Clinical Evaluation of NucliSENS Magnetic Extraction and NucliSENS Analyte-Specific Reagents for Real-Time Detection of Human Metapneumovirus in Pediatric Respiratory Specimens

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In this study, we evaluated the NucliSENS miniMAG (MM) and easyMAG (EM) nucleic acid extraction platforms (bioMérieux, Durham, NC) in combination with the NucliSENS EasyQ basic kit and analyte-specific reagents (ASRs) (bioMérieux) for the detection of human metapneumovirus (hMPV) in respiratory samples. Total nucleic acids from pediatric clinical samples (n = 653) and an hMPV-specific inhibition control (h-IC) were coextracted using the MM and/or the EM. Nucleic acid sequence-based amplification and real-time molecular beacon detection of hMPV were performed using a NucliSENS EasyQ analyzer (bioMérieux). Positive results were confirmed using an in-house-validated reverse transcriptase PCR ASR-based assay. The inclusion of the h-IC monitored the entire process, including the efficiency of nucleic acid extraction, amplification, and detection. The percentages of samples with inhibited amplification of the h-IC after initial NA extraction by EM and MM were 1.88% and 3.17%, respectively. After reprocessing of a new aliquot, the final h-IC inhibition rates were 0% (EM) and 1.06% (MM). The limit of detection of the assay was between 2 (EM extraction) and 10 (MM extraction) RNA copies/reaction, and specificity was 100% when testing viral respiratory isolates and clinical samples. hMPV was detected in 5.6% of pediatric samples tested and was also detected in three coinfections with respiratory syncytial virus (RSV). hMPV was the second most frequently detected respiratory virus in children of 0 to 2 years of age, after RSV. In summary, NucliSENS extraction and ASRs provided a sensitive and specific method for the detection of hMPV in respiratory samples.

Human metapneumovirus (hMPV) is an enveloped RNA virus of the Paramyxoviridae family, Pneumovirus subfamily (1, 7, 10–13, 21, 28–32). hMPV was first identified by van den Hoogen et al. in 2001 and is the first human pathogen in the Metapneumovirus genus (28, 29). Studies conducted over the last several years have demonstrated that worldwide hMPV infections account for between 5% and 10% of respiratory tract infections in children (1, 7, 8, 10, 11, 13, 21, 28, 30, 34). In addition, much like what is seen for respiratory syncytial virus (RSV), another member of the Paramyxoviridae family, patients with a compromised immune system and/or chronic obstructive pulmonary disease and the elderly are also at significant risk for hMPV disease (2, 7, 8, 10–13, 21, 32). Although hMPV can be isolated throughout the year, the major seasonality of hMPV overlaps with that of RSV. However, rates appear to be higher during the later winter to spring months, as RSV declines. The symptoms of hMPV respiratory disease overlap significantly with those of RSV, and often the two cannot be distinguished clinically (1, 7, 10, 11, 13, 28, 30, 34). Diseases associated with hMPV include both upper and lower respiratory tract disease, including bronchiolitis, pneumonia, croup, otitis media, and exacerbation of asthma. In health care settings (hospitals and chronic care settings, etc.), the failure to properly isolate patients with hMPV could lead to nosocomial transmission and the potential for increased morbidity and mortality (2, 4). Although specific antiviral therapy is currently not available for hMPV, the rapid identification of hMPV-infected patients is important for preventing nosocomial transmission, for understanding the clinical impact of hMPV infections in all age groups, and for understanding the clinical outcome for children with dual or multiple viral respiratory pathogens. Importantly, the rapid identification of a respiratory virus should aid in reducing the unnecessary use of antibiotics or at a minimum reducing the duration of use in persons without underlying clinical conditions that would necessitate the adjunctive use of antibiotics.

Currently, in most laboratories, the diagnosis of viral respiratory pathogens is limited to rapid antigen tests that identify influenza virus types A and B and RSV. In larger hospitals or university settings, where more-comprehensive clinical virology diagnostic services are available, traditional methods, such as direct immunofluorescence (DFA) and viral culture, do not routinely include the identification of hMPV. New hMPV DFA analyte-specific reagents (ASRs) (Diagnostic Hybrids, Athens, OH) are excellent tools for screening purposes but do not provide the same sensitivity as molecular methods (M. Lotlikar, R. Manji, L. Falk, F. Zhang, and C. C. Ginocchio, presented at the Clinical Virology Symposium, Clearwater Beach, FL, April 2007). hMPV can be grown in culture-based systems such as R-Mix (Diagnostic Hybrids, Athens, OH) (8; M. Lotlikar, R. Manji, L. Falk, F. Zhang, and C. C. Ginocchio, presented at the Clinical Virology Symposium, Clearwater Beach, FL, April 2007). hMPV can be grown in culture-based systems such as R-Mix (Diagnostic Hybrids, Athens, OH) (8; M. Lotlikar, R. Manji, L. Falk, F. Zhang, and C. C. Ginocchio, presented at the Clinical Virology Symposium, Clearwater Beach, FL, April 2007).
Beach, FL, April 2007) and LLC-MMK cells (9), but the average time to culture-based detection (7 to 14 days) is generally beyond a time frame (24 to 48 h) that would impact clinical management (discontinuation of inappropriate antibacterial therapy or initiation of appropriate antiviral therapy) or infection control procedures designed to prevent nosocomial transmission. Therefore, a rapid molecular diagnostic test is necessary to identify the majority of patients infected with hMPV.

