circulating at least 50 years. Antibody titers were also higher for children over 2 years when compared to children 6 months to 2 years of age, possibly related to boosting from reinfection.

Epidemiology

Since the initial discovery of hMPV, evidence of exposure and infection has been reported worldwide (2, 6, 7, 26, 28, 35, 36, 38, 42, 51, 63, 65, 71-73, 84, 86, 89, 93, 97, 104, 105, 107). A retrospective survey of archived respiratory specimens from children taken at outpatient visits using reverse transcription (RT)-PCR to detect hMPV RNA demonstrated 5% of upper respiratory and 12% of lower respiratory tract illness are caused by hMPV (118, 120). Other retrospective and prospective surveys of respiratory specimens from children with a respiratory illness in North America, South America, Europe, the Middle East and Asia, have shown 4-21% positive for hMPV infection (2, 6, 7, 20, 28, 33, 35, 36, 38, 42, 46, 76, 81, 85, 107). These studies have demonstrated that hMPV is responsible for a significant amount of acute respiratory illness in children, and is second only to respiratory syncytial virus (RSV) as a cause of lower respiratory tract disease.

Serological surveys to determine prevalence of exposure to hMPV show that over 80% of adults have evidence of immunoglobulin (Ig) G to the F or N protein of hMPV (54, 60). Seroprevalence in children demonstrates a drop-off of seropositivity from approximately 90% to 36% after one year, probably due to the loss of maternal antibodies, and then a gradual increase to greater than 90% positive over the age of 5 (70, 112). Another study in Saskatchewan detected a much lower seropositive level in children under 15 (overall 24%), but this increased to 99% by 16-30 years of age (71).

Although studied less frequently in adults, hMPV has been reported as the cause of respiratory illness in 0.8 to 4.5% of adult patients (43, 44, 50, 83). Increased risk factors for disease in adults appear to be advanced age, chronic obstructive pulmonary disease and other underlying cardiopulmonary complications (17, 44, 55, 57). Spread of hMPV is probably similar to RSV where young children, especially in day care settings, spread the virus quickly and bring it home to their older siblings and parents, and adults then develop a milder form of the disease.

Disease in the immunocompromised host can be especially devastating. One study reported 9% of respiratory illness in adults with hematologic malignancies was caused by hMPV, second to influenza and the same as RSV. The majority of these patients were stem cell transplant recipients (119). Another report showed that stem cell transplant recipients might have persistent asymptomatic hMPV infection, with 85.7% having hMPV RNA positive nasopharyngeal aspirates at least once during a one year study period (34).

In temperate climates, hMPV circulates predominately in the late winter and spring, overlapping with the seasonal distribution of RSV (1, 28, 81). However, other

communities have demonstrated year-round transmission, although with lower levels in late spring, summer and fall (42, 118, 120). There are two major genotypes of hMPV, and each genotype has two subgroups. Unlike influenza viruses, where a few distinct strains cause outbreaks each year, different hMPV lineages can co-circulate. It is not uncommon to have outbreaks occur in nearby communities caused by separate genotypes, and to have closely related strains circulate in far distant locations (1, 19, 90). In any given year, both genotypes and subgroups can be circulating, similar to that seen with RSV. A difference of illness severity between the two lineages has not been reported (61).

Given that the seasonal distribution of RSV and hMPV overlap in temperate areas, the potential for co-infection exists. Some studies have reported an increase in severity of illness with co-infection (61), but other studies show that they are uncommon and do not increase morbidity (20, 92, 115). hMPV has also been found to co-infect with the SARS virus, but this co-infection did not appear to increase pathogenicity (27).

Clinical manifestations

The clinical presentation of hMPV is indistinguishable from RSV, and involves both lower and upper respiratory tract infections (30). Rhinorrhea, fever and cough are the most frequently reported clinical findings (75). Otitis media, conjunctivitis and pharyngitis are common diagnoses in children with upper respiratory tract infection and bronchitis and bronchiolitis with lower respiratory tract infection (17, 42, 75, 76, 118, 120). Some studies have reported an association between wheezing and asthma exacerbations with hMPV infection (42, 117, 118). Lower respiratory tract infection with

hMPV is a frequent cause of hospitalization in children, second only to RSV, with infants and young children the most susceptible to severe infection (42, 81, 118). hMPV causes upper respiratory tract disease in approximately 1-5% of children, less commonly than other respiratory viruses such as influenza, parainfluenza, adenovirus and RSV (120). Adults with hMPV infection present with similar symptoms of upper and lower respiratory tract infection, and have diagnoses of bronchitis, pneumonia, and asthma exacerbations (45), and potentially exacerbations of chronic obstructive pulmonary disease (55).

There is limited evidence that hMPV can spread beyond the respiratory tract. hMPV RNA was detected by RT-PCR in brain and lung tissues of a previously healthy 14-month-old who died of encephalitis (99) and two other studies have described a total of six episodes of hMPV infection possibly related to encephalitis (49, 62). Given that other paramyxoviruses such as measles and Nipah virus are known to cross the blood-brain barrier, it is not unreasonable to speculate that hMPV may as well. To assess if hMPV causes a disseminated infection by screening other organs and blood has not been routinely performed and no conclusions can be made at this time.

Asymptomatic infection with hMPV has been reported. Falsey et al. reported nearly as many symptomatic as asymptomatic infections in adults using acute and convalescent sera compared to pre- and postseason sera collected from asymptomatic volunteers (44). However, Williams et al. reported only one hMPV infection from RT-PCR of nasal swabs in 86 asymptomatic children tested (120).

Diagnosis and treatment

hMPV can be diagnosed in cell culture, but grows poorly in only a limited number of cell lines with cytopathological effects taking over 10 days to manifest (23, 61). The most common method of diagnosis is currently the detection of viral RNA in RT-PCR. The nucleoprotein (N) and large polymerase (L) genes have been successfully targeted for genomic amplification. Real-time RT-PCR has also been developed that can detect all subgroups (74). Immunofluorescence is another rapid method to detect viral antigens, and hMPV-specific antibodies are available from commercial sources; however, this method may not be as sensitive as RT-PCR (61).

Beyond supportive care, there are few options for treatment. Ribavirin has been used for RSV, but is relatively ineffective partly due to its inability to decrease the host inflammatory reaction (61). Ribavirin has been tested against hMPV and can inhibit replication similarly as against RSV, although the effect in vivo has not been documented (122). Neutralizing monoclonal antibodies have been developed and tested in animals, and may provide a future method of prophylaxis for those at high-risk (110). Candidate vaccines are in development, but none are in clinical trials (24).

Paramyxovirus biology

Human metapneumovirus is a member of the family *Paramyxoviridae* and has been assigned to the subfamily *Pneumovirus* and the *Metapneumovirus* genus. The *Paramyxoviridae* are in the taxonomical order *Mononegavirales* which includes *Bornaviridae*, *Rhabdoviridae* and *Filoviridae* which all have linear, nonsegmented, single

stranded, negative sense RNA genomes. All viruses in this order, except the *Bornaviridae* and one *Rhabdoviridae* plant virus, replicate in the cytoplasm (66).

The *Paramyxoviridae* are enveloped viruses and have been defined by the fusion glycoprotein that causes viral cell fusion at neutral pH (67). Recently, hMPV has been shown to require low pH for fusion in vitro, and may represent a unique aspect of this paramyxovirus (101). Examples of *Paramyxoviridae* are listed in Table 1. The subfamily *Pneumovirus* differs from the subfamily *Paramyxovirinae* in the narrowness of the nucleocapsids, in the greater number of encoded proteins, and the structure of the attachment protein (67).

The *Paramyxoviridae* genomes are 15,000 to 19,000 nucleotides in length and all encode six genes that make the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), attachment (H, HN or G), and large (L) polymerase proteins (67). In addition, the *Rubulavirus* and *Pneumovirinae* genomes encode a small hydrophobic glycoprotein (SH). *Pneumovirinae* also have the M2 gene and the genus *Pneumovirus* has an additional 2 genes for nonstructural proteins NS1 and NS2 (Figure 1) (29, 67). The *Paramyxoviridae* have a lipid envelope that is derived from the host cell membrane and contains 2 major glycoprotein spikes (F, H/HN/G) and some members possess an additional SH glycoprotein. Inside the membrane is RNA encapsidated with the N protein. The L and P proteins are attached to the nucleocapsid core. The M protein underlies the lipid bilayer.

Paramyxovirus infection initiates with adsorption of the virus attachment protein to the cellular receptor, which differs by virus genus, and subsequent fusion of the virus

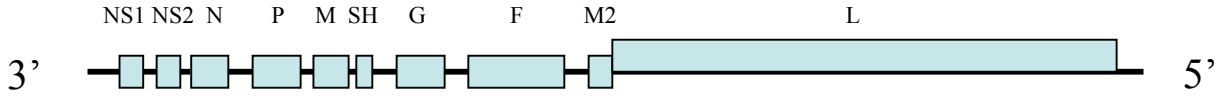
Table 1. **Examples of *Paramyxoviridae***

Subfamily	***Genus***	***Representative Viruses***
Paramyxovirinae	*Rubulavirus*	*Mumps virus*
		Human parainfluenza virus type 2, 4a, 4b
	Avulavirus	*Newcastle disease virus*
	Respirovirus	*Human parainfluenza virus type 1, 3*
		Sendai virus
	Henipavirus	*Hendra virus*
		Nipah virus
	Morbillivirus	*Measles virus*
		Rinderpest virus
Pneumovirinae	*Pneumovirus*	*Human respiratory syncytial virus (RSV)*
		Pneumomia virus of mice
	Metapneumovirus	*Human metapneumovirus (hMPV)*
		Avian metapneumovirus

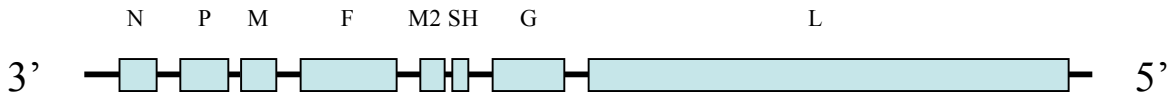
Figure 1. *Pneumovirus* and *Metapneumovirus* genome organization.

The *Pneumovirus* genome contains 10 open reading frames (ORFs) that encode 11 separate proteins and the *Metapneumovirus* genome contains 8 ORFs that encode 9 proteins. The M2 gene has two overlapping ORFs which encode two proteins. The genomes are flanked by 3' leader and 5' trailer sequences and have variable intergenic sequences.

Pneumovirus 15.2 Kb



Metapneumovirus 13.3 Kb



and cell membranes mediated by the fusion protein. The nucleocapsid is then released into the cytoplasm of the host cell. The RNA-dependent RNA polymerase transcribes the template in a stop-start manner to create viral mRNAs. Once viral proteins accumulate, new positive-sense antigenomes are produced that serve as templates to create new full-length, complementary, negative-sense genomic RNA with all the editing sites ignored. The nucleocapsid is then assembled and transported to the plasma membrane along with the matrix protein. The viral glycoproteins F, H/HN/G and SH are transported from the endoplasmic reticulum to the Golgi to the plasma membrane. Progeny virions are then released from the plasma membrane by budding (67).

Human metapneumovirus biology

Based on sequence identity, genomic organization and phylogenetic analysis, hMPV became the first mammalian virus to be classified a metapneumovirus. The sequences for the N, P, M, F, M2-1 and M2-2 ORFs had an overall amino acid identity of 80% between avian pneumovirus (APV) C and hMPV. The L and M2-2 ORFs had 64% and 56% homology, respectively. In contrast, RSV, the most closely related human pathogen, had sequence homology for these proteins between 22 and 44%. The SH and G proteins did not show sequence similarity to any viruses, and could explain the diverse host range between APV and hMPV (111).

There are genomic similarities and differences between RSV and hMPV. They both encode 3 envelope glycoproteins F, G and SH, although the order of the genes in the viral genome differs (Figure 1). Of these, the F gene is most conserved with 33% identity between them (111). The G gene of hMPV is smaller than that of RSV, and is the most

variable of all RSV and hMPV genes. Studies have demonstrated similarity of G between different subgroups of hMPV to be as low as 33%, with no homology to RSV G (8, 59). Human metapneumovirus lacks the two nonstructural genes, NS-1 and NS-2 that encode anti-interferon activity in RSV (61). It is unknown whether this has an impact on the pathogenesis of hMPV.

The F glycoprotein of hMPV is a type I transmembrane surface glycoprotein with a membrane anchor near its carboxyl terminus. It is synthesized as an inactive F_0 precursor that is activated by cleavage into two subunits, F_1 and F_2. The amino-terminus of the F_1 subunit contains the hydrophobic fusion peptide that initiates fusion. The F_1 subunit also contains two heptad repeats at the NH_2- and COOH-terminals. The F_2 subunit is extracellular and linked to F_1 by a disulfide bond (29, 68). A recent study demonstrated peptides from the heptad repeat region of F inhibit virus-cell fusion, supporting the hypothesis that hMPV F functions similarly to the F glycoprotein of other paramyxoviruses (77).

Unlike RSV, F_0 from hMPV only contains one cleavage activation site (RQSR) which does not conform to the consensus furin motif of RSV and APV (111), and trypsin is required for growth of hMPV in cell culture (17, 53, 103, 112). However, a naturally occurring strain variation with a serine to proline substitution (RQPR) did not require trypsin to grow in Vero cells, nor did recombinant viruses with this S101P substitution (98). The S101P viruses were also equally virulent in hamsters with replication restricted to the respiratory tract.

The hMPV G glycoprotein is a type II transmembrane glycoprotein with a hydrophobic region at the NH_2-terminal that comprises the signal peptide and membrane

anchor. Similar to RSV, it has a high content of serine, threonine and proline residues and migrates in gel electrophoresis as a diffuse band approximately 40 kDa larger than predicted, which is suggestive of a high carbohydrate content (29). The SH protein is a short integral membrane protein of 180 amino acids with an unknown function (11). Reverse genetics systems have demonstrated that the G and SH genes are not essential for virus viability for either APV or hMPV (12, 14, 80). Biacchesi et al. created recombinant hMPVs lacking the G gene, SH gene or both and demonstrated effective replication in cell culture. Except for the proteins encoded by the deleted genes, mutant viruses had a similar protein expression profile as that of the wild-type. Viruses without the G gene showed restricted replication in a hamster model compared to wild type, but still induced neutralizing antibodies and conferred protection to wild type hMPV challenge. Viruses without the SH gene replicated more efficiently in the hamster model (14).

A similar study was done by the same group using nonhuman primates and recombinant hMPV in which the SH, G or M2 gene was deleted. Replication occurred with G- and M2-deleted viruses but was reduced 6-fold and 160-fold in the upper respiratory tract and 3,200-fold and 4,000-fold in the lower respiratory tract, respectively. The SH deletion mutant replicated only slightly less than the wild-type. Therefore, none of these proteins are essential for replication, although G and M2 increase efficiency. All of the recombinant viruses were immunogenic and induced protective immune responses against challenge with wild-type hMPV (12).

Human metapneumovirus was originally described as having two serotypes with two genetic lineages for each (112, 114). Using a ferret model, van den Hoogan et al. raised antisera against isolates from prototype viruses from the two lineages and

demonstrated that serum obtained from an animal infected with one genotype could not neutralize a virus of the heterologous genotype in vitro, suggesting that they constitute separate serotypes (114). However, the amino acid identity between two prototypical strains (CAN98-75 and CAN97-83) ranges from 85% to 97% for the N, P, M, F, MS-1, MS-2 and L proteins, similar to RSV subgroups, suggesting that these two lineages are highly related (13). While the G gene is more divergent between the groups in hMPV than RSV, the F gene is more conserved.

To address this issue, Skiadopoulos et al. used the two strains in rodent and nonhuman primate models (103). In hamsters, the two lineages exhibited 48% antigenic relatedness based on reciprocal cross-neutralization assays, and infection with either strain provided protection against infection with the heterologous strain. Immunization with a recombinant human parainfluenza virus expressing the fusion protein from one strain resulted in serum antibodies that neutralized virus from both strains and protected from a challenge with either strain. Experiments with three nonhuman primate species, chimpanzees, African green monkeys and rhesus macaques, demonstrated that the two virus strains are 64 to 99% related antigenically. hMPV replicated efficiently in chimpanzees and African green monkeys and conferred protection from challenge with heterologous strains. These data suggest that the two genotypes may not be distinct serotypes, but the relevance to humans is still unknown.

Several studies have demonstrated two main lineages and four sublineages based on F and N sequence homology (1, 19, 73, 121). Australian researchers used the P gene to identify the four viral subtypes and create a global classification scheme. They denoted the four sublineages as A1, A2, B1 and B2 (74). The major difference between

the A and B genotypes are the polymorphisms in the G and SH proteins, with a 32 to 37% amino acid identity in G between A and B (1, 8, 88). No difference in severity among the various hMPV isolates has been found. The epidemiology of the circulating strains is complex, with viruses of all genotypes co-circulating, and dominant strains varying by location and year (1).

Immunity and human metapneumovirus

In RSV, the innate immune system, particularly pulmonary natural killer (NK) cells and cytotoxic T-lymphocytes (CTLs) are important in clearing primary infections, and are thought to confer short-term protection against reinfection during an epidemic (29). Respiratory tract secretory IgA antibodies also play a role in short-term protection, and over time with multiple reinfections, a more sustained secretory antibody response is obtained and may provide long-term protection (29). In RSV, the adaptive immune response is key to recovery from infection and the prevention of reinfection. The F and G glycoproteins have been used to induce serum antibody production, and were shown to be the only RSV proteins that induced long-term resistance to challenge. However, this protection is incomplete, probably partly due to decreased efficiency of antibody gaining access to the respiratory tract, with RSV-neutralizing antibodies providing better protection in the lower than the upper respiratory tract (29). The RSV F and G surface glycoproteins, but not SH, are significant neutralization antigens and are the major protective antigens. This is similar to other paramyxoviruses such as human parainfluenza virus (hPIV) types 1-4 where F and HN are the major neutralization and protective antigens (64).

Initial studies with recombinant hPIV1 vectors expressing the hMPV F protein and a chimeric human-bovine PIV3 expressing hMPV F protected hamsters from challenge with wild-type hMPV, demonstrating that F is a major neutralization antigen similar to RSV (103, 106). Skiadopoulos et al. also determined the contributions of all three hMPV surface glycoproteins in induction of neutralizing antibodies and protective immunity. Although the SH protein from RSV does not provide protection, hMPV SH is almost 2.5 times larger and could present a significant cell surface target. They created recombinant hPIV1 vectors that individually expressed F, G or SH and immunized hamsters with either one or two inoculums of each. To determine if serum antibodies were present, they conducted enzyme-linked immunosorbent assays (ELISAs) with hMPV-infected LLC-MK2 cells as the antigen coat. All animals that received rhPIV1-F developed a high antibody titer that was comparable to hMPV wild-type control inoculation. Two doses of rhPIV1-G resulted in one-tenth the titer of hMPV-binding antibodies, and immunization with SH protein did not induce any detectable antibodies. The rhPIV1-F also induced neutralizing antibodies and resistance to challenge, although not as great as in animals immunized with wild-type virus. In contrast, two doses of rhPIV1-G or rhPIV1-SH did not induce detectable neutralizing antibodies, although rhPIV1-G provided partial protection to challenge (102). These results confirm that F is the major antigenic determinant for hMPV.

