Development and Validation of an Enzyme-Linked Immunosorbent Assay for Human Metapneumovirus Serology Based on a Recombinant Viral Protein

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The human metapneumovirus (hMPV) is a respiratory viral pathogen belonging to the *Paramyxoviridae* family that has been associated with bronchiolitis and pneumonia in young children. We developed a simple enzyme-linked immunosorbent assay (ELISA) for hMPV serological testing using the nucleoprotein (N) from group A or B (N-A or N-B) as the antigen, and we evaluated it in both children and adults. The N proteins were first used in a Western immunoblot assay to identify hMPV-negative sera, which were then used to determine the cutoff value of the ELISA test. Subsequent evaluation of the ELISA-N test revealed that the mean reciprocal antibody titer of 20 randomly selected seropositive children was 143, compared to 69 for 20 seropositive adults. In a prospective evaluation of 71 adults with acute exacerbations of chronic obstructive pulmonary disease, 82 (81.6%) had prior hMPV antibodies and 3 (4.2%) had evidence of recent hMPV infection. In testing paired sera from adults (n = 4) with recent hMPV group A infection confirmed by reverse transcriptase PCR (RT-PCR), ELISAs using the N-A or N-B proteins were able to detect hMPV seroconversion. Moreover, testing of paired sera from three adults with a recent infection by the human respiratory syncytial virus confirmed by RT-PCR and serology did not reveal any increase in hMPV antibodies over time. The ELISA-N is a simple, objective, and specific serological test useful for detecting anti-hMPV antibodies following group A or B viral infections, which should permit a better understanding of the epidemiology of this virus.

The human metapneumovirus (hMPV) is a respiratory viral pathogen belonging to the *Metapneumovirus* genus which, along with the *Pneumovirus* genus (containing the human respiratory syncytial virus [hRSV]), is part of the *Pneumovirinae* subfamily within the *Paramyxoviridae* family. hMPV isolation was first reported in 2001 from young children with acute respiratory tract infections (ARTI) in The Netherlands (24), although seroprevalence studies showed that hMPV had been present for more than 50 years in that country. Limited reports have indicated that virtually all children are infected by 5 to 10 years of age (10, 24, 29). The virus has been associated with ARTI in all age groups, with more severe diseases such as bronchiolitis and pneumonia in young children, elderly subjects, and immunocompromised hosts (2, 4, 12, 17, 27). The importance of hMPV has been best studied in young children with severe ARTI, accounting for 5 to 10% of hospitalizations in that population (4, 11, 14). Although there is considerable overlap in the clinical features of hMPV and hRSV infections, the latter has been generally associated with a more severe outcome (4, 18, 26, 27).

The hMPV genome is a single strand of negative RNA of approximately 13 kb coding for nine proteins (23, 24). Phylogenetic analyses have demonstrated that hMPV is more closely related to avian metapneumoviruses (aMPV) than to hRSV. hMPV isolates can be separated into two major groups (A and B) and at least four subgroups based on sequence analysis of the fusion (F) and attachment (G) genes (2, 5, 20, 25). Nucleotide and amino acid sequence identities between two strains representing the two major hMPV groups were found to be 80 and 90%, respectively, which is similar to identities between hRSV groups A and B (1). However, whether those two genotypes represent different antigenic groups is still a matter of debate (22, 25).

Serological tests based on an enzyme-linked immunosorbent assay (ELISA) have been developed for the detection of aMPV antibodies. In those assays, different antigens were used, including lysed infected cell culture preparations as well as recombinant viral proteins, such as the matrix protein (M) and the nucleoprotein (N) (7, 15, 16). Those assays were found to be sensitive and specific for detecting aMPV antibodies, with the recombinant protein-ELISA test being more sensitive than assays using virus-infected Vero cells as the antigen (16). Until now, serological assays for hMPV have consisted of immunofluorescence assays or ELISA tests using hMPV-infected cells (2, 9, 10, 12, 24, 29). These assays are not suitable for large-scale epidemiological studies, and they have not been well validated with other related paramyxoviruses, such as hRSV, or for their ability to detect both hMPV genotypes. In this study, we describe the development and validation of an ELISA for detection of hMPV antibodies against both viral genotypes, based on a recombinant viral protein.

**Materials and Methods**

*Serum specimens.* Sera from children aged 0 to 4 years and from adults randomly selected from our clinical virology laboratory were first used to develop the assay and to determine its cutoff value. Subsequent validation of the assay was done using paired sera (collected 3 weeks apart) collected during a prospective study on the etiology of acute exacerbation of chronic obstructive pulmonary disease (COPD) in Canadian patients older than 40 years of age.
Production and purification of recombinant hMPV N proteins. The N genes of hMPV isolates CAN97-83 (group A) and CAN98-75 (group B) were amplified by reverse transcriptase PCR (RT-PCR) using primers N1 (5'-CGTATGATTCTCTGTTTTCAAGGATTGAC) and N2 (5'-GTCCTCTGAGGT TTCAATCTTTGACTGTC) for group A and N3 (5'-CGTACATATG ATGTCTCTCAGGATTTG) and N4 (5'-GTCCCTCAGTTAATCTACA TATCTTTG) for group B. The PCR products were cloned into the pET19b (+) vector (Novagen, Madison, Wis.), which contains a His6 tag downstream of the T7 promoter. The vectors were transformed into Escherichia coli strain BL21 (DE3), and the expression of the recombinant proteins was induced by 0.1 mM of isopropyl-β-D-thiogalactopyranoside. The recombinant proteins purified on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 12% polyacrylamide gels (Bio-Rad, Hercules, Calif.) to eliminate contaminating E. coli proteins. The 45-kDa band was excised from the gel, followed by protein extraction and elution with Nanosep 0.2-μm columns (Pall Life Sciences, Quebec City, Canada).