Several user-developed assays and commercial ASRs using reverse transcriptase PCR (RT-PCR) have been used for the detection of hMPV in clinical samples (6–8, 18, 19, 28, 32). Recently, a study by Dare et al. described the use of NucliSENS hMPV ASRs (bioMérieux, Durham, NC) for the detection of hMPV in immunosuppressed lung transplant recipients and in children evaluated for pertussis (8).

This study details our laboratory’s validation of an hMPV assay based on NucliSENS magnetic silica nucleic acid (NA) extraction (bioMérieux, Durham, NC), and NA sequence-based amplification (NASBA) (5, 8; E. Bufflier, H. Savelli, F. Jacobs, C. Moore, and P. van de Weil, presented at the Fourth European Meeting on Molecular Diagnostics, October 2005) using NucliSENS EasyQ basic kit v2 reagents (bioMérieux) and a NucliSENS hMPV primer-probe mix (bioMérieux). The extraction technology is based on the patented Boom method (3) but substitutes magnetic silica for the standard activated silica particles (26). The small, compact NucliSENS miniMAG instrument provides for the rotation of tubes and sample/buffer mixing during the extraction process (17, 23, 25). Removal of wash buffers and elution steps are performed manually. Recently, an automated version of the same process has been developed using the NucliSENS easyMAG instrument (bioMérieux) (16, 20, 26, 27). The primer-probe mix contains analyte-specific primers that target the matrix gene of hMPV and two molecular beacon probes, one for the detection of wild-type hMPV and one for the detection of an hMPV-specific internal control (h-IC). Parameters evaluated in the study included (i) a comparison of the performance of NucliSENS miniMAG and with that of easyMAG for the extraction of hMPV RNA from a variety of respiratory samples and control material spiked into a respiratory sample matrix, (ii) the determination of positive/negative cutoff values for the detection of hMPV and the h-IC, (iii) a validation of the performance of the h-IC, and (iv) assessment of the analytical and clinical sensitivity and specificity of the assay. (This study was presented in part at the 107th General Meeting of the American Society for Microbiology, abstr. C-076, Toronto, Canada, May 2007 [19a].)

MATERIALS AND METHODS

Sample types and characteristics. (i) h-IC RNA and hMPV in vitro-transcribed RNA standard. Generation of an h-IC RNA (bioMérieux, Grenoble, France) and hMPV in vitro-transcribed RNA standard (bioMérieux, Grenoble, France) were previously described (E. Bufflier, H. Savelli, F. Jacobs, C. Moore, and P. van de Weil, presented at the Fourth European Meeting on Molecular Diagnostics, October 2005). The primer binding sites are the same for the h-IC, and for the wild-type hMPV RNA target, permitting coamplification in a single-tube format. Verification of the hMPV RNA standard oligonucleotide length and purity and the determination of the RNA concentration was performed in-house using an Agilent 2100 Bioanalyzer instrument type G2938B (Agilent Technologies, Inc., Santa Barbara, CA) and an Agilent RNA 6000 Nano kit (Agilent). The concentration of the hMPV RNA standard was 3.36E12 copies/µl.

(ii) Viral isolates. hMPV strains A1 (NL/1/00), A2 (NL/17/00), B1 (NL/1/99), and B2 (NL/1/94) were kindly provided by J. Simon (Vironovative, Erasmus Medical Center, Rotterdam, The Netherlands). Local hMPV clinical isolates and viral isolates for specificity studies were provided by the North Shore University Hospital Clinical Virology Laboratory, Manhasset, NY. Isolates tested included adenovirus, parainfluenza virus types 1, 2, and 3, influenza virus types A and B, herpes simplex viruses types 1 and 2, varicella-zoster virus, cytomegalovirus, and enterovirus.