Unlike with other paramyxoviruses, including the most closely related human pathogen RSV, G does not appear to induce immunity in hMPV infection. The hMPV G and SH glycoproteins are heavily glycosylated, which could affect their antigenicity and immunogenicity. However, RSV G is also glycosylated and still induces neutralizing

antibody. It is possible that G and SH may be poor immunogens because they lack essential roles in infection and replication (12, 14). hMPV F and G may need to be expressed together to induce immunity; however that is not seen with other paramyxoviruses, and sera from naturally or experimentally infected nonhuman primates recognize hMPV F and not G in Western blot (15, 102).

Although immunization with a vector containing hMPV F protein can induce a protective antibody response, it cannot be determined whether this is due solely to the presence of neutralizing antibodies. Ulbrandt et al. obtained monoclonal antibodies from immunized mice and hamsters and found three that neutralized all four subgroup prototypes (110). Two of the monoclonal antibodies exhibited high-affinity binding to the F protein and protected hamsters against infection when given 24 hours before infection in both *in vitro* and *in vivo* models, demonstrating that antibody alone can be protective.

Serological tests to detect antibodies to hMPV have been developed. Initial tests used immunofluorescence assays or ELISA tests using hMPV-infected cells (17, 38, 39, 44, 112). Subsequently, efforts increased to develop tests based on recombinant expressed hMPV proteins, particularly the conserved N and F proteins. Leung et al. used cell lysates infected with vesicular stomatitis virus (VSV) expressing the hMPV F glycoprotein (70), and Ishiguro et al. developed an immunofluorescence assay (IFA) using insect cells infected with a recombinant baculovirus expressing hMPV F protein (60). Studies have shown that antibodies reactive to the hMPV nucleoprotein (N) can detect and distinguish between different *Metapneumovirus* (avian vs. human) and do not cross-react with *Pneumovirus* (3). Use of the N protein for antibody detection has been

performed by Hamelin and Boivin who purified His_6 tagged N protein expressed in recombinant *Escherichia coli* via nickel agarose and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and used it in ELISA to detect antibodies (54), and Liu et al. used lysates from insect cells infected with recombinant baculovirus expressing hMPV N protein in ELISA (71).

Reinfection with hMPV

Soon after the discovery of RSV as a significant cause of respiratory disease in children, it was determined that repeated infections could occur. Consequently, RSV is a common respiratory tract illness in adults (29). An early prospective study followed children from infancy through ten years and measured immunity by either the failure to become reinfected with RSV or a reduction in severity of clinical illness upon reinfection. They found that during epidemics, the attack rate decreased from 98% for the first infection, to 75% for a second infection and to 65% for a third. The time since the initial infection was important in determining protection, with no protection if exposed over one year after the first infection; however, severity was reduced with a third infection (56).

Another study determined the duration of immunity in adults after natural infection with RSV. Volunteers were challenged with RSV from the same strain group six times between 2 and 26 months after their natural infection. At 2 months, 47% experienced reinfection, which decreased to 30% by the time of their sixth re-exposure. Overall, 73% had two or more and 47% had three or more reinfections. The presence of higher neutralizing antibody titers to F and G correlated with protection against infection;

however, this protection was incomplete with a 25% risk of reinfection in those with the highest titers (52).

Experimental infection with various animal models has demonstrated that primary infections can protect against subsequent reinfection if challenged 4-6 weeks later (12, 103). In addition, immunization with recombinant virus or vectors expressing the fusion protein has also proven effective when challenged 6 weeks later (24, 106). Since these experiments did not prove long lasting immunity, van den Hoogen et al. designed an experiment with cynomolgus macaques to evaluate the length of protective immunity (113). Animals were inoculated with one of the two genotypes at 0, 6 and 10 weeks and then challenged with homologous or heterologous virus 11 months later. Other animals received homologous challenges 4, 6 and 12 weeks after a primary infection. While the animals were protected completely from homologous virus at 6 weeks and partially at 12 weeks, protection was greatly decreased 11 months after the last inoculation. None of the heterologous challenged animals had any protection, and one of three homologous challenged animals had lower viral genome copy numbers. This study confirmed waning immunity to hMPV after initial infection in this animal model, and suggests that lineages A and B represent different serotypes.

Data on the risk of natural reinfection with hMPV is limited. Many viruses with replication limited to the respiratory tract induce only transient immunity, unlike other systemic viruses (29). Since infection with RSV causes reinfection throughout life, it is expected that infection with hMPV would also induce only limited immunity. There have been few reports of hMPV reinfection in children. Soon after discovery of the virus, Pelletier et al. retrospectively tested respiratory specimens from an

immunocompromised 17 month-old child who died from an unknown respiratory disease (87). Specimens were also available from an infection 10 months previously. Both samples were positive only for hMPV, and were of different genotypes. Ebihara et al. also reported a case of a 9 month old child who was infected with two different strains of hMPV 19 days apart (37). Retrospective testing using PCR to detect viral RNA in respiratory specimens from previously undiagnosed cases of illness found 13 of 154 patients with evidence of infection twice during the study period (118, 120). This study is from the Vanderbilt Vaccine Clinic that enrolls healthy, full-term infants and follows them until 5 years of age to determine rates and impact of respiratory and enteric viruses. Of the 13 patients, reinfection occurred from one to 38 months apart, with an average of 17.6 months. They had genotype information for both infections on 6 of the 13 patients. Two were infected with different genotypes, two with different subtypes of the same genotype and two with the same subtype. In a study in Japan, out of 26 respiratory samples PCR positive for hMPV RNA, 26 acute and 10 convalescent serum samples were available for testing using indirect IFA with hMPV infected cells (38, 39). Twelve of the patients had undetectable IgG antibody in the acute sera, but IgM was positive in 11 of the 12. Three of these patients had convalescent sera available, and all seroconverted with positive IgG titers. Of the 14 with IgG positive acute titers, five of seven had at least a four-fold increase in their titers in the convalescent phase. Ages of the patients ranged from 6 months to 5 years 9 months. These data are suggestive of reinfection in approximately half of the detected cases. However, the number of paired sera available for testing was limited, and since most of these children would have been

previously infected with RSV, it is possible that there could be cross reactive antibodies to RSV creating false positives.

A prospective study using ELISA to test acute and convalescent sera and RT-PCR to test respiratory specimens determined differences in hMPV rates in various adult populations. Their cohorts included normally healthy adults >64 years, < 40 years, adults with underlying cardiopulmonary disease, and residents of a long-term care facility. Rates differed between the two years of the study, but overall respiratory disease was caused by reinfection with hMPV in 1.7% of healthy older adults, 2.9% of high-risk adults, 6.6% of young adults and 5.4% of long-term care facility residents (44).

The ability of hMPV to cause reinfections in immunocompetent hosts presents a challenge to vaccine development. Most reported cases of reinfections have been in immunocompromised patients, the very young, or adults. Infants and young children have had limited opportunity for multiple exposures and boosting, which as demonstrated for RSV, may be required for adequate immunity (52). Adults may have waning immunity after absence of exposure for many years, and may become reinfected when exposed to young children. Since most people show evidence of prior infection by the age of 5, the ability to mount an effective immune response later in childhood support immunization efforts. However, lack of an effective adaptive immune response would suggest exploration of new avenues for protection.

Hypothesis

The fusion glycoprotein of hMPV is highly conserved and a major immunologic determinant. Diagnostic and therapeutic interventions have been developed using F; however, there has been little characterization of the structure and properties of this important viral component in its native state. In contrast, the attachment glycoprotein is highly variable and is not essential for viral infection and replication, and provides very little protection after immunization. This is unusual for the paramyxoviruses and further research is necessary as to why this viral component is so different than in comparison to other paramyxovirus attachment proteins.

The impact of initial hMPV infections in early childhood and the capability of hMPV to cause reinfections in adult populations have been documented, especially in immunocompromised individuals or those with underlying illnesses. The ability of hMPV to cause reinfections in childhood has not been systematically determined. The F glycoprotein could be used in ELISA to calculate reinfection rates in paired sera samples to determine the impact hMPV has in older children.

The overall hypothesis of this dissertation is that using novel cloning techniques, recombinant hMPV F and G glycoproteins can be constructed and expressed which display tertiary structure and functional epitopes of natural hMPV glycoproteins. The hMPV F glycoprotein will prove to be an effective antigen in binding natural antibody and will express proper epitopes that can be utilized in an indirect ELISA to show that hMPV is a significant cause of illness in children who have previously been infected with the virus. Specific hypotheses include:

Hypothesis #1: Removal of the transmembrane domain will result in secretion of the F glycoprotein possessing native, antigenic features and that a similar construct of G will not produce the same level of antigenicity.

Hypothesis #2: Soluble F can be used in ELISA or Western blot assays to detect hMPV antibodies in animal and human specimens.

Hypothesis #3: Retrospective serosurvey of acute and convalescent serum samples from Thai school-children will provide evidence of near universal exposure with reinfection causing some acute illnesses.

Chapter 2 – Methods

Cell lines

MK2 cells, provided by Kevin Yim (Virion Systems, Inc.), and Vero cells were maintained in Eagle's minimal essential medium (Quality Biologicals, Gaithersburg, MD) supplemented with 10% cosmic calf serum (Hyclone, Logan, UT) 2mM L-glutamine, 100 units/ml penicillin and streptomycin and 10 µg/ml gentamicin (Quality Biologicals) (EMEM-10). Hela-USU and 293 cells were maintained in Dulbecco's modified Eagle's medium (Quality Biologicals) supplemented as described above (DMEM-10). These cultures were maintained at 37ºC in 7.5% CO_2.

Sf9 cells (Invitrogen, Carlsbad, CA) were maintained in Grace's insect medium (Invitrogen) supplemented with 10% Gibco™ fetal bovine serum (Invitrogen) and 10 µg/ml gentamicin. High Five™ cells (Invitrogen) were maintained in suspension culture with Express Five® Serum Free Media (Invitrogen) supplemented with 2 mM L-glutamine and 10 µg/ml gentamicin in a spinner flask at a concentration of 1-3 x 10^6 cells/ml. These cultures were maintained at 27ºC in the absence of CO_2.

Antibodies

The following antibodies were used in Western blot and immunoprecipitations: Polyclonal rabbit antiserum against hMPV F and G was purchased from Sigma Genosys (The Woodlands, TX) and obtained by immunization of rabbits with a synthetic peptide of either of the following sequences: CTGAPPELSGVTNNG, which corresponds to the cytoplasmic tail of hMPV F or KARVKNRVARSKC, which corresponds to the cytoplasmic tail of hMPV G. The peptide was conjugated to keyhole limpet hemocyanin

for immunization. Monoclonal antibody against hMPV F was kindly provided by Nancy Ulbrandt (MedImmune, Inc.).

Cloning of the hMPV F and G genes

The hMPV genes were amplified from the CAN97-83 strain of the virus kindly provided by Kevin Yim (Virion Systems, Inc.). LLC-MK2 cells at 90% confluency in six-well tissue culture dishes were infected with CAN97-83 at a multiplicity of infection (MOI) of 1. Cells were incubated for one hour at 37ºC with rocking every 15 minutes, then the virus removed and replaced with EMEM-10. The cells were incubated for 72 h then RNA was extracted using the RNEasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was created using RT-PCR and oligo dT primers. The primers were added to the RNA, incubated at 70ºC for 10 min, then the PCR reagents added and run at the following settings: 42ºC for 60 min, 70ºC for 15 min and 4ºC for 5 min.

Full-length F and G proteins were made using the cDNA. To obtain a full-length S-peptide tagged form of the hMPV F protein, the following oligonucleotides were used to generate clones: 5'-GGAATTCATGTCTTGGAAAGTGGTGATC-3' and 5'-CCATCGATCTAGCTGCTCATGTGCTGCGTTCGAATTTAGCAGCAGCGGTTTCTTTACTGTGTGGTATGAAGCCATTGTTTGTGAC-3'. To obtain a full-length S-peptide tagged form of the hMPV G protein, the following oligonucleotides were used to generate clones: 5'-GGAATTCATGGAGGTGAAAGTAGAGAAC-3' and 5'-CCATCGATCTAGCTGTCCATGTGCTGGCGTTCGAATTTAGCAGCAGCGGTTTCTTTGTTTTGCATTGTGCTTACAGATGCCTGTGG-3'. PCR was done with the

following settings: 94ºC for 5 min, then 30 cycles of 94ºC for 1 min, 50ºC for 30 sec, 72ºC for 2 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and then subcloned into pCRII-Blunt-TOPO (Invitrogen). These TOPO constructs were further subcloned into pCAGGS (82) as *Cla*I-*Eco*RI fragments and subcloned into phCMV1 (Gene Therapy Systems, Inc., San Diego, CA) as *Eco*RI fragments.

The TOPO construct was used as a template for PCR amplification with the following plasmids: 5'-GCGGTCGACATGTCTTGGAAAGTGGTGATC-3' and 5'-GAACGCCAGCACATGGACAGCTAGGGTACCGC-3' for hMPV F and 5'-GCGGTCGACATGGAGGTGAAAGRAGAGAAC-3' and 5'-GAACGCCAGCACATGGACAGCTAGGGTACCGC-3' for hMPV G. PCR was done with the following settings: 94ºC for 5 min, then 30 cycles of 94ºC for 30 sec, 65ºC for 30 sec, 72ºC for 3 min. The PCR products were digested with *Sal*I and *Acc*65I and then ligated to the vector pMCO2 (25).

For baculovirus expression, truncates of S-peptide tagged F and G proteins that lack the transmembrane domain and signal sequence were made using cDNA and the following oligonucleotides: 5'-CCGCTCGAGAAAGAGAGCTACCTAGAAGAATC-3' and 5'-GTCGACCTAGCTGTCCATGTGCTGGCGTTCGAATTTAGCAGCAGCGGTTTCTTTCCCTTTCTCTGCACTGCTTAGGATTCTG-3' for soluble hMPV F and 5'-CCGCTCGAGAACTACACAATACAAAAAACC-3' and 5'-GTCGACCTAGCTGTCCATGTGCTGGCGTTCGAATTTAGCAGCAGCGGTTTCTTTGTTTTGCATTGTGCTTACAGATGCCTGTGG-3' for soluble hMPV G. The PCR,

purification, and cloning into pCRII-Blunt-TOPO was performed as described previously. These TOPO constructs were further subcloned into pMelBac (Invitrogen) as *Xho*I-*Sal*I fragments.

For mammalian cell expression, a truncate of S-peptide tagged F protein that lacks the transmembrane domain was made using cDNA and the following oligonucleotides: 5'-CCGCTCGAGATGTCTTGGAAAGTGGTGATCAT-3' and 5'-GGATCCCTAGCTGTCCATGTGCTGGCGTTCGAATTTAGCAGCAGCGGTTTCTTTCCCTTTCTCTGCACTGCTTAGGATTCTG-3'. The PCR, purification, and cloning into pCRII-Blunt-TOPO was performed as described previously. These TOPO constructs were further subcloned into phCMV1 as *Xho*I-*Bam*HI fragments.

For mammalian cell expression, a truncate of G protein that lacks the transmembrane domain was made using cDNA and the following oligonucelotides: 5'-GATATCAACTACACAATACAAAAAACC-3' and 5'-TCTAGACTAGTTTTGCATTGTGCTTAC-3'. The PCR, purification, and cloning into pCRII-Blunt-TOPO was performed as described previously. These TOPO constructs were further subcloned into phCMV1-Hendra-S-Ig-κ, the Hendra virus G clone with 5' S-peptide tag and Ig κ linker in the phCMV1 vector, kindly provided by Yee-Peng Chan. The Hendra G gene was removed by digestion with *Xba*I and *Eco*RV and the hMPV G from the TOPO construct digested with the same enzymes to ligate into the phCMV1 vector.

Transfection and metabolic labeling

HeLa-USU, LLC-MK2 , Vero, NS0 and 293 cells in T-25 cm tissue culture flasks were transfected with expression plasmids using FuGene 6 transfection reagent (Roche, Indianapolis, IN) (HeLa-USU, LLC-MK2, Vero) or GenePorter2 (Genlantis, San Diego, CA) (NS0, 293) according to the manufacturer's instructions and incubated overnight at 37ºC. For pMCO2 transfections, the cells were then infected with vaccinia virus at an MOI of 10 and incubated at 37ºC for 6 h. For radiolabeling, the media was removed and replaced with minimal essential medium without methionine or cysteine containing 2.5% dialyzed fetal calf serum (Invitrogen) and 100 μCi/ml ^{35}S-cys/met Redivue Promix (Amersham Pharmacia Biotech, Piscataway, NJ), and incubated at 37ºC overnight. Cells were rinsed with phosphate buffered saline (PBS) then chased with DMEM or EMEM-10. If not radiolabled, 1ml of fresh media was added to each transfection after the overnight incubation and incubated an additional 24 h at 37ºC.

Baculovirus transfection and expression

The pMelBac vector containing sF or sG (4μg of DNA) was co-transfected with 0.5 μg of Bac-N-Blue™ DNA (Invitrogen) into Sf9 cells using Cellfectin® Reagent (Invitrogen) as per the manufacturer's instructions. The virus-containing supernatant was used to infect monolayers of Sf9 cells in plaque assays to isolate pure recombinant virus, and the presence of the sF or sG gene was confirmed through PCR amplification of the inserted gene. A high titer viral stock was obtained through repeated infections of confluent Sf9 cells and the titer determined through plaque assay. Optimization of protein expression was performed by trying different MOIs and duration of infection of

High Five™ cells in both adherent and suspension culture, with an MOI of 10 and 5-6 days demonstrating the highest level of protein expression.

Immunoprecipitation

Following metabolic labeling or transfection only, supernatants were removed, clarified by centrifugation and Complete protease inhibitor added (Roche). Cells were harvested by scraping, pelleted by centrifugation then resuspended in 200 µl of lysis buffer (100mM Tris-HCl, pH 8.0, 100 mM NaCl, 1.0% Triton X-100) containing Complete protease inhibitor. The lysates were incubated on ice for 10 min, and the nuclei removed by centrifugation. If using antiserum, the lysates or supernatants were precleared by incubation with Protein-G-Sepharose (Amersham Pharmacia Biotech) for 45 min at room temperature. Typically, 1 µl of antiserum or 60 µl of a 50% slurry of S-protein agarose (Novagen, San Diego, CA) was added to the lysate or supernatant and incubated at 4ºC overnight or for 1 h at room temperature. Protein-G-Sepharose beads were then added and incubated for 45 min with the protein-antiserum mixture. Protein G or S-protein agarose were washed twice with lysis buffer and once with lysis buffer containing 0.1% SDS and 0.1% sodium deoxycholate. Proteins were separated either by SDS-PAGE on a 10% polyacrylamide gel, or by a NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen). Proteins were visualized either through autoradiography (metabolic labeling) or Western blotting.

Gel electrophoresis, Coomassie staining and Western blotting

Purified proteins, cell supernatants or lysates were separated by SDS-PAGE on a 10% polyacrylamide gel, or by a NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen). Gels

were stained with Simply Blue SafeStain (Invitrogen) to visualize protein bands. For Western blot analysis, separated protein bands were transferred to nitrocellulose membranes and then blocked with 5% milk in PBS with 0.5% Tween 20 (PBS-T) (Sigma-Aldrich, St. Louis, MO) overnight at 4ºC or for 1-2 h at room temperature. The membranes were then incubated for 1 h at room temperature with antiserum, hMPV F mAb (110) or anti-S-peptide antibody conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX) at 1:25,000 dilution. Membranes were washed four times with PBS-T and incubated with horseradish peroxidase conjugated AffiniPure goat anti-Syrian hamster IgG, goat anti-Armenian hamster IgG (Jackson Laboratories, West Grove, PA), ImmunoPure goat anti-human IgG (Pierce Biotechnology, Rockford, IL) or rabbit anti-monkey IgG (Sigma-Aldrich) at 1:25,000 dilution for 1 h at room temperature, except for the anti-S-peptide antibody that could proceed directly to chemiluminescence. The remaining membranes were washed again four times with PBS-T and visualized by chemiluminescence (Super-Signal West Pico chemiluminescent substrate, Pierce Biotechnology).

