Published in final edited form as:

J Clin Virol. 2009 December ; 46(4): 314–317. doi:10.1016/j.jcv.2009.09.020.

Microneutralization Assay for the Measurement of Neutralizing Antibodies to Human Metapneumovirus

Ann R. Falsey, M.D.^{1,2}, Maria A. Formica, M.S.¹, and Edward E. Walsh, M.D.^{1,2}

¹Department of Medicine, Rochester General Hospital, University of Rochester School of Medicine and Dentistry, Rochester, NY

²Departments of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY

Abstract

Background—Human metapneumovirus (hMPV) is a newly discovered virus which causes respiratory illness in persons of all ages.

Objective—A simple and rapid method to determine neutralizing antibody titers against hMPV is needed to facilitate the development of vaccines and therapeutics for hMPV. Therefore, we sought to adapt the methodology used for RSV microneutralization assay (MNA) to measure neutralizing antibody titers against hMPV.

Study Design—Serial 2-fold dilutions of serum were made in 96 well microtiter plates and incubated with ~ 50 pfu of hMPV A or B strain for 60 minutes at room temperature. LLC-MK2 cells were added to the serum-virus mixtures and plates incubated at 35°C in CO₂ for 5 days. Plates were fixed with acetone; air dried, blocked and then developed with monoclonal antibody to the hMPV N protein followed by horse radish peroxidase labeled antibody and substrate. Neutralization titer was defined as the titer of serum that reduced color development by 50% compared to the positive control wells.

Results—Titers measured by MNA correlated well with those determined by standard plaque reduction assay ($R = 0.77$). Neutralization titers determined by MNA demonstrated excellent inter-assay variability (coefficient of variance = 7%). In addition, there was good correlation of antibody titers from 10 hMPV infected adults measured by MNA using either group A or group B hMPV ($R = 0.87$).

Conclusion—MNA is a simple and reproducible method for the measurement of serum neutralizing antibody against hMPV

© 2009 Elsevier B.V. All rights reserved.

Corresponding Author: Ann R. Falsey, M.D., Rochester General Hospital, 1425 Portland Avenue, #246, Rochester, NY 14621-3001, Phone 585-922-4339, ann.falsey@rochestergeneral.org Fax: 585-922-5168.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflicts of Interest:

Dr Falsey consults for Medimmune, GSK, Sanofi-pasteur, Astrazeneca, and ADMA Biologics and has received honoraria. She also serves on the advisory board of Quidel Inc. Dr Walsh consults for Astrazeneca and ADMA Biologics and has received honoraria. Ms. Formica has no conflicts of interest.

Background

Human metapneumovirus (hMPV) is an RNA virus in the pneumovirus subfamily of *Paramyxoviridae* first described in young children with bronchiolitis from the Netherlands.¹ Since its discovery in 2001, the virus has been shown to have world wide circulation and to be a common cause of respiratory illness in persons of all ages.² Similar to a related virus, respiratory syncytial virus (RSV), immunity to hMPV appears to be incomplete and re-infections occur throughout adulthood.³ Correlates of immunity have not been established, although antibodies to the fusion protein (F) have been shown to provide protective immunity in animal models.⁴ At the present time neutralizing antibody titers to hMPV are most commonly measured using standard plaque reduction assays.⁵ In these assays, plaques are identified under the microscope by visualizing typical cytopathic effect or immunostaining. Modifications to the traditional plaque assay using recombinant hMPV expressing green fluorescent protein allow automated reading of assays, however, this technology is not widely available.^{6, 7} A microneutralization assay (MNA) has been used successfully for the measurement of neutralizing antibodies to RSV in vaccine and epidemiologic studies.^{8, 9} This method is performed by adding suspended cells to an antibody-virus mixture followed by an enzyme immunoassay (EIA) readout to measure viral antigen production. Neutralizing antibody levels measured by MNA have been found to have better correlation with protection from RSV in animal models than titers measured by plaque reduction assay.¹⁰ However, development of a similar MNA for hMPV is complicated by the necessity of using trypsin in the viral growth media and slower viral growth characteristics.

Objectives

In this report, we describe the development of a MNA assay for use with trypsin dependent strains of hMPV as a simple and accurate method for the measurement of hMPV neutralizing antibody titers.

Study Design

Virus culture

LLC-MK2 cells and Vero cells were maintained in MEM, 5% fetal calf serum (FCS). The CAN 97-83 (group A) and CAN 98-75 (group B) strains of hMPV were kindly provided by Dr. Guy Boivin at Laval University Quebec, Canada. Viruses were grown in LLC-MK2 cells with viral growth media (VGM) consisting of 1% bovine serum albumin, 1% HEPES, 0.5% glucose and porcine pancreatic trypsin (2 units/ml). Viral stocks were quantified using a plaque assay.