(iii) Clinical study samples. Residual discard respiratory samples, collected between November 2004 and April 2005 and stored at −70°C, were from 653 children (number <2 years of age, 433; number between 2 and 5 years of age, 220) symptomatic for respiratory disease based on chart review. Samples were submitted to the North Shore University Hospital Clinical Virology Laboratory, Manhasset, NY, for routine viral testing by DFA (influenza virus A, influenza virus B, parainfluenza virus types 1, 2, and 3, adenovirus, RSV) and R-Mix rapid cell culture (Diagnostic Hybrids). Studies were performed under an Institutional Review Board-approved protocol. Specimens included nasopharyngeal (NP) aspirates (n = 104), NP swabs in viral transport medium (universal transport medium; Diagnostic Hybrids, Athens, OH) (n = 42), tracheal aspirates (n = 5), and sputum samples (n = 11).

(iv) Assay controls. Included in every run were a negative control (200 µl of respiratory sample matrix tested negative for hMPV) and a low-level hMPV-positive control. Positive controls consisted of a 200-µl aliquot from pooled hMPV-negative respiratory specimens added to a 2.0-ml NucliSENS lysis buffer tube (bioMérieux) and then spiked with either a clinical hMPV viral isolate or the hMPV RNA standard. Positive controls were titrated in NucliSENS lysis buffer and used at the lowest dilution or 1 dilution less than that which gave a 100% detection rate. Controls were processed as described below for clinical specimens.

ASR-based assay for the detection of hMPV. (i) Specimen processing and NA isolation. Clinical specimens and respiratory matrix specimens (used for specificity and sensitivity studies) were centrifuged at 2,000 rpm at 25°C for 10 min. Specimen supernatant was filtered using a 0.22-µm syringe-driven filter unit (Millipore, Bedford, MA). A 200-µl aliquot of supernatant was added to a microcentrifuge tube containing 24 µl of 10X DNase buffer (Promega, Madison, WI) and 200 µl of DNase I (Promega). Residual supernatant was stored at −70°C. Supernatants were incubated at 37°C for 40 min in a heat block without rotating or shaking. After DNase treatment, the entire volume was transferred to a 2.0-ml NucliSENS lysis buffer tube containing guanidine thiocyanate and Triton X-100. Lysis buffer tubes were vortexed and incubated at room temperature for 15 to 30 min, and then 20 µl of the h-IC was added to each tube. NA extraction was performed using the miniMAG and/or easyMAG instrument according to the manufacturer’s instructions. The miniMAG was used to extract a group of 387 samples, the easyMAG was used to extract a separate group of 181 samples, and 85 samples were extracted by both the miniMAG and the easyMAG. All NAS were eluted in 25 µl of RNase/DNase-free water, and 5 µl of the eluate was used in the amplification reactions. Therefore, theoretically, one-fifth of the hMPV isolation input concentration would be present in the amplification reaction. However, this is based upon the assumption that 100% extraction efficiency, although the actual amount is assumed to be less due to the loss of NASs during the extraction process. Residual eluates were stored at −70°C. (ii) NA amplification. The development of the NucliSENS primer and probe ASRs has been previously described (8, 24; E. Bufflier, H. Savelli, F. Jacobs, C. Moore, and P. van de Weil, presented at the Fourth European Meeting on Molecular Diagnostics, October 2005). Target amplification for 90 min at 41 ± 0.5°C and the continuous monitoring of emitted fluorescence were performed using a NucliSENS EasyQ analyzer (bioMérieux) and NucliSENS EasyQ Director 2.5 software (bioMérieux).

(iii) Molecular beacon detection of NASBA amplicons. During the 90-min amplification and detection process, the NucliSENS EasyQ analyzer reads the emitted fluorescence from both the hMPV-specific molecular beacon and the IC-specific molecular beacon every 20 s. The hMPV-specific molecular beacon (6-carboxyfluo- rescein) is read at a wavelength of 485 to 518 nm, and the IC-specific molecular beacon (ROX [6-carboxy-X-rhodamine]) is read at a wavelength of 576 to 604 nm. After the instrument normalizes against background fluorescence, two fluorescent signal curves (one for hMPV RNA and one for h-IC) are generated and interpreted by the NucliSENS EasyQ Director 2.5 software according to house-validated settings established during assay validation (sensitivity and specificity studies).