Protein purification

293 cells expressing S-peptide tagged sF or sG were grown to confluency in tissue culture roller bottles in DMEM-10 at 37ºC and CO_2 manually added to the bottles before incubation. The media was removed, the cells were rinsed twice with PBS and low serum Opti-MEM (Invitrogen) was added. The cells were incubated for 72 h and the supernatant clarified by centrifugation. An XK26 column (Amersham Pharmacia Biotech) was poured with 20 ml of S-protein agarose (Novagen, Inc.) and washed with 10

bed volumes of PBS. L-arginine (0.1 M) (Sigma-Aldrich) and Triton X-100 (0.1%) were added to the supernatant and it was passed through a low-protein binding filter. The supernatant was then passed over the agarose at a flow rate of approximately 5 ml/min and the column was washed with 10 bed volumes of PBS with 0.1% Triton X-100 and 0.1 M L-arginine and 5 bed volumes of PBS with 0.5 M NaCl, 0.5% Triton X-100, 0.1 M L-Arginine and 0.02 M Tris. The sF and sG were eluted with 1 bed volume of 0.2 M citric acid, pH 2 and neutralized with HEPES, pH 8. The eluate was then concentrated using 30 kDa Centricon centrifugal filter units (Millipore, Billerica, MA) and buffer exchanged into PBS with 0.2 M L-arginine and 0.01% Triton X-100. Protein concentrations were determined by SDS-PAGE and Coomassie staining with SimplyBlue SafeStain (Invitrogen) and densitometry analysis with Scion Image (Frederick, MD) software by comparison to known protein standards.

Molecular weight determinations

Six high molecular weight protein markers (Sigma-Aldrich) were separated on a Superdex 200 gel filtration column 10/300 in degassed PBS using an Amersham Pharmacia Biotech P-500 pump at a constant flow rate of 24 ml/h to determine their elution volumes (V_e). Void volume (V_o) was determined using blue dextran (1 mg/ml). Each eluted fraction of 1 ml was collected at 1 min intervals and monitored by UV absorption at 280 nm. A K_{av} for each protein was calculated using formula $K_{av} = (V_e - V_o)/ (V_t - V_o)$, where V_t is the total column bed volume. A calibration curve was generated by plotting the calculated K_{av} for each protein against the log of the known molecular weight. The sF and sG were separated on the calibrated column under

conditions identical to those used for the protein markers, and their elution volumes (V_e) and K_{av} were calculated. Molecular weights of sF and sG were determined by plotting on the calibration curve. Fractions containing sF and sG as determined by UV absorption were confirmed by SDS-PAGE and Western blotting and Coomassie staining.

Sucrose gradient analysis

Native forms of soluble hMPV F and G glycoproteins, purified by different methods, were analyzed by sucrose gradient centrifugation. Purified sF protein in PBS buffer with 0.2 M L-arginine and 0.01% Triton X-100 was purified as described above (sF_{A2}) and sF protein from the B1 lineage in PBS buffer provided by Nancy Ulbrandt (MedImmune, Inc.) (sF_{B1}) was purified as previously described (110) . In addition, low-serum culture medium (20 ml of Opti-MEM) from two T-150 cm^2 cell-culture flasks confluent with 293 cells expressing sF and sG (48 h at 37ºC), was collected, clarified by centrifugation, buffer-exchanged into PBS, concentrated using 30-kDa Centricon centrifugal filter units (Millipore), and further concentrated by Centriprep (Millipore). The final volume of 0.4 ml was divided into two equal portions of 0.2 ml.

One portion of the unpurified sG and $sF_{A2,}$ and 50 µg of the purified sF_{A2} and sF_{B1} was cross-linked with the reducible reagent 3,3'-dithiobis-(sulfosuccinimidylpropionate) (DTSSP; Pierce Biotechnology) at a final concentration of 5 mM for 30 min at room temperature. The reaction was quenched with 100 mM Tris (pH 7.5) for 15 min at room temperature, and the samples were layered onto the sucrose gradients.

The sucrose gradients were prepared by underlying 6 ml of 5% sucrose with 6 ml of 20% sucrose in polyallomer 14- by 95-mm tubes (Beckman Coulter, Inc., Fullerton,

CA). The sucrose was dissolved in a 100mM Tris, 100mM NaCl solution. A linear sucrose gradient was generated with a gradient master (Biocomp, Frederickton, NB, Canada) at an angle of 81.5º for 1 min 55 seconds at a speed of 15 rpm. The cross-linked and uncross-linked preparations of sG and both versions of sF_{A2} and sF_{B1} were overlaid on top of the gradients and centrifuged at 40,000 rpm for 20 h using an SW40 rotor (Beckman Coulter, Inc.). Fractions of approximately 800 µl were collected from the bottom of the gradient using a Beckman fraction recovery system and automated fraction collector. A portion (10 µl) of each fraction was boiled in sample buffer with and without 5% β-mercaptoethanol, and the proteins were separated by electrophoresis and analyzed by Coomassie staining or Western blotting. sG was first immunoprecipitated with S-protein agarose before addition of sample buffer.

Serum samples

Control human serum samples from children infected with hMPV or without hMPV infection were kindly provided by Dr. Jeffrey Kahn (Yale University) (70). For development of standards, single donor human serum was purchased (Innovative Research, Southfield, MI) and calibrated to known human positive sera. Pre- and post-immunization sera after infection with CAN97-83 (lineage A2) or CAN98-75 (lineage B2) from each of the following species was kindly provided by Dr. Mario Skiadopoulos (NIH): Chimpanzee (post day 28), Rhesus macaque (post day 28), African green monkey (post day 43), Cynomolgus macaque (post day 28), rabbit (post day 71) and hamster (pooled from the two lineages, post day 200).

A prospective study of dengue virus transmission and disease in primary school children was conducted in Thailand from 1998 to 2002. The study site is in Kamphaeng Phet Province, 358 km northwest of Bangkok, in subdistrict Muang, which has a population of 198,943 according to the year 2000 census (40, 41). Children were enrolled from 12 elementary schools and baseline demographic and blood samples taken. Active case surveillance of the cohort schools started on 1 June and ended 15 November each year, concurrent with peak dengue transmission. School absence or visit to a school nurse was used as an indicator of possible illness. After notification, the village health worker traveled to the home of the child to evaluate the reason for absence. If there was a history of fever within seven days of the absence or an oral temperature of 38ºC or greater, the child was brought to the public health nurse for symptom questionnaire, physical examination and blood draw. A convalescent blood sample was also collected approximately 14 days later. Sera were stored at -20ºC at the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand. The Walter Reed Institute of Research (WRAIR) Human Use Committee and the Thai Ethical Review Committee, Ministry of Public Health, Nonthaburi, Thailand approved the protocol under which the samples were collected. The Uniformed Services University Institutional Review Board approved the use of the serum samples for this study as an exempt protocol.

Enzyme-linked Immunosorbent Assay (ELISA)

An ELISA was developed to detect anti-hMPV antibodies in animal or human sera using sF as the antigen. Ninty-six well microtiter plates (Immulon II) were coated

with purified sF protein in 50 mM sodium bicarbonate and 20 mM sodium carbonate buffer (pH 9.6) at a concentration of 50 ng of protein/well and incubated overnight at 4°C. The plates were washed six times with PBS-T and blocked overnight at 4°C or for 1 h at 37°C with PBS-T with 5% bovine serum albumin (BSA) (Sigma-Aldrich) added. The plates were washed 6 times with PBS-T and dilutions of serum in PBS-T containing 1% BSA were added and incubated for 1 h at 37°C. The plates were washed 6 times and incubated for 1 h at 37°C with alkaline phosphatase-conjugated Affini-pure goat anti-mouse IgG, goat anti-Syrian hamster IgG, goat anti-rabbit IgG, goat anti-human IgG, goat anti-human IgM (Jackson ImmunoResearch), or rabbit anti-monkey IgG (Sigma-Aldrich) diluted 1:5000. The plates were washed again six times with the same solution and antibodies were detected by adding 100 ul of PNPP substrate (Pierce Biotechnology) to each well and incubating for 30 min at room temperature and then adding 50 ul of 2 N NaOH to each well to stop the reaction. The absorption at 405 nm was measured on a VersaMax microtiter plate reader (Molecular Devices, Sunnyvale, CA).

To test the ability of guanidine hydrochloride to separate the antigen-antibody bond in antibodies of different age and maturation, 0.5 M of guanidine hydrochloride (Sigma-Aldrich) was added to the PBS-T 1% BSA solution and the sera was incubated in this solution on the ELISA plate for 2 h at room temperature while rocking gently.

Animal immunization

BALB/c mice were purchased from a commercial breeder (Jackson Laboratory, Bar Harbor, ME) and fed a standard diet and water ad libitum. Animals were

anaesthetized by isoflurane inhalation prior to immunization. Mice in groups of four were immunized with either 10 μg of sF, s10 μg of sG, or 5 μg each of sF and sG all adjuvanted 1:1 with TiterMax Gold (Sigma). The immunization was split equally and given by intramuscular and intraperitoneal injection. The mice were immunized three times 28 days apart and bled by tail vein on day 0, day 43 and day 66.

Statistical analysis

Mean optical densities (OD) were compared using two-way analysis of variance (ANOVA) with main effects of antigen and dilution to compare the two antigens after adjusting for dilution (SPSS v. 14 for Windows). Differences in antibody avidity after administration of guanidine were tested using repeated measures ANOVA with both guanidine and convalescent vs. acute as within-subject factors. Wilks' lambda statistic was used to compare the guanidine difference between acute and convalescent sera (SPSS v. 12 for Windows). Comparison between untested samples, tested samples and positive samples were tested using t-tests for continuous variables and Chi-squared tests for categorical data (Excel 2007). *P*-values <0.05 are considered statistically significant.

Chapter 3 – Characterization of Soluble, Human Metapneumovirus Fusion and Attachment Proteins

Introduction

Fusion glycoproteins are major antigenic determinants for all the paramyxoviruses, as well as for other viruses such as human immunodeficiency virus, Ebola virus and influenza which have similar functional fusion glycoproteins. Paramyxovirus attachment glycoproteins can also be antigenic, including the G glycoprotein in closely related RSV. However, the G glycoprotein of hMPV is not crucial, both for infection and replication of the virus and for inducing immunity in the host.

During the course of my studies, 2 recent studies have created soluble fusion proteins by deleting the transmembrane domains (32, 110), and one has used soluble F as a vaccine to induce neutralizing antibody formation in a cotton rat model (32). Expressing G in a recombinant PIV model demonstrated limited ability to induce neutralizing antibody after vaccination of hamsters (102). Limited research has been published on the native conformations of these glycoproteins, with soluble F appearing to be expressed as a trimer (32). Although the neutralization and protective antigens of hMPV remain to be determined, it is likely that the high degree of divergence of the G protein between and within hMPV subgroups will result in limited cross-neutralization and cross-protective antibody responses. We hypothesize that removal of the transmembrane domain of F will result in secretion of glycoprotein with native, antigenic

features and that a similar construct of G will not demonstrate the same level of antigenicity.

Results

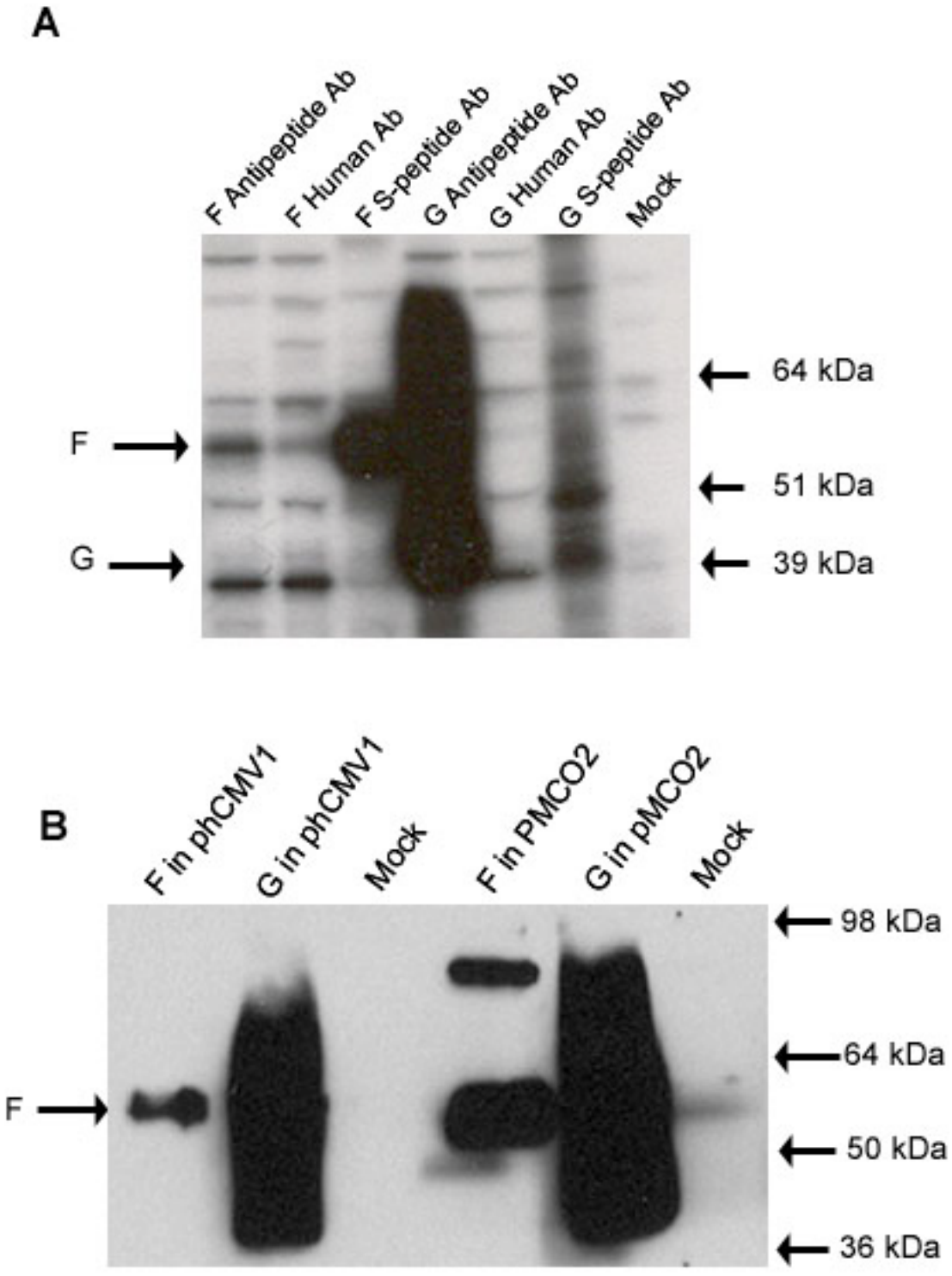
Generation and expression of full length hMPV F and G in mammalian cells. We cloned the F and G native viral sequences from CAN97-83 into the vaccinia vector pMCO2 and the mammalian vector pCAGGS and transfected into HeLa-USU and LLC-MK2 cells. The construct did not express well in HeLa-USU cells or with the pCAGGS vector in either cell line since the proteins could not be detected in cell lysates by immunoprecipitation and Western blotting or radioimmunoassay. However, the full-length proteins expressed with pMCO2 were readily detectable by radioimmunoassay in LLC-MK2 cells (Figure 2A).

Cell lysates from pMCO2-F or G transfected cells were immunoprecipitated with hMPV anti-peptide antibodies or hMPV positive human sera followed by protein G-Sepharose or with S-protein agarose beads. The predicted molecular mass of hMPV F monomer based on the nucleotide sequence is 58 kDa (32) and for G is 26 kDa (8). Figure 2A demonstrates bands corresponding to hMPV F and G migrating at the expected range, with G running as a smear from approximately 35 to 100 kDa, suggestive of a high carbohydrate component, as previously described (8, 29). F appears to have reacted to hMPV-positive human sera, whereas G does not, as expected.

The F and G ORFs were then subcloned into the mammalian expression vector phCMV1, transfected into LLC-MK2 cells and tested by immunoprecipitation with S-protein agarose and Western blotting with anti-S-peptide antibody (Figure 2B). The

Figure 2. Expression of full-length hMPV F and G glycoproteins.

LLC-MK2 cells were transfected with pMCO2 constructs containing the hMPV full-length F or G genes or pMCO2 vector only, infected with vaccinia and metabolically labeled. Lysates were immunoprecipitated with anti-peptide Ab against the cytoplasmic tail, human antisera or S-protein agarose, analyzed by SDS-PAGE and autoradiography (A). LLC-MK2 cells were transfected with pMCO2 or phCMV1 constructs containing the hMPV full-length F or G genes, or vector alone. The cells transfected with pMCO2 were infected with vaccinia prior to harvest. Lysates were immunoprecipitated with S-protein agarose and analyzed by Western blotting using anti-S-peptide antibody (B).

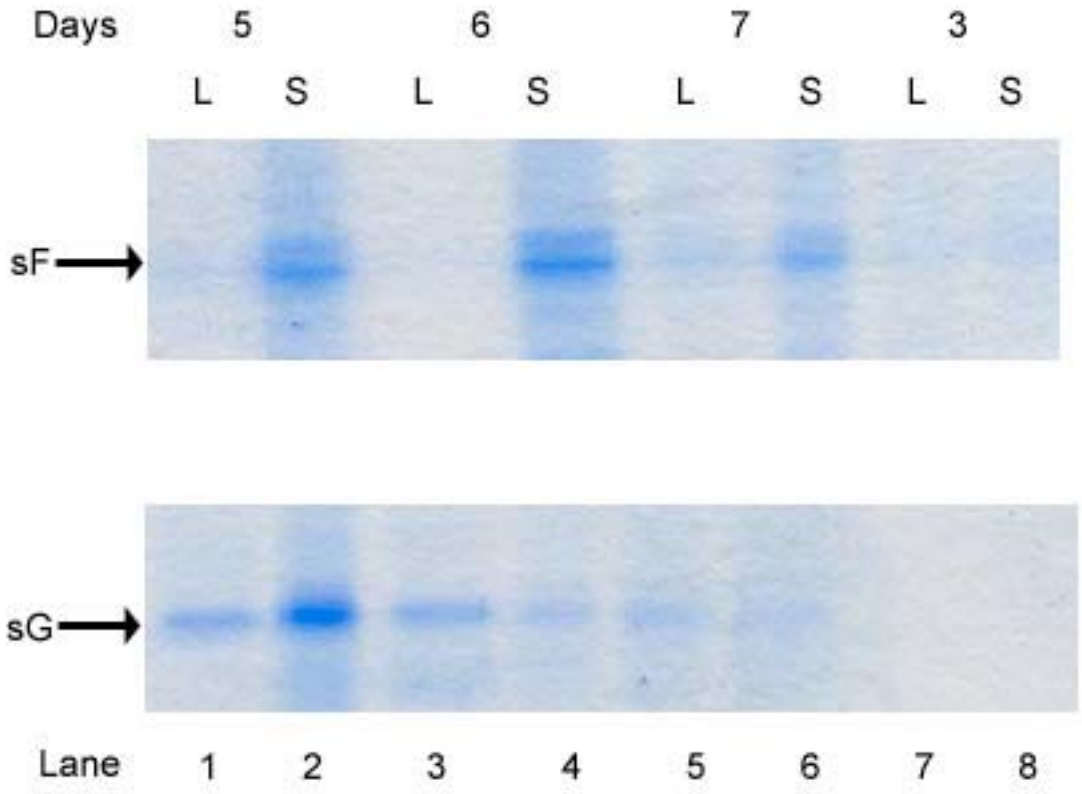

A
F Antipeptide Ab
F Human Ab
F S-peptide Ab
G Antipeptide Ab
G Human Ab
G S-peptide Ab
Mock
64 kDa
51 kDa
39 kDa
F
G
B
F in phCMV1
G in phCMV1
Mock
F in PMCO2
G in pMCO2
Mock
98 kDa
64 kDa
50 kDa
36 kDa
F

immunoprecipitated proteins revealed bands corresponding to the correct molecular size, with F in pMCO2 also showing a band of approximately 110 kDa, possibly reflecting a dimer, which has been previously reported (32). Again, G is seen as a smear from approximately 36 to 95 kDa.