Plaque Assay

Twenty-four well plates were set with 2×10^5 Vero cells to achieve ~80% confluence the next day. After monolayers have been established, 200ul of serial 10-fold dilutions of hMPV were added in duplicate to the monolayers and allowed to adsorb for 1 hour at room temperature (RT). The inoculum was then aspirated and wells overlaid with 0.5% methylcellulose in VGM. Plates were incubated at 35°C in a CO₂ incubator for 6 days. Monolayers were rinsed, methanol fixed and stained with a monoclonal antibody directed against the hMPV N protein, followed by peroxidase labeled goat anti-mouse immunoglobulin and TruBlue substrate (KPL Kierkegaard and Percy Laboratories, Inc.) and plaques counted under a microscope.

Human sera

Serum samples were obtained from 20 subjects who were enrolled in a prospective study of respiratory illness and had hMPV infection documented by non-group specific reverse

transcription polymerase chain reaction (RT-PCR) or a ≥ 4 -fold rise in serum hMPV specific IgG by EIA.³ Acute and convalescent sera were tested by MNA using CAN 97-83 and CAN 98-75 hMPV. The neutralizing activities of 10 paired samples were also measured using standard plaque reduction assay. All sera were heat inactivated at 56°C for 30 minutes to eliminate serum complement.

Neutralization Assays

Plaque Reduction Neutralization Assay—Serum neutralizing antibody was measured using a modification of a plaque assay as described by Williams, et al.⁵ Twenty-four well plates were set with 2×10^5 cells/ml Vero cells to achieve ~80% confluence the next day. Ten serial 2-fold dilutions of sera starting at 1:50 were incubated at RT for 60 minutes with an equal volume hMPV to provide approximately 50 plaque forming units (pfu)/ 200uL. Two hundred microliters of the serum-virus mixtures were added in duplicate to cell monolayers and allowed to adsorb for 3 hours at RT. Positive control wells consisted of virus without sera. Inoculums were aspirated and wells were overlaid with 0.5% methylcellulose in VGM. Plates were incubated and developed in the same manner as noted above. Plaques were counted and 50% plaque neutralization titers calculated.

Microneutralization Assay

Nine serial 2-fold dilutions (1:50 –1:12,800) of serum were made in 50ul VGM with trypsin in 96 well microtiter plates. Fifty microliters of virus containing ~ 50 pfu of hMPV A or B strain was added to the serum dilutions and allowed to incubate for 60 minutes at RT. Positive control wells of virus without sera and negative control wells without virus or sera were included in triplicate on each plate. While the serum-virus mixtures incubated, a single cell suspension of LLC-MK2 cells was prepared by trypsinizing (Gibco 0.5% bovine pancrease trypsin in EDTA) a confluent monolayer and suspended cells were transferred to a 50 ml centrifuge tube, topped with sterile PBS and gently mixed. The cells were then pelleted at 200g for 5 minutes, supernatant aspirated and cells resuspended in PBS. This procedure was repeated once and the cells were resuspended at a concentration of 3×10^5 /ml in VGM with porcine trypsin. Then, 100 ul of cells were added to the serum-virus mixtures and plates incubated at 35°C in CO₂ for 5 days. The plates were fixed with 80% acetone in phosphate buffered saline (PBS) for 15 minutes at RT, air dried and then blocked for 30 minutes containing PBS with 0.5% gelatin and 2% FCS. A monoclonal antibody to the hMPV N protein was diluted in PBS with 0.5% gelatin/ 2% FCS/0.5% Tween 20 and incubated at RT for 2 hours. Wells were washed and horse radish peroxidase conjugated goat anti-mouse IgG added, followed by another 2 hour incubation. After washing, O-phenylenediamine dihydrochloride was added and the neutralization titer was defined as the titer of serum that reduced color development by 50% compared to the positive control wells (Figure 1)

Results

Twenty acute and convalescent serum pairs were analyzed using the microneutralization assay using CAN 97-83 (MNA-A) and CAN 98-75 (MNA-B) strains of hMPV. Twenty samples (10 acute and convalescent pairs) were run in three assays using CAN 97-83 hMPV performed on different days with excellent inter-assay variability (coefficient of variance = 7%). The mean log₂ titers for MNA-A and MNA-B values were 11.53 ± 1.96 and 11.90 ± 2.10 , respectively. The correlation of MNA-A and MNA-B values was high with a correlation coefficient of 0.87 (Figure 2). There was also a good correlation between the rise in titer from acute to convalescent values for MNA-A and MNA-B ($r=0.83$). Overall, there was 70% concordance of sero-response (≥ 4 -fold rise) with 16/20(80%) having a ≥ 4 -fold rise by MNA-A and 18/20 (90%) by MNA-B. Because the RT-PCR assay used for diagnosis was not group specific, the infecting hMPV strain for the illnesses was not known.