Western blot analysis. The purified N proteins from hMPV group A (N-A) and group B (N-B) were separated by SDS-PAGE with a 12% polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was blocked for 30 min with phosphate-buffered saline (PBS) containing 0.05% Tween 20 at 37°C. Serum samples (diluted 1:20 in PBS–0.05% Tween 20) were added, and the membrane was incubated for 1 h at 37°C. The membrane was washed three times with PBS–0.05% Tween 20 and incubated for 1 h at 37°C with horseradish peroxidase-conjugated AffiniPure goat anti-human immunoglobulin A (IgA) plus IgG plus IgM (H + L; Jackson ImmunoResearch Laboratories, West Grove, Pa.) diluted 1:10,000. Detection was done by adding an O-dianisidine solution [10 mM KH2PO4, 10 mM Na2HPO4, 0.05% hydrogen peroxide, 0.01% O-dianisidine solubilized in absolute methanol].

ELISA for the hMPV N proteins. Ninety-six-microwell plates were coated with 100 μl of 1-μg/ml purified hMPV N-A or N-B proteins in 0.1 M sodium bicarbonate buffer (pH 9.6) and incubated overnight at room temperature. The plates were blocked for 30 min at 37°C with PBS containing 0.05% Tween 20. Serial twofold dilutions (starting at a 1:20 dilution) of serum samples in PBS–0.05% Tween 20 as well as three negative controls shown to be nonreactive by Western blotting were subsequently selected as negative controls. Those sera were tested by ELISA for both the N-A and N-B proteins. A seroconversion (≥4-fold increase in antibody titer) was observed for the same patients against the N-B protein. In contrast, no increase in hMPV titers was observed over time with the ELISA N-A test in three adults with acute exacerbations of COPD who had recent hRSV infections (two group A and one group B), which were confirmed by both RT-PCR for the fusion gene (3) and by seroconversion using the complement fixation test (Fig. 2).

RESULTS

Establishment of the cutoff value for the hMPV ELISA test. Twenty-four sera from children aged 0 to 4 years as well as 30 sera from adults randomly selected from our clinical virology laboratory were first tested by Western blotting using the hMPV N-A protein to identify hMPV-negative sera. Three specimens which did not react with the N-A protein in Western blotting were subsequently selected as negative controls. Those three control sera gave similar results when evaluated in duplicate by both the ELISA N-A (mean optical density [OD] of 0.2 ± 0.05) and the ELISA N-B (mean OD of 0.25 ± 0.04) tests. The cutoff value of each assay was defined by adding 3 standard deviations (SD) to the mean OD of the three negative samples. The end point serological titer of subsequent sera was determined by reporting the highest dilution with an OD greater than or equal to the assay's cutoff value.

Evaluation of sensitivity and specificity of the hMPV ELISA test. The ability of the hMPV ELISA test to detect seroconversion following acute infections by hMPV and hRSV was then evaluated using acute- and convalescent-phase sera collected 3 weeks apart from subjects with ARTI. Four adults aged 49, 83, 63, and 39 years (patients 1 to 4) from our hospital had a recent hMPV group A infection that was confirmed by RT-PCR for the N gene (8). Paired sera from these subjects were tested by ELISA for both the N-A and N-B proteins. A seroconversion (≥4-fold increase in antibody titer) was observed for the four patients by using both N proteins (Fig. 1). The increases in antibody titer against the N-A protein following the infection were 16-, 128-, 8-, and 4-fold for patients 1, 2, 3, and 4, respectively, whereas they were 8-, 16-, 4-, and 4-fold for the same patients against the N-B protein. In contrast, no increase in hMPV titers was observed over time with the ELISA N-A test in three adults with acute exacerbations of COPD who had recent hRSV infections (two group A and one group B), which were confirmed by both RT-PCR for the fusion gene (3) and by seroconversion using the complement fixation test (Fig. 2). Based on the previous evaluations, the hMPV ELISA N-A test was selected for further studies.