Generation and expression of soluble and secreted F and G glycoproteins. We initially employed a baculovirus system to generate soluble F and G glycoproteins. The baculovirus expression system can provide a method for the production of large quantities of native, glycosylated, antigenic eukaryotic proteins that can be used in immunoblot and enzyme immunoassays. Baculovirus expression had previously been employed for immunoflourescence using full-length hMPV F from cell lysates (60). We subcloned the S-peptide-tagged F and G ORFs without the transmembrane (TM) domains and signal sequences (sF and sG) in frame with the mellitin promoter of the baculovirus transfer vector pMelBac. We co-transfected the vector with *Autographa californica* viral DNA into insect cells to allow recombination and the creation of recombinant virus containing the F or G ORFs that can infect insect cells. High Five™ cells were infected with an MOI of 10 and harvested every 24 hours to determine the optimal time point for protein expression. Maximum expression for both sF and sG occurred from 5 to 6 days. Coomassie stain of lysates and supernatants demonstrated proteins migrating at the appropriate size with a higher concentration in the supernatant (Figure 3). However, any large scale expression attempts using larger tissue culture plates or cells optimized in

Figure 3. Baculovirus expressed soluble F and G glycoproteins.

High Five™ cells in 6-well tissue culture plates were infected at an MOI of 10 with recombinant baculovirus containing ORFs for sF or sG and incubated for 7 days. The cells were lysed and the lysate (L) and supernatant (S) were immunoprecipitated with S-protein agarose, separated on SDS-PAGE and Coomassie stained. The top panel shows sF and the bottom panel shows sG. Lanes 1 through 6 are protein expression from days 5 through 7. Lanes 7 and 8 are uninfected cells in the top panel, and cells infected with wild-type baculovirus in the bottom panel.


Days
5
6
7
3
L
S
L
S
L
S
L
S
sF
sG
Lane
1
2
3
4
5
6
7
8

suspension in spinner flasks resulted in small amounts of secreted protein, and virtually none recoverable by column purification. After several attempts and discussions with other scientists who had unsuccessfully tried hMPV sF baculovirus expression, this line of approach was abandoned.

Because we had success expressing the full-length constructs of F and G with the mammalian expression vector phCMV1, we subsequently attempted expression of soluble F and G in this system. S-peptide-tagged sF without the TM domain and cytoplasmic tail was subcloned into this vector. Since G is a type II membrane glycoprotein, the TM domain is at the N terminus of the protein and possibly overlaps with the signal sequence for endoplasmic reticulum targeting (21). Therefore, removing the TM domain could prevent expression and secretion of the protein. Previously, soluble G from Hendra virus (sHeV G) had been expressed using a pSecTag2 expression vector that encodes an Ig κ leader sequence (21). The sHeV G had the S-peptide tag fused in frame between the Ig κ leader sequence and the sHeV G ORF along with two 15-amino acid linker elements, one between the Ig κ leader sequence and the S-peptide tag and one between the tag and the G coding sequence. Subsequently, this sHeV G with κ leader sequence and S-peptide tag had been subcloned into the phCMV1 vector. The Hendra ORF was ligated out of this vector and replaced with the hMPV sG from TOPO as described in the Methods. Diagrams of the hMPV sF and sG constructs are shown in Figure 4.

Figure 4. hMPV sF and sG constructs in phCMV1 vector.

The transmembrane domains and cytoplasmic tails were deleted and S-peptide tag added by PCR. The sG sequence was cloned in frame with the Ig κ leader sequence. The Ig κ leader and linkers were taken from previously constructed soluble Hendra virus G.


hMPV sG
N-
hMPV G truncated to remove transmembrane domain and cytoplasmic tail
-C
hMPV sF
N-
hMPV F truncated to remove transmembrane domain and cytoplasmic tail
-C
Ig κ-chain leader sequence: METDTLLLWVLLLWVPGSTGD
15 aa linker: AAQPARRARRTKLGT
S-peptide tag: KETAAAKFERQHMDS
6 aa linker: NSADIQ

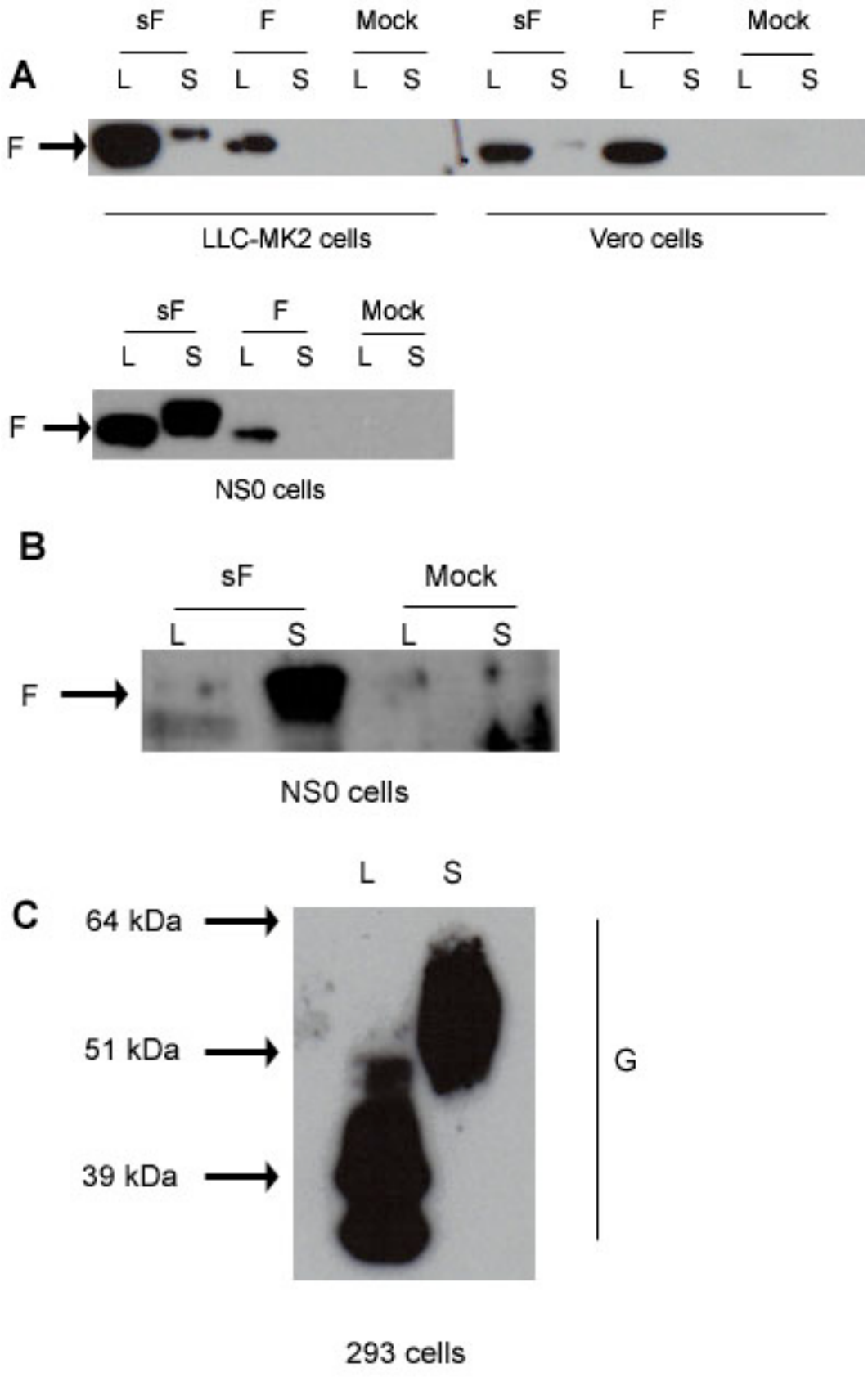
The sF phCMV1 vector was transfected into the two commonly used cell lines for hMPV infection and propagation, LLC-MK2 and Vero. Expression of F glycoprotein in the supernatant was demonstrated for both cell lines, although there appeared to be more protein in the cell lysate (Figure 5A). Ulbrandt et al. had success expressing hMPV sF in the mouse myeloma cell line NS0 (110). Transfection of this cell line with the phCMV1 sF vector resulted in high levels of expression of sF in the supernatant (Figure 5A). We saw a similar pattern after transfecting 293 cells. The secreted sF glycoprotein has an apparent molecular weight slightly larger than full-length F and sF recovered from cell lysates. This could be due to a glycosylation site that is exposed when the TM is removed and post-translationally modified when expressed. This alteration may not normally occur with cell-surface F expression. However, the construct was efficiently secreted and detected when probed with serum from known hMPV-infected patients (Figure 5B), demonstrating that the protein is still an effective antigen.

NS0 and 293 cells transfected with phCMV1 expressing sF were stabilized using geneticin and cloned through limited dilutions. Comparison of the expression levels revealed more protein secreted by 293 cells, and we chose these cells to continue protein expression and purification.

We also transfected the sG phCMV1 construct into 293 cells and created a stable cell line. Western blotting revealed sG in the lysate running from the predicted molecular mass of 26 kDa to approximately 50 kDa, and secreted glycoprotein running from approximately 45 to 65 kDa (Figure 5C), consistent with posttranslational modification and glycosylation.

Figure 5. Expression of recombinant sF and sG glycoproteins.

LLC-MK2, Vero, NS0 or 293 cells were transfected with phCMV1 containing the hMPV sF (A, B) or sG (C) construct and incubated for 40 hours at 37ºC. Lysates were prepared in buffer containing Triton X-100 and both lysates (L) and supernatants (S) were clarified by centrifugation and immunoprecipitated with S-protein agarose. The proteins were resolved on 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with anti-S-peptide antibody (A, C) or human sera from an hMPV positive patient (B). The mock lanes indicate cells transfected with empty phCMV1 vector.


A
sF
F
Mock
sF
F
Mock
L S L S L S L S L S L S
F
LLC-MK2 cells
Vero cells
sF
F
Mock
L S L S L S
F
NS0 cells
B
sF
Mock
L S L S
F
NS0 cells
C
L S
64 kDa
51 kDa
39 kDa
G
293 cells

The soluble F and G glycoproteins were purified from both serum-containing and low-serum media by running the supernatant over an S-protein agarose column. The concentrated glycoproteins from low-serum media appeared highly pure, and sF had an approximate molecular size of 58 kDa, and sG approximately 56 to 60 kDa in SDS-PAGE gel stained with Coomassie blue. (Figure 6).

Characterization of soluble hMPV F and G. To evaluate whether the secreted sF and sG are oligomeric, we determined their molecular size using size exclusion chromatography with a Superdex 200 analytical grade column XK26. A 50 µg aliquot of purified sF or sG was passed over the column and fractioned as described for the molecular weight standards in the Methods. The elution profiles of the purified sF and sG are shown in Figure 7A. Samples from all areas of elevation in the elution profile were analyzed by SDS-PAGE under reducing conditions and Western blotting to determine the presence of sF or sG proteins versus contaminating serum proteins (Figure 7B). Coomassie stains of the fractions confirmed the Western blot results. The plot of the protein standards on the calibration curve and the placement of the sF and sG peaks are depicted in Figure 7C. Both proteins were present in only one peak of the elution profile, suggesting that one species predominates.

The estimated molecular size of the glycoproteins based on the elution profile is 113 kDa for sF and 265 kDa for sG, with the range for all protein containing fractions of 50-120 kDa for sF and 48-275 kDa for sG. The peaks correspond to sizes that are larger

Figure 6. Purified sF and sG glycoproteins.

293 cells stably transformed with phCMV1 containing the hMPV sF or sG ORF were incubated in roller bottles with DMEM-10, then switched to Opti-MEM when 75% confluent. After 3 days, the media was harvested, clarified by centrifugation, and protease inhibitor added. The supernatant was run over a column of S-protein agarose, eluted in acid, neutralized, buffer exchanged and concentrated. 5 µl of each concentrate was analyzed by SDS-PAGE and Coomassie staining.

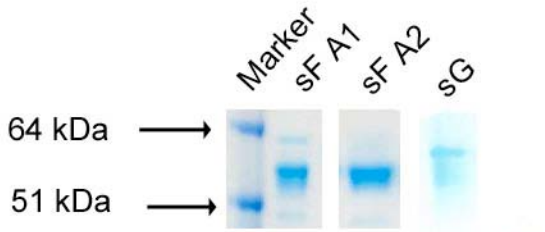
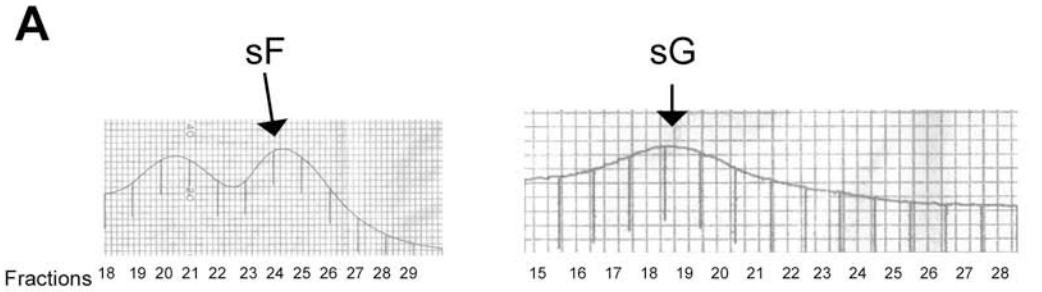
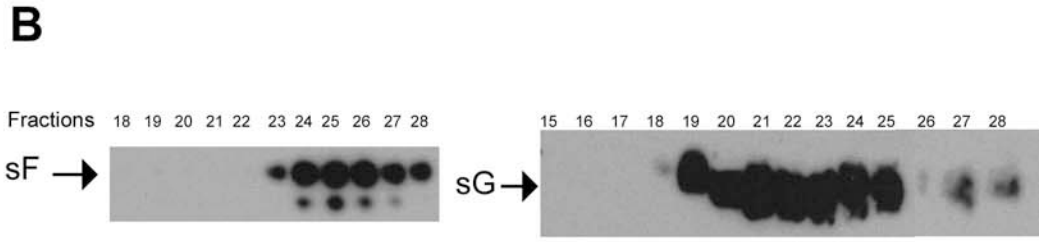
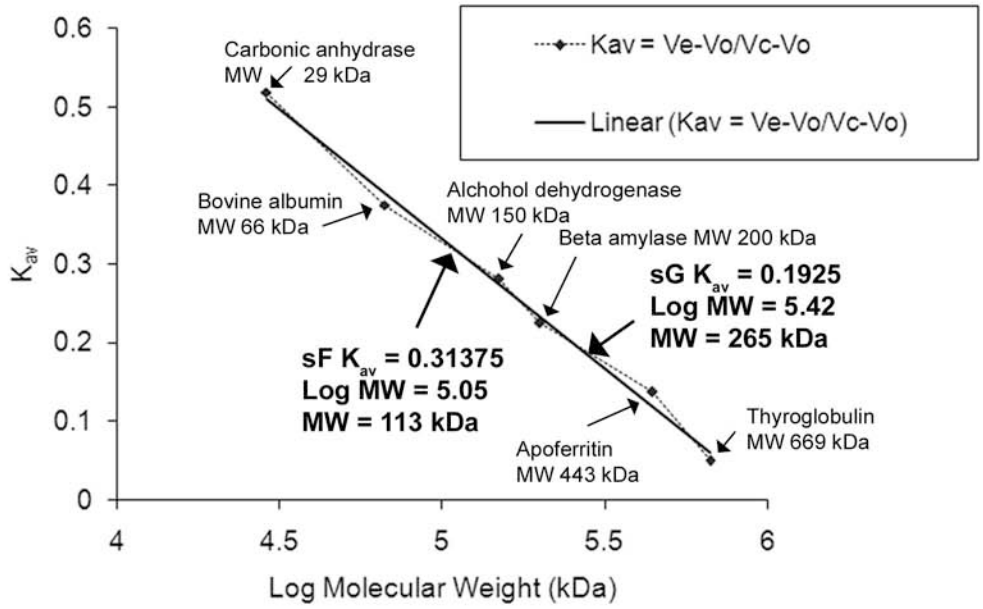

Marker
sF A1
sF A2
sG
64 kDa
51 kDa

Figure 7. Size exclusion chromatography of hMPV sF and sG.

Molecular weight samples were separated on a Superdex 200 size exclusion column to generate a calibration curve. Purified sF and sG were separated on the column and fractioned. Fractions from peaks in the elution profiles (A) were tested for the presence of sF and sG by separation on SDS-PAGE and Western blotting with anti-S-peptide antibody (B). The molecular weight estimates of the peaks based on the calculated K_{av} are plotted on the calibration curve (C).