Ten of the 20 acute and convalescent sera tested by MNA-A were also assayed by plaque reduction assays using CAN 97-83 hMPV (Group A) in three separate assays. Neutralizing titers were slightly more variable as measured by plaque reduction than MNA (coefficient of variation = 9%). Correlation of the average neutralizing titers for the 3 assays of the 10 acute and convalescent determined by both plaque reduction and MNA was good with a correlation coefficient of 0.77 (Figure 3) although mean titers were higher by plaque reduction than MNA (13.25 ± 1.09 vs. 10.80 ± 0.80 , $p = .0001$ by students t-test). This difference was primarily due to higher convalescent titers as determined by plaque reduction. Although MNA and plaque reduction responses correlated well ($r = .76$), the magnitude of rise was somewhat greater for plaque reduction compared to MNA (3.38 ± 2.1 vs. 1.95 ± 1.3 , $p = .09$). Seven subjects demonstrated a ≥ 4 -fold rise and one subject showed a drop in titer by both assays. Two subjects had a ≥ 4 -fold rise in titer by plaque reduction with only a modest rise by MNA.

Discussion

Adapting the MNA methodology used for RSV to hMPV was complicated by the nature of LLC-MK2 cells and the need for trypsin in the growth media of hMPV. In the RSV assay, EDTA is used to disperse Hep-2 cells but was not effective for LLC-MK2 cells and resulted in an poorly adherent irregular monolayer. While the use of bovine pancreas trypsin produced a homogenous single cell suspension of LLC-MK2 cells, a monolayer would not set in the 96 well plates with the trypsin present when added to the virus-sera mixture. By centrifuging the LLC-MK2 cells and removing the bovine trypsin, an even adherent, monolayer resulted despite the presence of a low concentration of porcine trypsin in the hMPV growth media.

With minor modifications of the RSV MNA methodology, we found that MNA for hMPV is technically easy to perform and results in reproducible values over a wide range of antibody titers. Because the readout does not require laborious manual plaque counting with a microscope, the MNA can be used for studies which require rapid screening of large numbers of sera for hMPV neutralizing activity. Selection of susceptible volunteers for human challenge studies, screening donors to develop a plasma pool of high hMPV titer immunoglobulin, and clinical trials of hMPV vaccine candidates would be facilitated by a rapid and accurate assay such as the MNA.

Another potential advantage of the MNA compared to the standard plaque reduction assay may be better prediction of in-vivo protection from hMPV infection similar to the RSV MNA.¹⁰ Although hMPV neutralizing titers measured by plaque reduction correlated with MNA titers, some differences were apparent. In the MNA, antibody remains in the culture media throughout the assay, whereas, after the initial period of neutralization antibody is removed in the plaque reduction assay. The continued presence of antibody throughout the course of the assay neutralizes virus released from infected cells as well as prevents viral spread by inhibiting cell to cell fusion. Functionally distinct antibodies may be measured by MNA (i.e., anti-binding + anti-fusion) compared to plaque reduction which may primarily measure anti-binding antibody. In addition, the plaque assay measures only visible plaques, in contrast to the MNA, which quantitates total viral antigen production in the monolayer. These attributes of the MNA may best mimic in-vivo virus neutralization and thus, correlate better than plaque reduction assays with protection from hMPV infection.

Genetic analyses of hMPV indicate two major genotypes (Group A & B) can be defined and that the greatest diversity is found in the G protein.¹¹⁻¹³ However, it is not clear if genotypes translate into antigenically distinct serotypes. Most, but not all investigators, have demonstrated cross protection from infection with heterologous strains during animal challenge studies.^{11, 14} Using reverse genetics, the individual contributions of the F, G and SH proteins of hMPV have been evaluated in the hamster model.^{11, 15} The F protein was found to

be highly immunogenic and protective, whereas the SH and G proteins were not. Thus, the neutralizing activity of sera may be driven primarily by antibody to the conserved F protein without a significant role of the antigenically more diverse G protein.¹⁴ Thus, it is not unexpected we found a high degree of correlation between neutralizing antibody titers to group A and group B strains of hMPV in naturally infected adults. Although our diagnostic RT-PCR does not identify the infecting strain of hMPV, subjects had similar responses to both strains. Comparable observations have been noted with RSV infected adults, although neutralizing activity of antibodies directed against the RSV G protein have been demonstrated.¹⁶ Because our results may not be applicable to children with primary infection, studies of strain specific immune responses are needed in children.

Conclusions

In summary, MNA is a simple and reproducible method for the measurement of serum neutralizing antibody against hMPV. This assay should be useful in studies of disease pathogenesis and facilitate clinical studies of hMPV vaccine candidates.