Sero logical titers in children and adults. Forty randomly selected and hMPV-seropositive sera from 20 children ≤4 years old and from 20 adults were tested by the ELISA N-A test to determine mean antibody titers in the two populations. As shown in Fig. 3, the reciprocal of the mean antibody titer for the seropositive children was 143 compared to 69 for the adults. More specifically, none of the adults had a titer of >160, compared to 4 of 20 (20%) children.

hMPV seroprevalence and seroconversion in adults with exacerbations of COPD. Paired sera (collected 3 weeks apart) from a prospective study of patients with acute exacerbations of COPD were tested by the ELISA N-A test. Fifty-eight (81.6%) of the 71 acute-phase sera were positive (at a dilution 1:20) for hMPV antibodies at the onset of disease and three (4.2%) patients had evidence of a seroconversion, i.e., a ≥4-fold rise in antibodies in previously seropositive individuals (1/640 to 1/5,120, 1/320 to 1/1,280, and 1/320 to 1/1,280). Interestingly, two of the three adults with a seroconversion had a positive RT-PCR test for hMPV on nasopharyngeal aspirates.
DISCUSSION

We have developed an objective, simple, and specific ELISA test using the recombinant nucleoprotein (N) to allow the detection of hMPV antibodies. Using this assay, we observed that mean antibody titers for hMPV were higher in children than in adults and that the seroprevalence for this virus was approximately 82% in adult patients ≥40 years old with COPD. Importantly, we demonstrated that our ELISA-N test was specific for hMPV, as antibodies against hRSV (another human respiratory virus which belongs to the same subfamily as hMPV but to a different genus) did not cross-react with the hMPV nucleoprotein. Furthermore, we showed that seroconversions following acute hMPV infections confirmed by RT-PCR could be detected by either the N-A or the N-B recombinant proteins.

Until now, serological assays for hMPV have used infected cells as the antigen and none have been developed based on a recombinant protein (2, 9, 10, 12, 24, 29). It has been demonstrated that recombinant-protein ELISAs were more sensitive than those using virus-infected cells or inactivated virus as the antigen for detecting aMPV antibodies (15, 16). Among the hMPV proteins, the nucleoprotein (N) along with the matrix (M) and the fusion (F) proteins are the most conserved between representatives of the two hMPV groups (1). In contrast, the G and SH proteins are the most variable, with only 37 and 59% amino acid identity between the two groups. A study by Gulati et al. revealed that the N protein was better than M for detecting antibodies against the three aMPV groups (16). The N antigen has also been successfully used to develop serological assays for bovine and human RSVs and the coronavirus associated with the severe acute respiratory syndrome (6, 21, 30). In pneumoviruses, the N gene is also among the most expressed genes, and its related protein has been shown to be immunogenic. Furthermore, antibodies against N appear early and predominate throughout the infection (6, 21, 28). Consistent with those properties, we hypothesized that the N protein was a good antigen to use in developing a serological test for hMPV.

The few serological surveys realized with immunofluorescence assays using infected cells have shown that hMPV infec-
tion is almost universal in childhood (10, 24, 29). Falsey et al. also detected a high seroprevalence for this virus in adults by ELISA using viral cell lysates (12). We compared mean antibody titers in those two populations and observed that hMPV titers were higher in young children (0 to 4 years), consistent with a primary infection early in life. We also found out that 81.6% of adult patients with COPD were seropositive for hMPV. Assuming that virtually all children are infected by 5 to 10 years of age (10, 24, 29), this result indicates that adults can lose their antibodies or that titers were below the cutoff value of our assay. In any case, adults can be reinfected by hMPV as indicated in previous studies (2, 12) and in the present work, where 3 (4.2%) of 71 adults with exacerbation of COPD demonstrated a seroconversion for this pathogen in the ELISA N-A test. This percentage is consistent with that in a study reporting that hMPV was responsible for 4.5% of ARTI within a population of healthy adults and high-risk subjects with underlying cardiopulmonary diseases (12). Patients with COPD are more susceptible to severe respiratory infections than the general population, and other studies would be required to confirm the rate of hMPV (re)infection in healthy adults. At least one case of hMPV reinfection has been reported by our group in a young immunocompromised child (19). Whether reinfections in adults are generally caused by a different or the same hMPV genotype remains to be determined.

Serological testing for hMPV only permits a retrospective diagnosis, and a seroconversion or a $\geq 4$-fold rise in antibody titers must be demonstrated to confirm recent infection. We have used antigens from both viral groups (N-A and N-B) to detect recent hMPV group A infections and, as expected, the increase in titers was higher with the ELISA N-A test. However, there was clearly an important cross-reactivity with the N-B protein, indicating that those two proteins are antigenically related (22) and that antibodies can recognize conserved epitopes from both N proteins. Based on those results, we conclude that any of the two N proteins can be used as the antigen for the ELISA serological test.

In addition to other diagnostic tests, such as PCR, a serological assay realized on two paired sera is a nice adjunct to perform comprehensive etiological studies. This is particularly true during reinfections of adult patients, for whom viral excretion is of limited duration. Indeed, a combination of PCR...
and serology has been shown to have greater sensitivity than each test alone for hRSV diagnosis in adults (13). Such an approach could also be of interest for hMPV, as shown by our small study in COPD patients, where we were able to confirm one additional hMPV infection by serology compared to PCR.

In summary, our ELISA-N test is a fast, sensitive, and specific serological assay which will be of benefit in better understanding the epidemiology of hMPV in various populations of patients.

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