A
sF
sG
Fractions 18 19 20 21 22 23 24 25 26 27 28 29
15 16 17 18 19 20 21 22 23 24 25 26 27 28


B
Fractions 18 19 20 21 22 23 24 25 26 27 28
15 16 17 18 19 20 21 22 23 24 25 26 27 28
sF
sG


C
Kav = Ve-Vo/Vc-Vo
Linear (Kav = Ve-Vo/Vc-Vo)
Carbonic anhydrase MW 29 kDa
Bovine albumin MW 66 kDa
Alchohol dehydrogenase MW 150 kDa
Beta amylase MW 200 kDa
sG Kav = 0.1925
Log MW = 5.42
MW = 265 kDa
sF Kav = 0.31375
Log MW = 5.05
MW = 113 kDa
Apoferritin MW 443 kDa
Thyroglobulin MW 669 kDa
Kav
0.6
0.5
0.4
0.3
0.2
0.1
0
4
4.5
5
5.5
6
Log Molecular Weight (kDa)

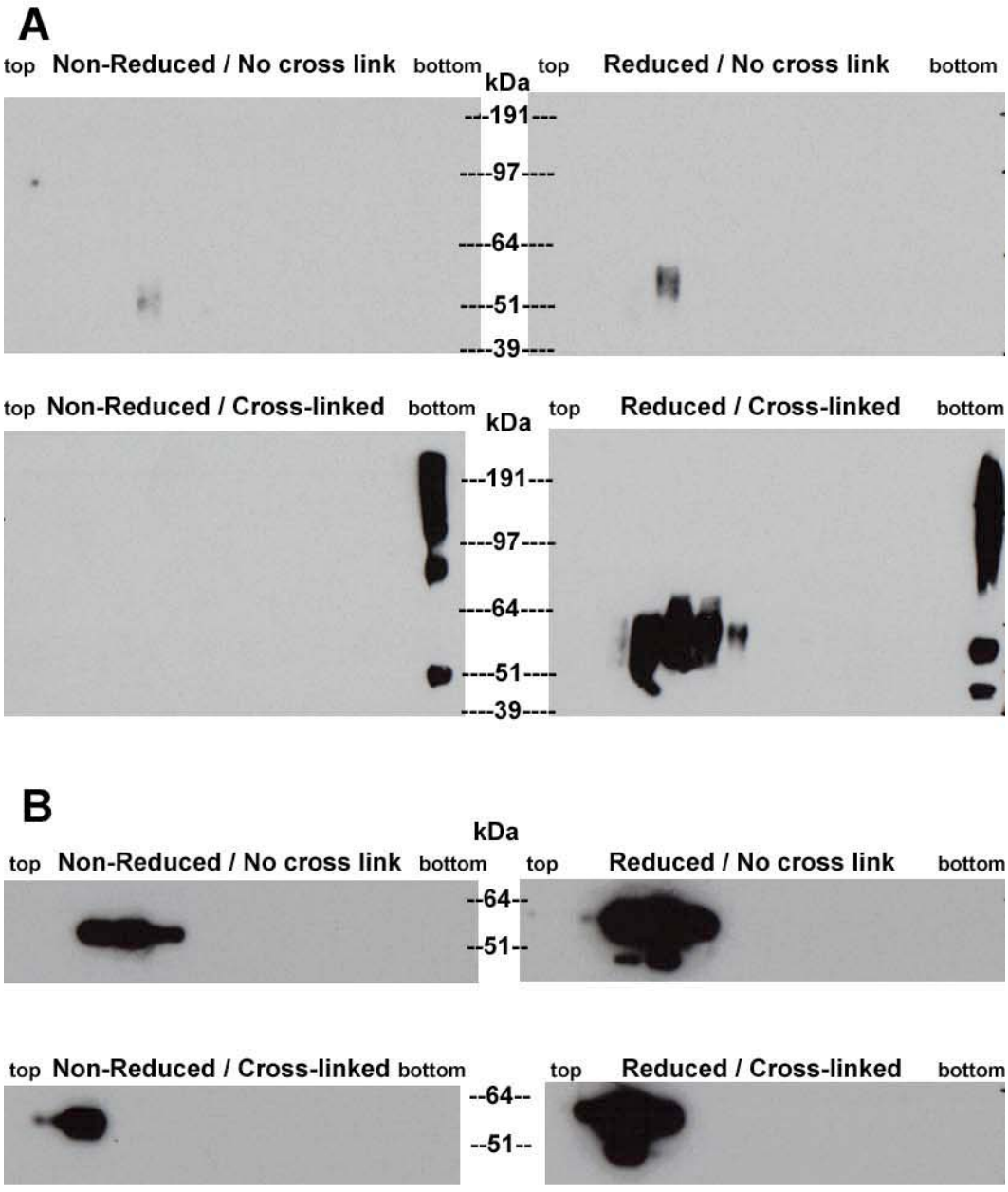
than expected for monomeric sF and sG. It has been reported that engineered soluble virus membrane glycoproteins have overestimated molecular size calculated from size exclusion chromatography. Specifically, gp120 from human immunodeficiency virus type 1 and Hendra virus G protein have apparent molecular masses of twice that expected based on SDS-PAGE (21, 123). Given this prediction, it appears that sF glycoprotein is a monomer and sG possibly a dimer. The apparent homogeneous monomeric form of hMPV sF was indeed unexpected, yet for certain experiments it may be exceedingly beneficial such as in crystallization and structural determinations.

To further characterize the purified proteins, we used sucrose gradient centrifugation. One-half of a 100 μg sample of protein was cross-linked with the reducible reagent DTSSP at a final concentration of 5 mM, and then overlaid and centrifuged on a 5-to-20% continuous sucrose gradient. The gradients were fractioned, and 2 10 μl samples of each were boiled in sample buffer, one set with and one set without β-mercaptoethanol. For sG, we immunoprecipitated all the fractions with S-protein agarose prior to addition of sample buffer. The four sets of fractions were then analyzed on 4-12% NuPAGE gels and Western blot.

Figure 8A shows sG fractions denatured with or without cross-linker and with or without the presence of a reducing agent. From these data, we can determine that there is one species of sG, with an additional large aggregate running in the bottom of the gradient when cross-linked. All the samples were subjected to boiling and a denaturing gel, and this may explain the inability to maintain a higher molecular weight structure in the non-reduced, non-cross-linked blot. The lack of bands in the cross-linked, non-

Figure 8. Oligomeric and monomeric forms of sG and sF.

Protein preparations of sG (A) and sF (B) either cross-linked with the reducible cross-linking reagent DTSSP or untreated were layered onto 5-20% continuous sucrose gradients and fractioned. For sG, the fractions were precipitated with S-protein agarose first, then the sG agarose beads and 20 µl of the sF fractions were divided into two tubes and boiled in NuPAGE sample buffer with and without the reducing agent β-mercaptoethanol. They were resolved by 4-12% NuPAGE gels, transferred to nitrocellulose membranes and probed with anti-S-peptide antibody. The top and bottom of the fractions are indicated.


A
top Non-Reduced / No cross link bottom
top Reduced / No cross link bottom
kDa
---191---
----97----
----64----
----51----
----39----
top Non-Reduced / Cross-linked bottom
top Reduced / Cross-linked bottom
kDa
---191---
----97----
----64----
----51----
----39----
B
kDa
top Non-Reduced / No cross link bottom
top Reduced / No cross link bottom
--64--
--51--
top Non-Reduced / Cross-linked bottom
top Reduced / Cross-linked bottom
--64--
--51--

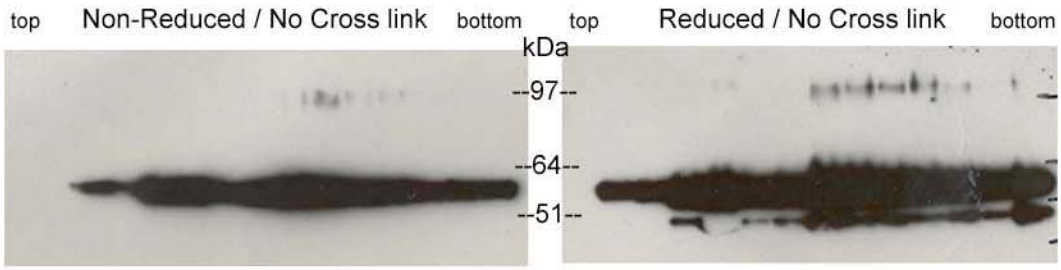
reduced blot may be because the cross-linker created higher molecular weight aggregates that could not resolve on the gel without the presence of reducing agent. It is also possible that the aggregated protein may not release from the S-protein agarose into the sample buffer without reducing agent present. The amount of protein present in the cross-linked, reduced blot is much greater than that seen in the non-cross-linked samples. It is possible that without the cross-linker the protein did not maintain a single species in the sucrose gradient and with differing amounts of glycosylation, the protein was distributed throughout the fractions and were not at high enough concentration to be detected in this Western blot. The largest amount of protein may have existed as a dimer, even without the cross-linker, and this may be represented by the one band that is seen in both the non-cross-linked blots. With cross-linker, more protein is visible in a range of bands in the same general area of the gradient. Other paramyxovirus attachment proteins exist as dimers and tetramers, and most data point to a dimer of dimers (21, 29).

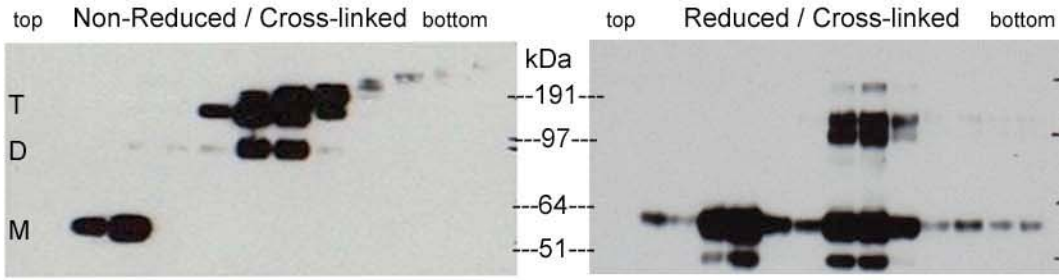
Figure 8B shows sF cross-linked and uncross-linked in non-reducing and reducing conditions. There is only one species present that runs in the upper fractions of the gradient which could represent a monomer. The only difference between reduced and non-reduced is the presence of smaller proteins in the reduced blot, probably representing F_1. Cross-linking does not change the oligomeric profile. This is consistent with one species, probably a monomer that does not cross-link to higher molecular forms.

As all other paramyxovirus fusion proteins that have been characterized form trimers in the native state, we analyzed sF hMPV protein from the B1 lineage (sF_{B1}), kindly provided by Nancy Ulbrandt as a comparison. As seen in Figure 9, sF_{B1} protein

Figure 9. Oligomeric forms of sF_{B1}.

Protein preparations of sF_{B1} either cross-linked with the reducible reagent DTSSP or non-cross-linked were layered onto 5-20% continuous sucrose gradients and fractioned. The fractions were divided into two tubes and boiled in NuPAGE sample buffer with and without the reducing agent β-mercaptoethanol. They were resolved by 4-12% NuPAGE gels, transferred to nitrocellulose membranes and probed with monoclonal F antibody. The top and bottom of the fractions are indicated. The species indicated in the non-reduced, cross-linked blot are: M, monomer; D, dimer; T, trimer.


top
Non-Reduced / No Cross link
bottom
top
Reduced / No Cross link
bottom
kDa
--97--
--64--
--51--


top
Non-Reduced / Cross-linked
bottom
top
Reduced / Cross-linked
bottom
kDa
---191---
---97---
---64---
---51---
T
D
M

appears in all fractions of the non-cross-linked gradient, with two main areas of concentration. When reduced, a lower band appears, probably representing F_1. There are also bands visible in both the reduced and non-reduced gels running at approximately 97 kDa that may represent a dimer. With the addition of cross-linker, the sF_{B1} protein clearly retains its oligomeric structure, with separate fractions consistent with monomers, dimers and trimers, and even larger species in the bottom fractions. When reduced, the majority of oligomeric forms are reduced to monomers.

It is unclear why the version of sF that we made (sF_{A2}) only forms monomers whereas sF_{B1} is oligomeric. It is unlikely that the different genetic lineage is the cause. There are multiple differences in the construction and preparation of the two proteins as outlined in Table 2. sF_{A2} is in a buffer containing L-arginine based on the ability of L-arginine to prevent protein aggregation during purification (109). To test whether L-arginine is a potential cause of failure to form oligomeric species, 293 cells stably expressing sF were grown in reduced-serum media and the supernatant directly concentrated and buffer exchanged in PBS as described in the Methods. This procedure also eliminated using S-protein agarose and elution with low pH for purification. Half of the protein obtained from this method was cross-linked and the cross-linked and un-cross-linked proteins were analyzed as previously described. There was no difference in the analysis between this method of protein purification and buffer exchange and the previous method. Therefore, it may be the cellular processing, shorter truncation, the presence of the S-peptide tag or some other element disrupting the protein interactions.

Table 2. Differences between sF_{A2} and sF_{B1}.

sF_{A2}	sF_{B1}
Truncated after residue 487	Truncated after residue 490
Tagged with S-peptide	Tagged with 6-His
Expressed in 293 cells	Expressed in NS0 cells
Purified with S-protein agarose, pH 2.0	Purified with mAb, pH 2.8
PBS buffer with Triton X-100 and L-Arginine	PBS buffer

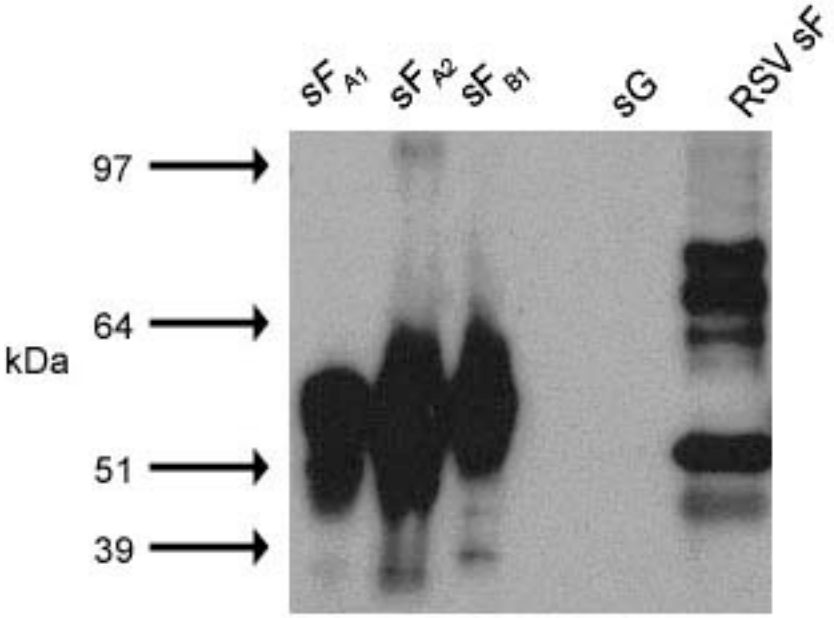
Despite this unexpected result, sF_{A2} still appears to be an effective antigen as demonstrated previously with F mAb and positive human sera.

Antigenicity of sF and sG. As demonstrated previously, hMPV positive human serum reacts to sF_{A2} from the supernatant of transfected cells (Figure 5B). We further tested the purified sG and sF_{A2}, and two versions of soluble F (sF_{A1}, sF_{B1}) and RSV sF provided by Dr. Nancy Ulbrandt. Positive human control sera reacted to all three types of sF, and to RSV F, but not to sG (Figure 10). It is expected with the high homology of F between different genotypes that a patient would have antibodies equally reactive to F from either genotype. However, since G has low homology between genetic lineages of hMPV, and it is unknown what genotype caused the hMPV infection in the control sera, it is impossible to say whether there are no detectable antibodies to G because G is not an effective immunogen or because the patient was infected with a different type. In addition, there has been controversy as to whether the two major lineages of hMPV are separate serotypes (113). To test the reactivity of known lineage infections to both sF and sG, to further demonstrate the antigenicity of the monomer sF_{A2}, and to test the possibility of cross-reactivity to the most closely related human pathogen RSV, we obtained pre- and post-immunization sera from animals that had been inoculated with lineages A2 and B2, as described in the Methods.

Hamsters were inoculated with both CAN97-83 (A2) and CAN98-75 (B2), whereas the rabbits and non-human primates were inoculated with either strain. Using the purified hMPV F proteins from genotypes A1 and B1 and RSV F from Dr. Ulbrandt

Figure 10. Detection of human antibody to hMPV sF.

Purified proteins from hMPV sF_{A1}, sF_{A2}, sF_{B1}, sG and RSV F were resolved on a 4-12% NuPAGE gel and transferred to a nitrocellulose membrane. The membrane was probed with known positive hMPV human control sera. Bound antibodies were reacted with a peroxidase-conjugated goat anti-human IgG and visualized by chemiluminescence.


sF A1
sF A2
sF B1
sG
RSV sF
97
64
51
39
kDa

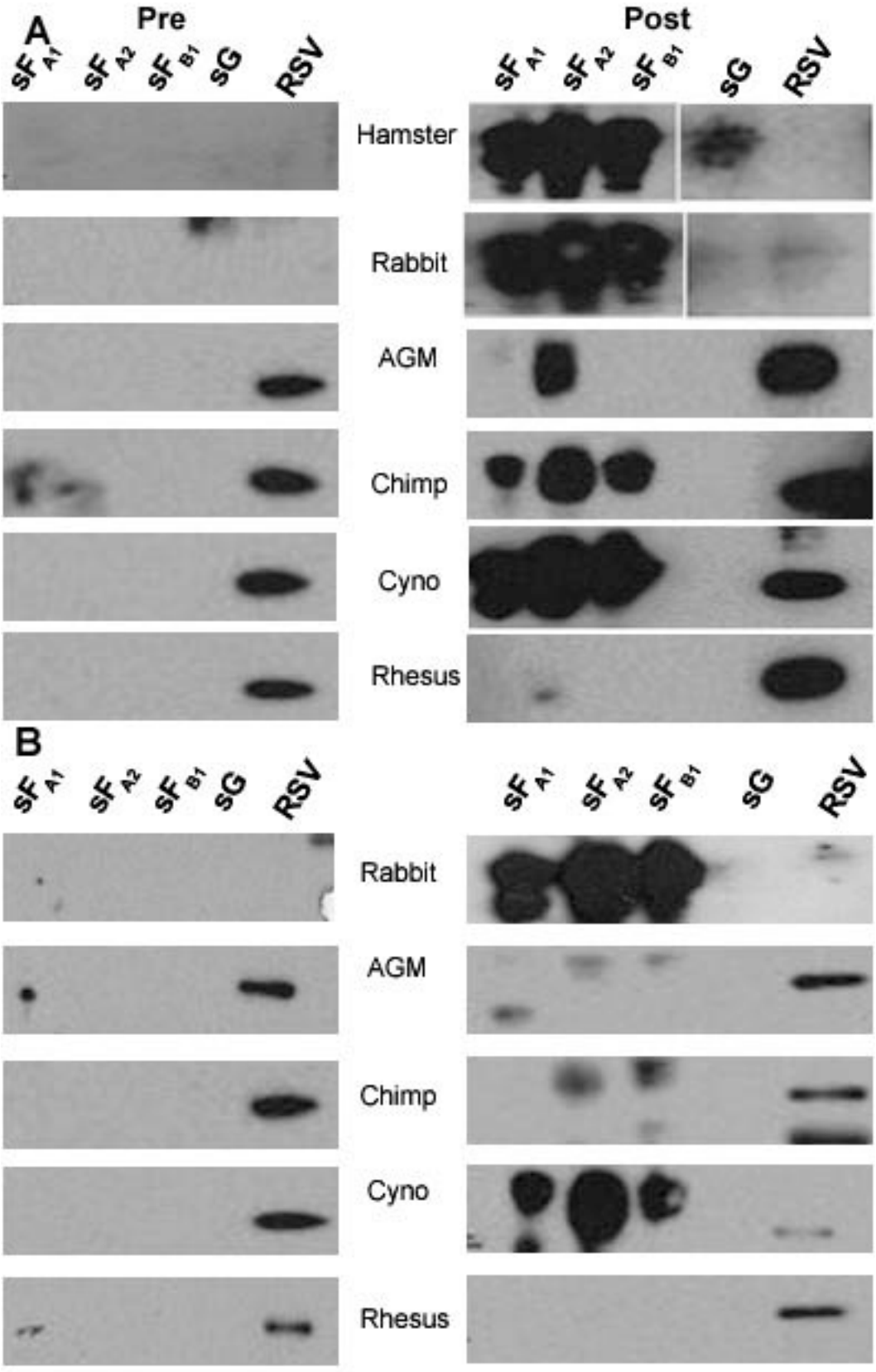
and purified hMPV F and G that we constructed from genotype A2, we tested the reactivity of the different animal sera by Western blotting. As seen in Figure 11, all of the non-human primates had pre-existing antibody to RSV. For the hamster and rabbit, exposure to either strain of hMPV did not induce antibodies cross-reactive to sF from RSV. After infection with lineage A2, most of the animals developed antibody reactive to all three types of sF protein (Figure 11A). Interestingly, the rhesus macaque and African green monkey (AGM) reacted only to sF_{A2}, suggesting either that species may play a role in the antibody response to different virus lineages or antigenic sites on this monomeric protein may be more accessible to antibody. Although exposed to the same exact virus strain from which sG is derived, only the hamster demonstrated antibodies to sG.