Acknowledgments

The authors wish to thank Patricia A. Hennessey and Mary Criddle for collection of serum samples and Jamie Bear for technical assistance.

References

1. van den Hoogen BG, de Jong JC, Groen J, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med* 2001;7(6):719–724. [PubMed: 11385510]
2. Kahn JS. Epidemiology of human metapneumovirus. *Clin Microbiol Rev* 2006;19(3):546–557. [PubMed: 16847085]
3. Walsh EE, Peterson DR, Falsey AR. Human metapneumovirus infections in adults: Another piece of the puzzle. *Arch Intern Med* 2008;168:2489–2496. [PubMed: 19064834]
4. Herfst S, Fouchier RA. Vaccination approaches to combat human metapneumovirus lower respiratory tract infections. *J Clin Virol* 2008;41(1):49–52. [PubMed: 18054841]
5. Williams JV, Tollefson SJ, Johnson JE, Crowe JE Jr. The cotton rat (*sigmodon hispidus*) is a permissive small animal model of human metapneumovirus infection, pathogenesis, and protective immunity. *J Virol* 2005;79(17):10944–10951. [PubMed: 16103146]
6. de Graaf M, Herfst S, Schrauwen EJ, van den Hoogen BG, Osterhaus AD, Fouchier RA. An improved plaque reduction virus neutralization assay for human metapneumovirus. *J Virol Methods* 2007;143(2):169–174. [PubMed: 17420056]
7. Biacchesi S, Skiadopoulos MH, Yang L, Murphy BR, Collins PL, Buchholz UJ. Rapid human metapneumovirus microneutralization assay based on green fluorescent protein expression. *J Virol Methods* 2005;128(1–2):192–197. [PubMed: 15955576]
8. Anderson LJ, Hierholzer JC, Bingham PG, Stone YO. Microneutralization test for respiratory syncytial virus based on an enzyme immunoassay. *J Clin Microbiol* 1985;22(6):1050–1052. [PubMed: 4066916]
9. Falsey AR, Walsh EE. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in ambulatory adults over age 60. *Vaccine* 1996;14(13):1214–1218. [PubMed: 8961507]
10. Siber GR, Leszczynski J, Pena-Cruz V, et al. Protective activity of a human respiratory syncytial virus immune globulin prepared from donors screened by microneutralization assay. *J Infect Dis* 1992;165(3):456–463. [PubMed: 1538152]
11. van den Hoogen BG, Herfst S, Sprong L, et al. Antigenic and genetic variability of human metapneumoviruses. *Emerg Infect Dis* 2004;10(4):658–666. [PubMed: 15200856]
12. Bastien N, Normand S, Taylor T, et al. Sequence analysis of the N, P, M and F genes of canadian human metapneumovirus strains. *Virus Res* 2003;93:51–62. [PubMed: 12727342]
13. Biacchesi S, Skiadopoulos MH, Boivin G, et al. Genetic diversity between human metapneumovirus subgroups. *Virology* 2003;315:1–9. [PubMed: 14592754]

14. Skiadopoulos MH, Biacchesi S, Buchholz UJ, et al. The two major human metapneumovirus genetic lineages are highly related antigenically, and the fusion (F) protein is a major contributor to this antigenic relatedness. *J Virol* 2004;78(13):6927–6937. [PubMed: 15194769]
15. Skiadopoulos MH, Biacchesi S, Buchholz UJ, et al. Individual contributions of the human metapneumovirus F, G, and SH surface glycoproteins to the induction of neutralizing antibodies and protective immunity. *Virology* 2006;345(2):492–501. [PubMed: 16300813]
16. Walsh EE, Falsey AR. Age related differences in humoral immune response to respiratory syncytial virus infection in adults. *J Med Virol* 2004;73(2):295–299. [PubMed: 15122807]

Figure 1.

Sample graph of MNA results from a single 96-well plate containing 3 individual serum samples shown by lines with squares, circles and triangles. Optical density (OD) of the positive control wells is shown by the top line and the negative control by the bottom line on the horizontal axis. Neutralizing titer is defined as the intercept of the sample line with the line representing 50% reduction in OD.

Figure 2. Correlation of MNA titers to Group A and B hMPV

Correlation of 40 sera (20 acute and convalescent pairs) measured by MNA using CAN 97-83 (group A) and CAN 98-75 (group B) hMPV. Titers are expressed as log 2. $R = 0.87$.

Figure 3. Correlation of Neutralizing Titers measured by MNA vs. Plaque Reduction

Correlation of the mean neutralizing titers of 20 serum samples (10 acute and convalescent pairs) determined in 3 MNA and 3 plaque reduction assays. Assays were performed using CAN 98-75 hMPV and titers are expressed in log 2.