After infection with lineage B2, fewer animals demonstrated an antibody response (Figure 11B). The rabbit and cynomolgus macaque had readily identifiable antibody responses to all three sF proteins, whereas the chimpanzee and AGM had weaker responses and undetectable antibody to sF_{A1} in the chimpanzee. No antibodies were detected to any of the sF proteins for the rhesus macaque, confirmed in repeated testing.

Original studies done with these animals demonstrated high levels of virus replication in chimpanzees and AGMs, and low replication in rhesus macaques (103). However, all of the monkeys and the chimpanzees produced neutralizing antibodies, and as F has been shown to be the major antigenic determinant, and immunization with F alone can induce neutralizing antibodies and protection against challenge, it is unusual

Figure 11. Western blot analysis of pre- and post-infection animal sera.

Purified sF_{A1}, sF_{A2}, sF_{B1} and sG from hMPV and RSV sF were resolved on 4-12% Nu-PAGE gels and transferred to nitrocellulose membranes. The membranes were probed with animal sera from pre and post infection with hMPV A2 virus (A) or hMPV B2 virus (B) and bound antibodies were reacted with a peroxidase-conjugated goat anti-Syrian hamster, goat anti-rabbit, or rabbit anti-monkey IgG and visualized by chemiluminescence. The hamster was infected with both A2 and B2 lineages, the other animals were infected with only one. The non-human primate species are: AGM, African green monkey; chimp, chimpanzee; cyno, cynomolgus macaque; rhesus, rhesus macaque.


A
Pre
Post
SF A1
SF A2
SF B1
SG
RSV
Hamster
Rabbit
AGM
Chimp
Cyno
Rhesus
B
SF A1
SF A2
SF B1
SG
RSV
Rabbit
AGM
Chimp
Cyno
Rhesus

that antibodies to F were not detected in Western blot. Poor or lack of binding could be due to denaturing of the protein in the SDS gel and subsequent loss of conformation-dependent binding sites. We attempted to determine if binding would occur if the protein and antibody were allowed to bind prior to resolution in a denaturing gel. We incubated protein and sera and then immunoprecipitated with Protein-G-sepharose or -agarose and resolved on 4-12% Nu-PAGE gels and probed with F mAb. Despite multiple attempts using different Protein-G beads, wash regimens and protein concentrations, we were unable to reduce background binding of protein to the beads. Signals were consistently weaker for pre-exposure sera compared to post-exposure sera; however, protein without sera had a stronger signal than with post-exposure sera, indicating that without the presence of antibody, the protein readily bonded to the sepharose or agarose beads.

To further test the reactivity of antibodies to the hMPV surface proteins, to compare the sensitivity of the sF_{A1} oligomer and the sF_{A2} monomer, and to determine if there is cross reactivity to RSV, the sera were tested in ELISA. For all animals, infection with either genotype resulted in increased antibody titers to antigen (sF_{A1} and sF_{B1}). The chimpanzee and cynomolgus macaque had significantly higher titers to both sF genotypes after infection with lineage A. Previous studies have demonstrated increased virus titers in animals artificially infected with the A genotype, so the elevated antibody titers could be a reflection of the virus titer (103).

Interestingly, all of the non-human primates appeared to have low levels of pre-existing antibody to hMPV sF, even though they were previously tested in neutralization assays and were negative. However, many captive chimpanzees are naturally positive for

hMPV (103), and it is possible that they had been exposed but their titers were not high enough to neutralize virus, or they had non-neutralizing antibodies.

A direct comparison of the antibody reaction to the sF_{A1} oligomer and the sF_{A2} monomer after immunization with the B2 lineage in non-human primates showed a significant difference using 2-way ANOVA for the AGM, cynomolgus and rhesus monkeys, but not for chimpanzees (Figure 12). Despite this, the sF_{A2} antigen effectively detected antibody, and in the animal most closely related to humans, the native conformation of the glycoproteins bound equally to antibody.

Testing the sera using sG as the antigen in indirect ELISA demonstrated that all of the animals infected with the same A2 lineage did develop an antibody response to sG, but not if they were infected with the B2 lineage, as is expected with only 33% homology to G between the lineages. The antibody response was weaker than to F, and detectable only at dilutions of 1:200 and lower, but still of interest given the limited evidence of G being antigenic.

All of the non-human primates had pre-existing antibodies to RSV, but infection with hMPV did not increase antibody titers reactive to sF from RSV in ELISA except at low dilutions after infection with hMPV B2 in the cynomolgus macaque, generally demonstrating a lack of cross-reactivity to this antigen in these animal models. However, both the hamster and rabbits, which had no pre-existing RSV antibody, did develop antibodies reactive to RSV sF in ELISA after infection with hMPV (Figure 13) suggesting that antibodies in these species may be less specific, or that repeated exposures increases antibody specificity.

Figure 12. Comparison of sF_{A1} and sF_{A2} antigens for detection of antibody in ELISA.

Glycoproteins sF_{A1} (oligomeric) or sF_{A2} (monomeric) were coated onto Immulon II 96-well plates at a concentration of 50 ng/well so that half of each plate was coated with sF_{A1} or sF_{A2}. Pre and post hMPV B2 infection sera from a rabbit (A), rhesus macaque (B), cynomolgus macaque (C), African green monkey (D) and chimpanzee (E) were tested in 11 serial dilutions starting at 1:64 against both the A1 and A2 antigens. The plates were reacted with alkaline phosphatase-conjugated goat anti-rabbit or rabbit anti-monkey antibodies and antibodies detected with PNPP substrate. Error bars depict the standard error of two replicates per dilution. A two-way analysis of variance (ANOVA) with main effects of antigen and dilution was used to compare the two antigens after adjusting for dilution: (A) $p \leq 0.0001$, (B) $p = 0.0001$, (C) $p \leq 0.0001$, (D) $p \leq 0.0001$, (E) $p = 0.121$. Abbreviations: Pre / A1, pre-infection sera tested against A1 antigen; Post / A1, post-infection sera tested against A1 antigen; Pre / A2, pre-infection sera tested against A2 antigen; Post / A2, post-infection sera tested against A2 antigen.

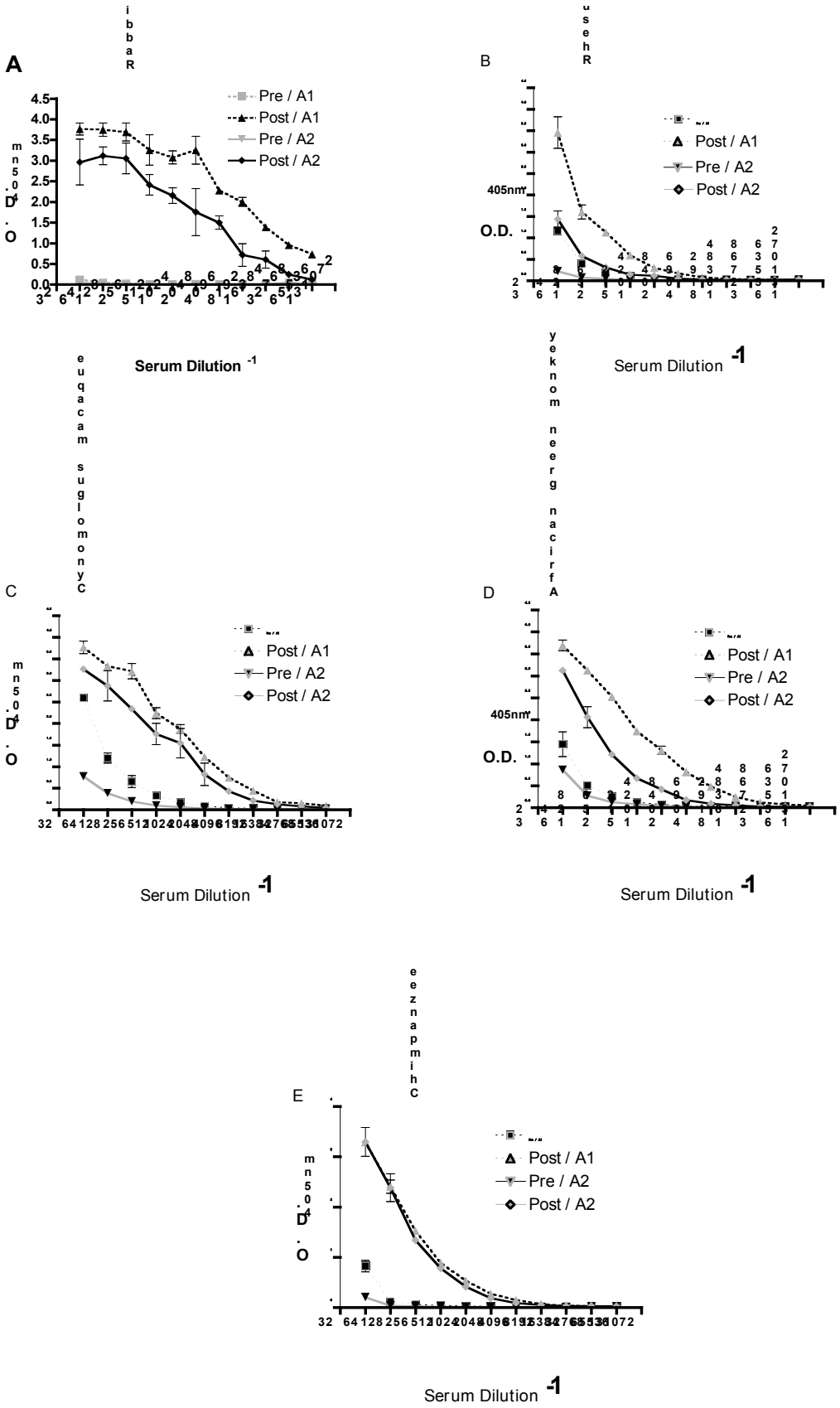
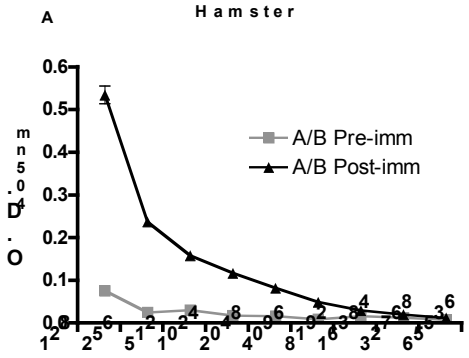
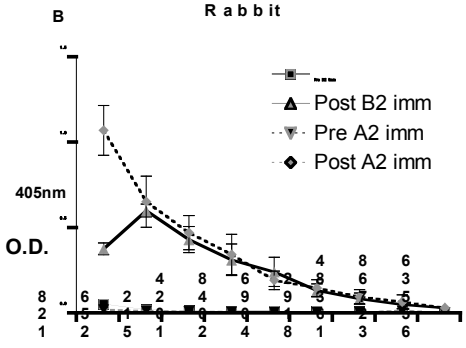
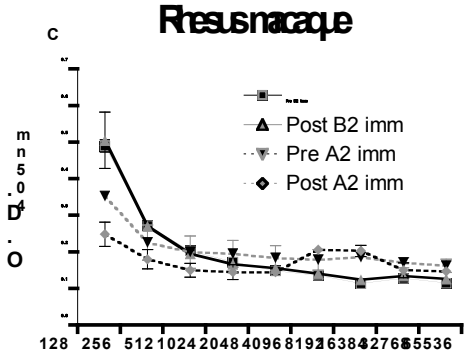

A
B
C
D
E
Pre / A1
Post / A1
Pre / A2
Post / A2
405nm
O.D.
Serum Dilution -1

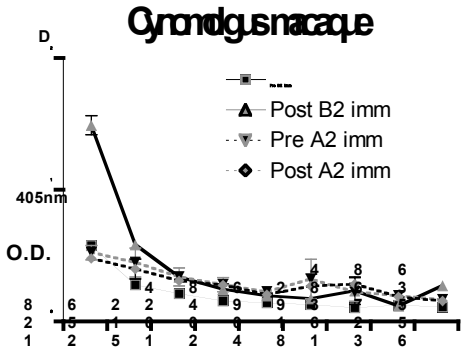
Figure 13. Detection of antibodies reactive to RSV F after immunization with hMPV.

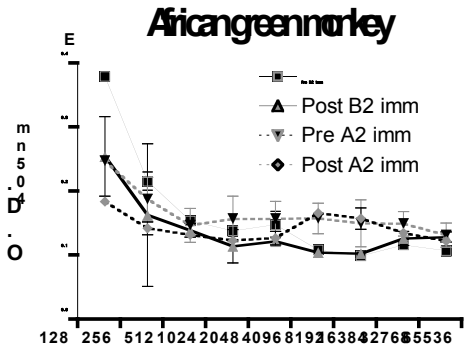
sF glycoprotein from RSV was coated onto Immulon II 96-well plates at a concentration of 50 ng/well. Pre and post hMPV A2 and B2 infection sera from a hamster (A), rabbit (B), rhesus macaque (C), cynomolgus macaque (D), African green monkey (E) and chimpanzee (F) were tested in 9 serial dilutions starting at 1:200 against the RSV antigen. The plates were reacted with alkaline phosphatase-conjugated goat anti-Syrian hamster, goat anti-rabbit or rabbit anti-monkey antibodies and antibodies detected with PNPP substrate. Error bars depict the standard error of two replicates per dilution. The hamster was infected with hMPV from both genotypes, the other graphs represent two animals each infected with one genotype.

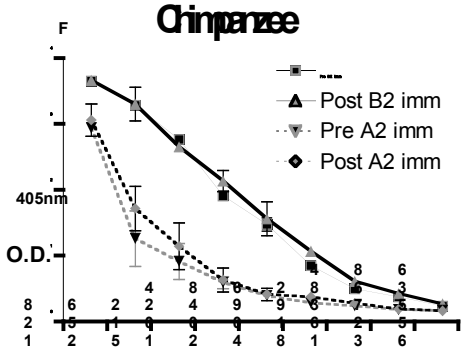

A
Hamster
A/B Pre-imm
A/B Post-imm
Serum Dilution -1


B
Rabbit
Post B2 imm
Pre A2 imm
Post A2 imm
405nm
O.D.
Serum Dilution -1


C
Rhesus macaque
Post B2 imm
Pre A2 imm
Post A2 imm
Serum Dilution -1


D
Cynomolgus macaque
Post B2 imm
Pre A2 imm
Post A2 imm
405nm
O.D.
Serum Dilution -1


E
African green monkey
Post B2 imm
Pre A2 imm
Post A2 imm
Serum Dilution -1


F
Chimpanzee
Post B2 imm
Pre A2 imm
Post A2 imm
405nm
O.D.
Serum Dilution -1

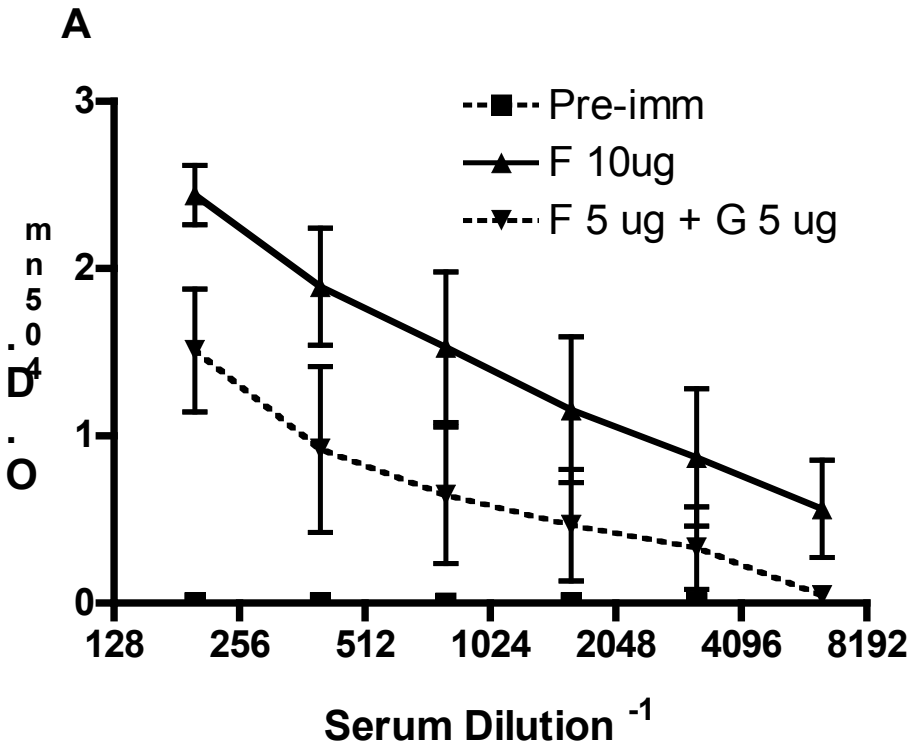
Immunizations with sF and sG. Previous studies have demonstrated that hMPV F protein, in soluble, purified form or expressed from a recombinant virus, can induce neutralizing antibodies and protect against challenge (102, 103, 110, 113). Only one study tested neutralization and protection after exposure to G expressed by recombinant virus and found no neutralizing antibodies and protection to be one-tenth of that provided by immunization with F (102). Further, no previous study has tried a combination of the two surface glycoproteins to induce immunity.

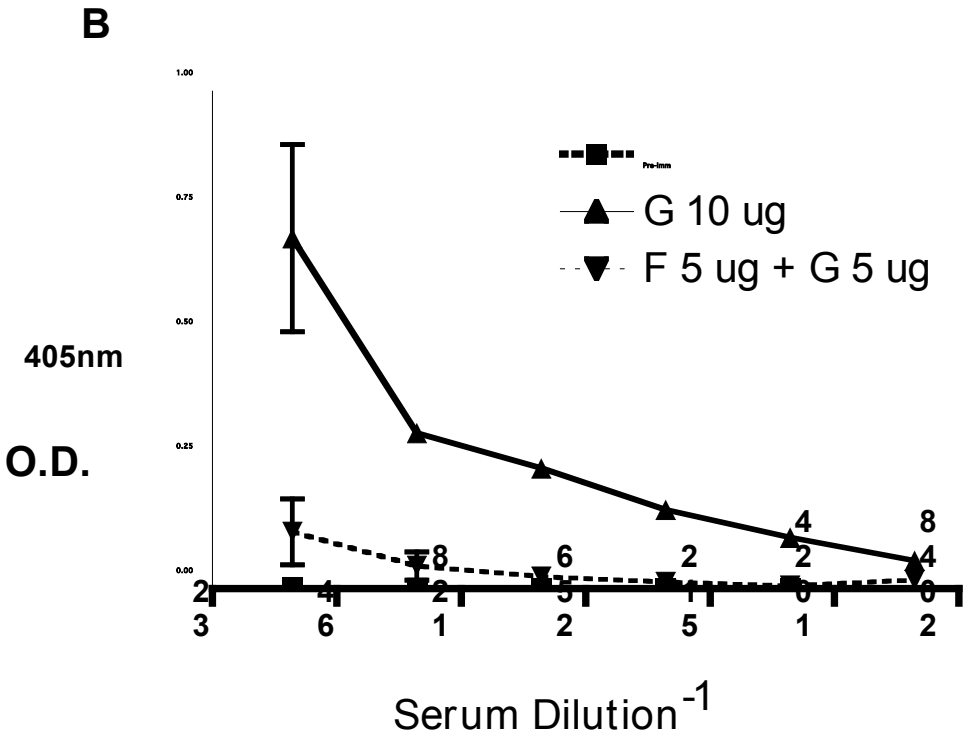
Purified, soluble F and G proteins were used to immunize mice to test their ability to induce neutralizing antibodies. Three groups of four mice were immunized at 0, 28 and 56 days with 10 μg of sF, 10 μg of sG or 5 μg of each. Aliquots of their pre-bleed sera were mixed and compared to post-immunization sera by ELISA. Figure 14 shows that mice immunized with sF, whether alone or in combination with sG, had a robust immune response to sF_{A1} glycoprotein. Those immunized with both sF and sG only received 5 μg of sF and they had decreased titers in comparison with the mice that received 10 μg. Mice immunized with sG also had a detectable immune response to sG antigen, although much less than that seen with sF, and they had a similar dose response.

The F antigen used in ELISA did not have the S-peptide tag, so the antibody response is directed solely to the F glycoprotein. The only available sG antigen is S-peptide tagged, so to ensure that the response detected was not to the tag, the same sera were tested in ELISA to S-peptide-tagged Hendra F. After the second immunization, there was a response to sG and not to the S-peptide tag, but after the third immunization, a similar response to S-tagged sG and S-tagged Hendra sF developed. At this time, it is

Figure 14. Antibody responses after sF and sG immunization.

sF_{A1} and sG glycoproteins were coated onto Immulon II 96-well plates at a concentration of 50 ng/well. A mixture of pre immunization sera from all of the mice and post immunization sera (sF, sG, sF+sG) were tested in 6 serial dilutions starting at 1:200 against sF_{A1} antigen and 1:50 against sG antigen. The sF and sF+sG groups had 4 mice and the sG group had 3 mice. The plates were reacted with alkaline phosphatase-conjugated goat anti-mouse antibodies and detected with PNPP substrate. Each dilution was performed in duplicate, averaged and the average of all the mice in the group is plotted, with error bars depicting the standard error of all the mice in the group.


A
Pre-imm
F 10ug
F 5 ug + G 5 ug
O.D. mn504
3
2
1
0
128
256
512
1024
2048
4096
8192
Serum Dilution -1


B
Pre-imm
G 10 ug
F 5 ug + G 5 ug
405nm
O.D.
1.00
0.75
0.50
0.25
0.00
Serum Dilution -1

impossible to determine how much of the detected antibody response is to the G glycoprotein and how much is to the S-peptide. We have seen similar responses in mice immunized against S-peptide tagged Hendra G. Neutralization assays are planned in collaboration with MedImmune, Inc. and should assist in determining whether antibodies to sG are present and whether in high enough concentration to have any neutralizing capacity.

Discussion

We generated clones of full-length hMPV F and G genes and expressed them in mammalian cells. Recombinant proteins were detected by immunoblotting transfected cell lysates using anti-peptide antibodies generated against the cytoplasmic tail and convalescent human serum for hMPV F. The recombinant proteins appeared generally as monomers, with F running in a denaturing, reducing gel at the expected size of 58 kDa. However, when expressed in the vaccinia vector pMCO2, there appeared to be both monomeric and potentially dimeric forms. This has previously been reported for both RSV and hMPV F (32, 95) and is thought to be an artifact, as paramyxovirus fusion proteins primarily exist as trimers.

Glycoprotein G expressed as a smear from approximately 35 to 100 kDa, much larger than the 26 kDa calculated mass for the unmodified form. It has previously been reported that one species of approximately 80-100 kDa is detected using an antipeptide antibody directed against the C-terminus of hMPV G (14). When using serum raised against the G cytoplasmic tail on the N-terminus, a ladder of bands from 13 to 100 kDa is detected. The S-peptide tag in our preparation is on the N-terminus, as is the cytoplasmic

tail to which the anti-peptide antibody was generated. Using antibodies against both the S-peptide tag and the anti-peptide antibody, we see the same pattern, indicating that not only is G heavily glycosylated, but could also be represented by C-terminally truncated species.

We generated a truncated, soluble form of the F glycoprotein of hMPV by removing the transmembrane domain and stably transforming a mammalian cell line with an expression vector. This recombinant protein is antigenic as seen by probes with human and multiple animal species antisera. It is also immunogenic in mice. Although expected to be a trimer in native form, sF_{A2} appears to be a monomer. Cseke et al. demonstrated that although the predicted molecular mass of the trimer is 168 kDa, most of the protein they purified could pass through a 100,000-molecular-mass cutoff membrane, and hypothesized that trimerization might be concentration and solution dependent (32). We compared our protein with that made by Ulbrandt et al., which did appear to form monomers and trimers and higher molecular weight species in sucrose gradient fractions. With the use of a reducible cross-linker, the oligomeric forms became more stable in a denaturing gel, and revealed that the majority of the monomers and trimers are present as separate species that do not cross-link to higher molecular forms. There are multiple differences between the protocols for development of these two proteins, including the length of sF_{A2}, growth in different mammalian cell lines in different conditions, purification by S-peptide-tag vs. mAb, and the presence of the S-peptide tag itself, which is nine residues longer than the 6-His-tag, and could potentially interfere with protein binding. Regardless, sF_{A2} has proven to be an effective antigen and

immunogen. As a monomer, it may expose more epitopes than a trimer, and could possibly elicit an increased antibody response. More testing needs to be done to determine the antigenicity of this protein, including comparing neutralizing antibody responses and protection induced after immunization with monomeric and trimeric forms.

Generation of soluble and secreted type II membrane glycoproteins can be problematic because of the need to retain a signal peptide and removal of the TM anchor domain, which can sometimes overlap. Besides hMPV, the other paramyxoviruses that possess a G envelope glycoprotein as attachment glycoproteins are RSV, Hendra virus and Nipah virus. RSV spontaneously produces both membrane-attached and secreted, truncated versions of G by using alternative methionine start sites (94), but hMPV does not. Attempts in this laboratory by other scientists to make soluble Hendra G by sequentially deleting the TM similarly to what occurs naturally in RSV were unsuccessful, but replacing the signal sequence, cytoplasmic tail and TM with an Ig κ leader sequence resulted in secreted, soluble Hendra G. We used this same approach and also obtained secreted, soluble hMPV G, although the expression was less than that seen for hMPV sF.

Many animal models have proven useful in demonstrating replication and development of immunity to hMPV. All of the pre-immunization animal sera used in this study were tested for the presence of neutralizing antibodies to hMPV. Interestingly, captive chimpanzees were shown to naturally have a high seroprevalence (19 of 31 positive) for hMPV based on the presence of neutralizing antibodies, either from infection from human handlers or animal-to-animal transmission (103). This is similar to

that seen in chimpanzees for RSV (31). AGMs and rhesus macaques did not demonstrate seroprevalence for hMPV in these initial tests. The detection of low levels of antibody pre-immunization in all the non-human primates for hMPV in ELISA is interesting, and may demonstrate waning immunity from an older infection or remnants of maternal antibodies, neither of which are high enough to neutralize virus, or the presence of non-neutralizing antibody.

To determine whether there is cross-reactivity of hMPV antibodies to RSV antigens in ELISA is important in developing a serological test for prevalence and incidence of infection. In testing an ELISA based on hMPV F for human samples, Leung et al. tested RSV-specific antibody against their VSV-hMPV F conjugate and found no cross-reactivity (70); however, the reverse, testing known hMPV positive sera against RSV antigen, has not been done. All of the non-human primates had pre-existing antibodies to RSV F in both Western blot and ELISA, and only one had mildly increased titers in ELISA post-infection with just one genotype, arguing that there is little non-specific increase in antibodies to RSV after exposure to hMPV, and that the new hMPV antibodies do not cross-react to RSV F. However, in the hamster and rabbit model, where there were no pre-existing RSV antibodies, infection with hMPV resulted in detectable antibodies to RSV F in ELISA, which must represent ability of the antibody to cross-react to RSV F, which has 33% homology with hMPV F. This could be due to a less specific antibody response in animals that are not the normal hosts of this virus, or the generation of antibodies with lower specificity with first exposure to the virus compared to the non-human primates with pre-existing hMPV antibody.

Immunization of mice with sF and sG elicited antibodies to each of these antigens, with a much greater response seen to F. The only previous study that attempted immunization with a recombinant virus expressing hMPV G showed a decreased neutralizing antibody response compared to F. Virus neutralization assays will determine if there is any neutralization capability after immunization with sG, or if there is improvement in neutralization after immunization with sF and sG compared to sF alone. Although our sF construct appears to be a monomer, it is antigenic and immunogenic and could facilitate structural studies of this important paramyxovirus protein.

Chapter 4 – Human Metapneumovirus Reinfection in Kamphaeng Phet, Thailand

Introduction

Human metapneumovirus causes a significant amount of childhood respiratory illness and has been documented worldwide. Despite apparent near-universal exposure during early childhood, the impact of hMPV later in life is still under debate. Reports of reinfection are not uncommon, but are mostly case reports. The largest study to test specifically for reinfection was in adult volunteers and revealed 4.5% of 984 illnesses were caused by hMPV (45). In addition, sera from 217 asymptomatic adult volunteers taken at the beginning and end of two winter seasons showed an asymptomatic infection rate of 4.1%, mostly in adults under 40 years of age.

Incomplete immunity has also been demonstrated for RSV. Repeat infections are usually milder and are less likely to result in lower respiratory tract infections and hospitalization. As people age, multiple reinfections induce higher levels of IgA and a more sustained secretory antibody response. In particular, IgA antibodies may play a role in long-term protection more than serum antibodies, especially in the upper respiratory tract (29). It is postulated that susceptibility to hMPV reinfection functions in a similar manner. As children age, the ability to resist infection increases with multiple exposures. It is possible that due to these exposures, older children may not only be less susceptible to reinfection than younger children but could also be less susceptible than adults who may have waning immunity. However, the known ability of pneumoviruses to cause disease in those previously exposed leads us to believe that reinfection may be more

common than reported, especially for mild infections. We hypothesize that schoolchildren ages 7 to 16 will have evidence of almost 100% exposure to hMPV and those with mild, febrile illnesses will have evidence of hMPV reinfection and will demonstrate reinfection rates similar to those seen for adults.

Results

Study population demographics. Serum samples from a prospective dengue study were collected as detailed in the Methods. During the dengue study, all paired serum samples were tested for evidence of recent infection with dengue and Japanese encephalitis viruses. A total of 2,557 paired acute and convalescent sera were negative for flavivirus infection and available for this study. For these samples, the ages ranged from 6 to 16 years with an average age of 9 years, 11 months and 50.6% were male. For all the patients in the serum bank, 73.9% reported cough, rhinorrhea or both, and the average temperature at the acute visit was 100.6ºF. We tested 1,380 (54%) of the samples. All of the available samples taken in 1999 and 2000 were tested, and a random sample of those collected in 2001 and 2002. Samples from 1998 were not all available, and we tested all the sera collected from 1 June-29 July, 18-22 September and 21-29 October in 1998. There was no statistically significant difference between the gender and age of those tested and untested (Table 3).

Testing of ELISA standards. Known hMPV positive and negative human sera kindly provided by Dr.Jeffrey Khan were used to develop standards for the serosurvey. As almost everyone has been exposed to hMPV by the age of 5, anonymous human sera

Table 3. Characteristics of children with samples in the serum bank, those tested for hMPV and those positive for an acute hMPV infection

	Untested Samples n=1177	*Negative Tested Samples n=1312*	*Positive Samples n=68*	*p-Value**
Enrolled children				
Age range (years)	6-15	7-16	7-14	
Mean age (years) (1 SD)	10.1 (1.5)	9.9 (1.6)	10 (1.6)	$p > .05$
Male/female sex (ratio)	594/583 (1.02)	664/648 (1.03)	36/32 (1.13)	$p > .05$
Clinical symptoms				
Cough	72.3%	62.5%	85.3%	***p* < .001**
Rhinorrhea	57.0%	49.8%	66.2%	***p* < .01**
Mean temperature ºF[§]	100.8	100.5	100.0	***p* = .007**

* 3-way comparison between untested, tested and positive samples for age and sex. 2-way comparison between tested and positive samples for clinical symptoms. χ^2 test for categorical data and t-test for continuous variables.

[§]Temperature at time of health care worker visit.

SD = Standard deviation

from adults was purchased and tested against the known positive sera to ensure having an adequate amount for the serosurvey. We pooled three with the highest titers and used these sera as the positive and negative standard (Figure 15). Dilutions of the standard from 1:64 to 1:16,384 were run on every ELISA plate and a standard curve generated. The point where the curve started to steeply climb, usually at the 1:4,096 dilution, is the optical density (OD) reading we considered positive for all samples on that plate.

Sera screening and confirmatory testing reveal evidence of reinfection with hMPV. All the sera were tested in indirect ELISA using sF_{A1} glycoprotein as the antigen in initial dilutions of 1:100, 1:500 and 1:5000. All acute and convalescent paired specimens that were both positive at the same 1:100 or 1:500 dilutions and negative at 1:5000 were considered evidence of previous infection without reinfection. If both the acute and convalescent titers were negative at 1:100 or if both were positive at 1:5000, the results were inconclusive and the samples were selected for further testing. If at least the convalescent sample was positive at 1:5000 and there was a two-fold difference in the OD value at 1:5000 between the acute and convalescent sample, they were considered presumptive for reinfection and also retested. The selected samples were retested in serial dilutions from 1:500 to 1:16,000 and again from 1:64 to 1:262,144 if necessary. Table 4 depicts the flow diagram of testing and results. There were 66 presumptive reinfections, 2 new infections and 2 patients with no evidence of hMPV exposure**,**

Figure 15. Standard curve for serosurvey.

sF_{A1} glycoprotein was coated onto Immulon II 96-well plates at a concentration of 50 ng/well. Known hMPV negative and positive sera and a mixture of three sera from anonymous donors were tested in 11 serial dilutions starting at 1:64. The plates were reacted with alkaline phosphatase-conjugated goat anti-human antibodies and detected with PNPP substrate. Each dilution was performed in duplicate and the means with standard error bars are plotted.

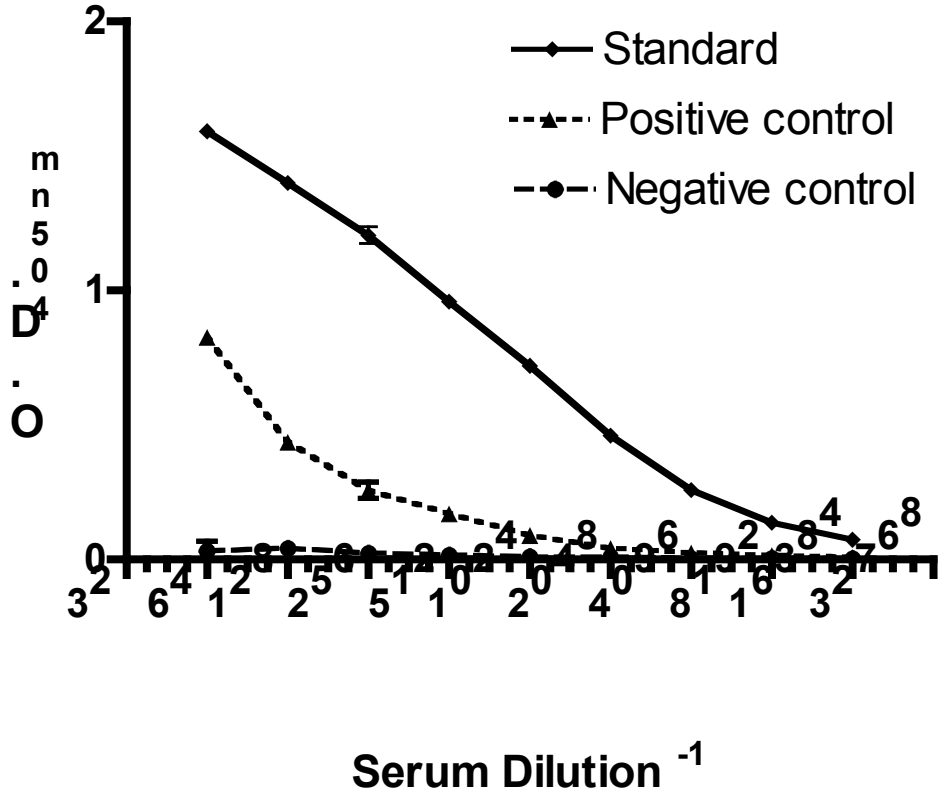
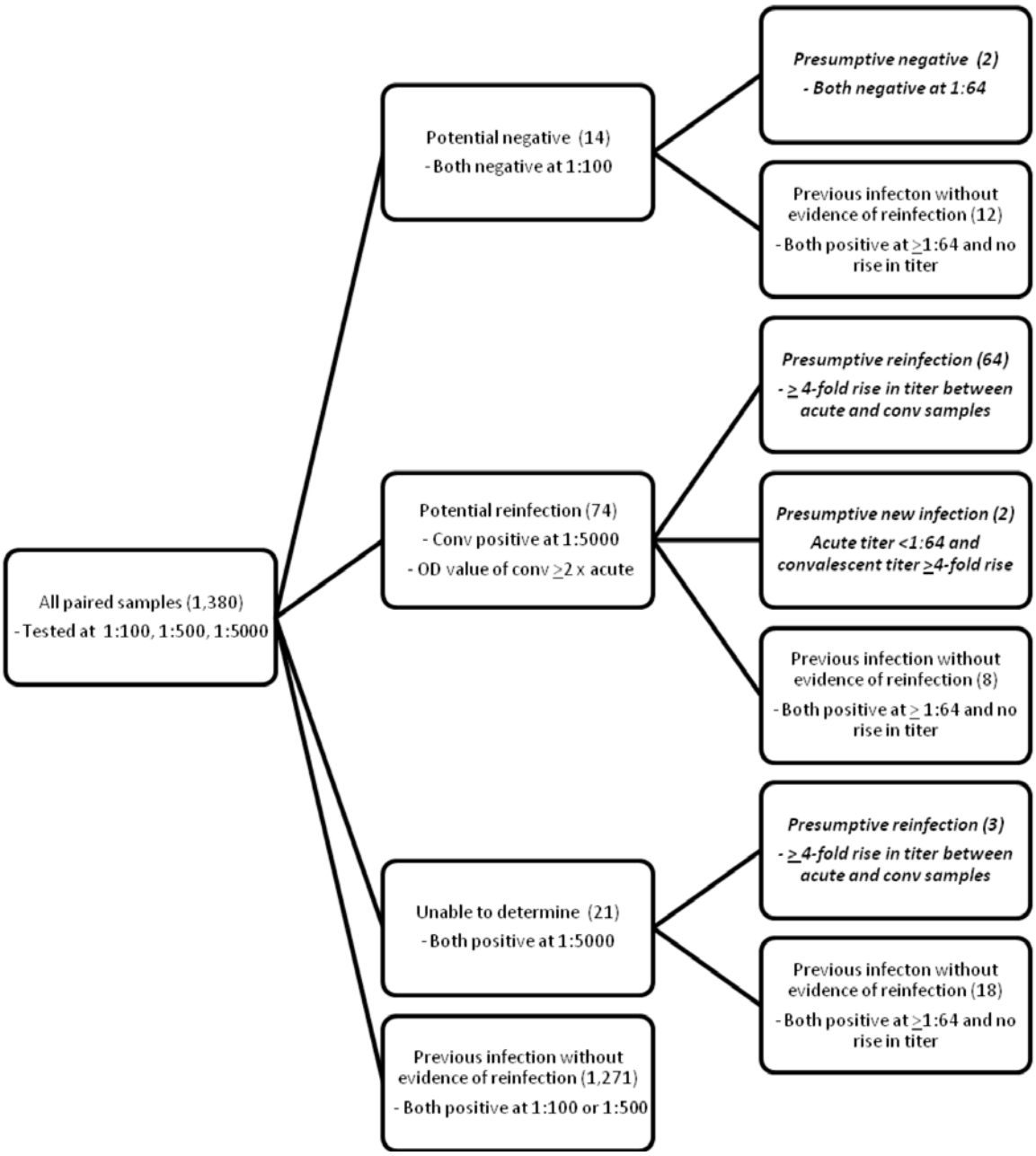

Standard
Positive control
Negative control
O.D. 450nm
2
1
0
Serum Dilution -1

Table 4. Flow diagram of serum sample testing



for an overall previous infection rate of 99.7%, and a reinfection rate of 4.9%. Table 5 lists the results by year. As the samples were initially tested using protein provided by MedImmune, Inc. (sF_{A1}), all of the negative sera, new infections and reinfections were retested using newly created sF_{A2} with the same results.

The paired specimens that were negative in ELISA and the paired specimens that showed a potential new infection were further tested by Western blotting. An example of each one is shown in Figure 16. All the samples were positive for antibody to RSV F, but negative for three different subtypes of hMPV F in the acute sera. The two new infections demonstrated antibody to all three F subtypes in the convalescent sera. Although we cannot rule out the possibility of antibody below the limits of detection in the acute sera, it appears that 4 volunteers were negative for exposure prior to their illness, and two of these had a new infection with hMPV.

Human antibodies bind equally to F from both genetic lineages but poorly to G. As there is debate whether the two genetic lineages represent different serotypes, it is possible that the ELISA could be negative if the patients were infected with lineage B and we used sF lineage A as antigen. Leung et al. demonstrated no difference in OD readings in ELISA using cell lysates infected with recombinant VSV expressing A or B lineages as antigen (70). To verify that our assay was equally sensitive for the two lineages, we tested 12 paired sera with evidence of reinfection and 48 paired sera with only evidence of a prior exposure in serial dilutions using the sF_{B1} protein as antigen and obtained the same result for every sample.

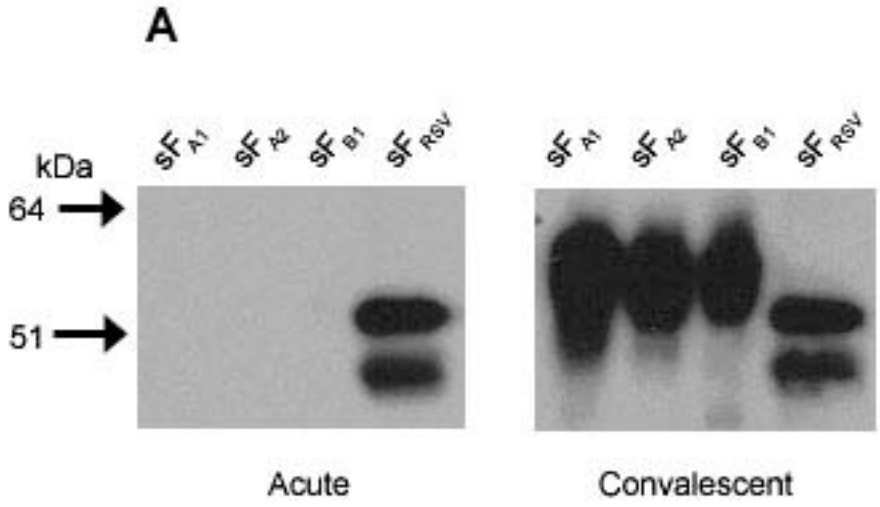
Table 5. Results of human serosurvey

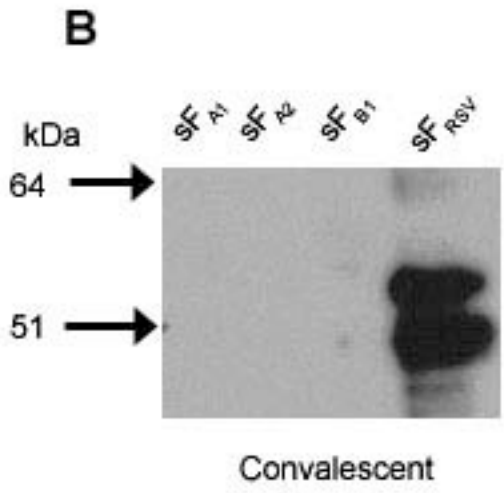
Year	*Number tested of total*	*≥ 4-fold rise in titer*
1998	390/1,083 (36.0%)	14 (3.6%)*
1999	326/341 (95.6%)	1 (0.3%)
2000	364/372 (97.8%)	48 (13.2%)*
2001	157/461 (34.1%)	2 (1.3%)
2002	143/300 (47.7%)	3 (2.1%)
Total	1,380/2,557 (54.0%)	68 (4.9%)

* One case a presumptive new infection with negative acute sera.

Figure 16. Western blot of presumptive hMPV negative and new infection sera.

Purified hMPV sF_{A1}, sF_{A2}, and sF_{B1} and RSV sF were resolved on 4-12% Nu-PAGE gels and transferred to nitrocellulose membranes. The membranes were probed with human sera as follows: (A) acute and convalescent sera from a patient after the acute sera tested negative but the convalescent sera tested positive for hMPV sF antibody in ELISA, or (B) convalescent sera from patient with paired sera that both tested negative in ELISA for hMPV sF antibody. Bound antibodies were reacted with a peroxidase-conjugated goat anti-human IgG and visualized by chemiluminescence.


A
kDa
64
51
sF A1
sF A2
sF B1
sF RSV
sF A1
sF A2
sF B1
sF RSV
Acute
Convalescent


B
kDa
64
51
sF A1
sF A2
sF B1
sF RSV
Convalescent

As G only has 32-37% amino acid identity between the A and B genotypes (1, 14, 88), and we do not know the lineages that caused the infections, it is impossible to know whether to expect a change in reactivity to G in ELISA after reinfection. However, it is known that different strains can cause annual outbreaks of infection and they can co-circulate, so it would be unusual to have all of the infections over a 5 year period caused by the same lineage. In addition, as described in Chapter 3, sera from non-human primates immunized with the same strain of virus do react to G. To determine if an antibody reaction to the G glycoprotein exists, we screened 50 of the reinfections at 1:50 and 1:2500 dilutions. Six had two times or greater the optical density reading in both dilutions between acute and convalescent titers. Without virus isolation, it is impossible to determine if the rise in titer to G is secondary to infection with a similar A2 (CAN97-83) lineage from which the antigen was derived. However, it is significant that antibodies directed against the G glycoprotein are present and increase in titer to infection in some of the patients.

Specificity of hMPV ELISA. Antibodies can cross-react to antigens with similar epitopes. RSV is the most closely related human pathogen and the F glycoprotein has 30-43% homology to hMPV F (9). It is possible that reinfection with RSV could cause a rise in RSV antibody titer detectable by hMPV F antigen. Studies have shown that polyclonal antibody to hMPV nucleoprotein (N) cross-reacts to all the metapneumoviruses, but not to RSV (3). Hamelin and Boivin tested an ELISA based on recombinant N protein against sera from three adults with recent documented RSV infection and found no increase in titers to hMPV (54). Leung et al. demonstrated that anti-RSV antiserum did

not bind to plates coated with lysates from cells infected with rVSV-hMPV-F, and serum which had RSV antibodies subtracted did not bind differently to hMPV F compared to serum with RSV antibodies (70). However, no one has tested RSV cross-reactivity to purified hMPV F glycoprotein in ELISA, and RSV-naïve rabbits and hamsters did develop cross-reactive antibodies to RSV after infection with hMPV as described in Chapter 3.

Another possible occurrence is a non-specific clonal expansion of antibodies to hMPV after infection with another virus since most patients have been previously exposed to hMPV and maintain memory cells. To test this theory, we initially assayed 32 sera in our hMPV ELISA from dengue positive patients confirmed by virus isolation and neutralization. Only 2 out of 14 from 1998 and 0 of 18 from 2001 had increased titers to hMPV sF between acute and convalescent sera. To further determine the potential of a non-specific response and to test for cross-reactivity, we assayed the previous 68 and the two new dengue reinfection positive convalescent sera against RSV F. As a control, we used serial dilutions of concentrated human RSV antibody, kindly provided by Dr. Jorge Blanco (Virion Systems, Inc.) on each plate to generate a standard curve. Only 19 of the 70 had a four-fold or greater rise in antibody titer to the RSV antigen, and 16 of 19 demonstrated a greatly reduced titer elevation when compared to hMPV (Table 6). While we cannot rule out cross-reactivity or non-specific clonal expansion of antibodies to a different infection, it is also possible that these represent co-infections with more than one virus.

Table 6. Day of sample collection and IgG antibody titers to hMPV and RSV

		Day of illness sample taken		Titer of hMPV IgG antibody			Titer of RSV IgG antibody		
Age (y)	Sex	Acute	Conv	Acute	Conv	Fold increase	Acute	Conv	Fold increase
9	F	1	15	<1:64†	1:8192	>128	**1:200**	**1:6400**	**32**
8	M	--*	--*	1:500	1:4000	8	1:1600	1:1600	1
8	M	2	16	1:64	1:32768	512	**1:800**	**1:6400**	**8**
12	F	1	14	1:500	>1:16000	>32	1:3200	1:6400	2
12	M	2	14	1:500	>1:16000	>32	1:1600	1:1600	1
12	F	2	14	1:1000	>1:16000	>16	1:3200	1:3200	1
8	M	2	14	1:128	1:4096	32	1:400	1:400	1
8	F	4	28	1:64	1:4096	64	1:400	1:800	2
10	M	3	17	1:500	1:8000	16	1:1600	1:1600	1
8	M	3	18	1:1000	1:16000	16	1:1600	1:1600	1
10	M	2	16	1:500	1:8000	16	1:400	1:800	2
11	F	2	21	1:500	1:8000	16	1:800	1:800	1
9§	M	2	16	1:64	1:32768	512	**1:200**	**1:3200**	**16**
12	F	1	14	1:500	1:8000	16	1:1600	1:3200	2
9	F	1	18	1:256	1:65536	256	**1:800**	**1:3200**	**4**
10§	M	2	15	1:1000	>1:16000	>16	1:1600	1:1600	1
14	M	2	21	1:500	1:2000	4	1:3200	1:1600	0.5
14	F	2	18	1:500	1:2000	4	1:200	1:200	1
8	F	1	11	1:128	1:65536	512	**1:600**	**>1:6400**	**>8**
10	M	2	18	1:500	1:16000	32	1:1600	1:1600	1
11	F	2	17	1:500	>1:16000	>32	**1:800**	**1:3200**	**4**
10	M	2	15	1:500	1:4000	8	1:1600	1:1600	1
12	F	2	13	1:500	>1:16000	>32	**1:800**	**>1:3200**	**>4**
7	F	2	16	1:128	1:65536	512	1:200	1:200	1
10	M	2	16	1:128	1:16384	128	1:400	1:400	1
9	M	1	14	1:1000	>1:16000	>16	1:400	1:400	1
9	M	1	15	1:1024	1:65536	64	1:400	1:400	1
9	M	1	13	1:500	1:16000	32	1:200	1:400	2
9	M	2	14	1:500	>1:16000	>32	**1:400**	**1:6400**	**16**
9	F	2	14	1:256	1:32768	128	1:800	1:1600	2
11	F	1	12	1:500	1:2000	4	1:1600	1:1600	1
12	F	1	13	1:500	1:8000	16	1:800	1:400	0.5
8	F	1	13	1:500	>1:16000	>32	1:400	1:800	2
12	F	2	13	1:1000	>1:16000	>16	1:1600	1:800	0.5
9	F	5	20	1:500	>1:16000	>32	1:800	1:800	1
8	M	4	18	1:500	>1:16000	>32	1:400	1:800	2
10	M	6	18	1:500	>1:16000	>32	1:800	1:1600	2
8	M	2	17	1:1000	1:8000	8	1:400	1:400	1
12	M	2	17	1:1000	>1:16000	>16	1:1600	1:3200	2
8	M	1	15	1:128	1:65538	512	**1:800**	**1:6400**	**8**
10	M	2	15	1:500	1:8000	16	1:400	1:400	1
8	M	2	14	1:500	>1:16000	>32	**1:800**	**1:3200**	**4**

Age (y)	Sex	Day of illness sample taken: Acute	Day of illness sample taken: Conv	Titer of hMPV IgG antibody: Acute	Titer of hMPV IgG antibody: Conv	Fold increase	Titer of RSV IgG antibody: Acute	Titer of RSV IgG antibody: Conv	Fold increase
8	F	3	15	1:500	>1:16000	>32	1:400	1:800	2
9	F	2	14	1:500	>1:16000	>32	1:800	1:800	1
11	M	2	14	1:500	1:8000	16	1:400	1:400	1
11	M	1	13	1:500	1:4000	8	1:200	1:200	1
8	F	5	18	<1:64†	1:16384	>256	**1:1600**	**>1:6400**	**>4**
8	M	2	16	1:500	1:2000	4	**1:800**	**1:6400**	**8**
10	M	2	17	1:500	1:4000	8	1:200	1:200	1
11	M	2	16	1:500	1:2000	4	1:200	1:200	1
10	M	2	24	1:256	1:32768	128	**1:400**	**1:3200**	**8**
10	F	2	14	1:1000	>1:16000	>16	**1:800**	**1:3200**	**4**
12	M	2	14	1:1000	>1:16000	>16	1:400	1:400	1
11	F	1	13	1:256	1:131072	512	1:1600	1:3200	2
8	F	2	16	1:500	>1:16000	>32	**1:800**	**>1:6400**	**>8**
12	F	2	16	1:256	1:131072	512	1:1600	1:1600	1
10	F	2	15	1:1024	1:16384	16	1:400	1:400	1
10	F	3	15	1:16384	1:65536	4	1:1600	1:3200	2
12	M	3	17	1:1000	>1:16000	>16	1:800	1:800	1
9	M	1	15	1:256	1:32768	128	**1:400**	**1:1600**	**4**
10	F	1	21	1:500	1:4000	8	1:400	1:800	2
10	F	4	19	1:2000	1:8000	4	**1:800**	**1:6400**	**8**
12	M	1	15	1:4000	1:16000	4	1:3200	1:6400	2
8	F	3	16	1:1000	>1:16000	>16	1:200	1:200	1
13	M	2	9	1:1000	1:16000	16	1:800	1:400	0.5
9	F	1	16	1:500	1:4000	8	**1:200**	**>1:6400**	**>32**
11	M	3	18	1:500	1:2000	4	1:800	1:1600	2
8	M	3	13	1:64	1:8192	128	**1:200**	**1:1600**	**8**
8	M	2	22	1:500	>1:16000	>32	1:800	1:1600	2
9	M	2	15	1:500	>1:16000	>32	1:1600	1:1600	1

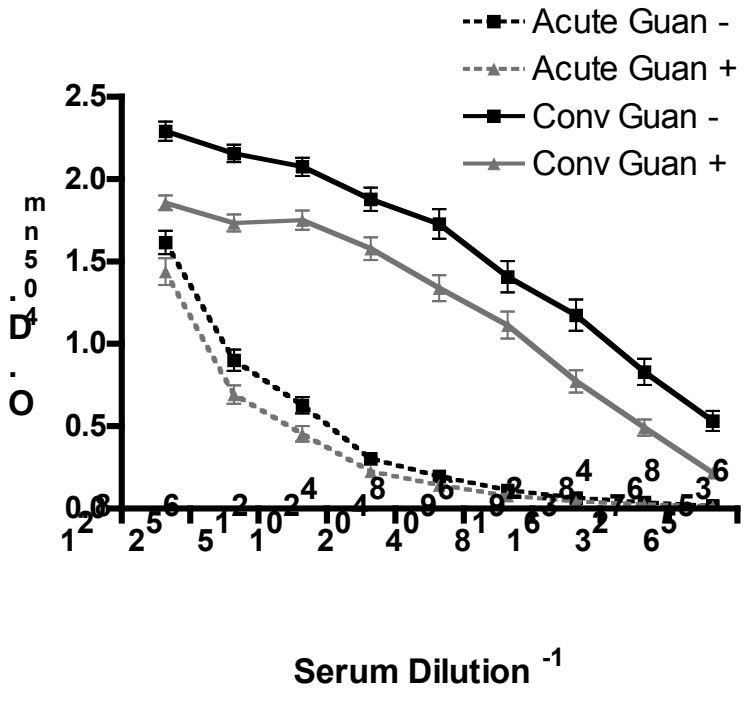
Cases with $\geq$ 4 fold-rise in titer for RSV are in bold.
*Unknown exact date of illness onset, convalescent sample taken 14 days after acute sample.
†First apparent infection with hMPV.
§Dengue positive cases.

Convalescent antibodies demonstrate decreased avidity. Antibody avidity has been demonstrated to gradually increase over time (58). Avidity can be used as a marker of relative antibody age to further demonstrate that an increase in antibody titer in convalescent sera is secondary to new antibody development and not an error in measurement. IgG avidity can be measured by the resistance to dissociation of the antigen-antibody complex to a disruptive agent. Inouye et al. used guanidine to disrupt antigen-antibody binding for rubella, Japanese encephalitis virus and rotavirus (58). Using a similar technique, we tested whether we could measure a detectable difference in acute and convalescent antibody avidity in 25 of the reinfection positive sera that were negative in RSV F ELISA. Averages of the 25 sera replicates are graphed in Figure 17 and exhibit a significant difference in antibody avidity between acute and convalescent samples. These data demonstrate newly formed antibody between the two blood draws; however, it cannot be determined if these new antibodies are the result of reinfection with hMPV or a non-specific increase in antibody from exposure to a different virus. This second explanation is less likely as all of these serum pairs did not react to RSV antigen in ELISA.

Epidemiology of hMPV in the study population. We found the majority of reinfections in 1998 and 2000. We were not able to test a representative sample of the study period in 1998, but 48 of the 68 new or re-infections occurred in 2000, accounting for 13.2% of all febrile illnesses in the study population in that year. 32 of these cases occurred in a discrete 2 week time period, suggesting a localized outbreak (Figure 18).

Figure 17. Guanidine assay for antibody avidity.

sF_{A2} glycoproteins were coated onto Immulon II 96-well plates at a concentration of 50 ng/well. Acute and convalescent sera from 25 hMPV positive and RSV negative cases were tested in 9 serial dilutions starting at 1:200 with and without 0.5 M guanidine added in the dilution buffer and incubated for 2 hours at room temperature. The plates were reacted with alkaline phosphatase-conjugated goat anti-human antibodies and detected with PNPP substrate. Each dilution was performed in duplicate and averaged and the mean of all the averaged results are plotted, with error bars depicting the standard error. Differences in antibody avidity after administration of guanidine were tested using repeated measures ANOVA with both guanidine and convalescent vs. acute as within-subject factors. Wilks' lambda was used to determine if there is a statistically significant difference between the change in acute and convalescent titers for each dilution with and without guanidine.



Dilution	*P-Value*
1:200	0.001
1:400	<0.001
1:800	0.002
1:1600	<0.001
1:3200	<0.001
1:6400	<0.001
1:12800	<0.001
1:25600	<0.001
1:51200	<0.001

Figure 18. Epidemic curve of hMPV cases in 2000.

Cases of illness from July to November with a 4-fold or greater rise in titer to hMPV sF were plotted by date.