Assay validation studies. (i) Establishment of hMPV assay positive/negative cutoff values. (See the supplemental material.) To establish an algorithm for the interpretation of the hMPV assay results that would provide an accurate cutoff value for the identification of true positive and true negative clinical samples, data from several experiments were evaluated. Initially, 20 hMPV-negative res-
piratory specimens and a low-level positive control were tested and results evaluated using the NucliSENS EasyQ Director 2.5 generic default settings (detection threshold, $\geq 1.100$; IC lambda threshold, $\geq 1.100$; growth threshold, 86%; maximum time to primer depletion, 180 min). After background fluorescence was assessed, titered stocks of the hMPV isolates, in vitro-transcribed hMPV RNA, and viral isolates used for specificity studies (all spiked into NucliSENS lysis buffer tubes containing 200 $\mu$L of an hMPV-negative respiratory matrix background and h-IC) were used to determine the optimal settings by which a clinical sample containing hMPV and the h-IC would be deemed positive by the NucliSENS Director 2.5 software. Parameters were adjusted to ensure the results were optimal without generating false-positive and false-negative results within the scope of detection of the assay. The parameters were reevaluated at the completion of the clinical study and verification of hMPV-positive clinical samples.

(ii) Determination of LOD. Limit of detection (LOD) studies were performed using both miniMAG and easyMAG NA eluates generated from the extraction of replicate aliquots from single-dilution series made in NucliSENS lysis buffer and respiratory sample matrix negative for hMPV. Samples included (i) 60 replicates of the hMPV A1 strain (concentration $= 0.5 \times 10^{-1}$ 50% tissue culture infective dose [TCID$_{50}$] determined at Vironovative) with a dilution series ranging from 0.313 to 6.25 TCID$_{50}$/isolation (iso) input (approximately 0.125 to 1.25 TCID$_{50}$/amplification [amp] reaction), tested over four independent runs; (ii) 72 replicates of an hMPV B1 strain (concentration $= 0.5 \times 10^{-4}$ TCID$_{50}$ determined at Vironovative) dilution series ranging from 0.156 to 6.25 TCID$_{50}$/iso (approximately 0.0312 to 1.25 TCID$_{50}$/amp), tested over four independent runs; and (iii) 80 replicates of serial dilutions ranging over 10, 25, 50, 100, and 250 RNA copies/iso (cps/iso) (approximately 2, 5, 10, 20, and 50 RNA copies/amp) [cps/amp] tested in four independent runs.

(iii) Determination of assay specificity. Specificity of the hMPV assay was determined using (i) clinical isolates of hMPV ($n = 54$) harvested from R-Mix cell culture (Diagnostic Hybrids, Athens, OH); (ii) adult and pediatric respiratory samples ($n = 54$) positive for hMPV by DFA using in-house-validated ASRs (Diagnostic Hybrids); (iii) clinical isolates of other viruses commonly isolated from respiratory samples, as previously listed; and (iv) clinical study samples ($n = 653$) obtained from the virology laboratory at North Shore University Hospital, Manhasset, NY. Viruses harvested from cell culture (20 $\mu$L of culture supernatant) were added to 200 $\mu$L of pooled respiratory sample matrix tested negative for hMPV and processed as described above.

(iv) Evaluation of h-IC. To monitor all steps of the assay, including NA isolation, amplification, and detection, h-IC was added to each sample prior to NA extraction. An initial h-IC cutoff value (minimum value that indicates no potential amplification inhibition) was based on previous laboratory experience with similar types of assays (RSV and enterovirus) and the generic parameters used by the Director 2.5 software. Values falling below the cutoff value would be indicative of either amplification inhibitors in the sample or amplification/reagent failure. Samples with a positive wild-type hMPV result could have either a valid or an invalid h-IC result (due to competition from wild-type hMPV RNA amplification) with an h-IC value that was below the acceptable level, the routine procedure was to process, extract, and amplify a new aliquot of the sample. Initial h-IC cutoff values were reevaluated during the study based upon the h-IC values generated from testing the clinical samples. Further validation of the performance of the h-IC was also tested using 10 viral culture cell lysates containing hMPV (see the supplemental material).

To evaluate the potential for false-negative results due to competition that may occur when simultaneously amplifying wild-type hMPV RNA and h-IC, LOD studies were performed with and without the presence of the h-IC. Replicates ($n = 6$) of a dilution series of hMPV RNA containing 10, 25, 50, 100, and 250 RNA cps/iso (approximately 2, 5, 10, 20, and 50 hMPV RNA cps/amp) were added to NucliSENS lysis buffer containing 200 $\mu$L of respiratory sample matrix. h-IC was added to only one set of replicates. Both sets of replicates were extracted using the easyMAG and tested simultaneously with the hMPV assay as described above.

Clinical performance studies. (i) Detection of hMPV in clinical samples. Pediatric respiratory samples ($n = 653$) were processed and NAs extracted and tested for hMPV as described above. hMPV prevalence rates were stratified by patient age, and rates were compared to all those of other viruses identified from the samples by DFA and R-Mix culture.

(ii) Evaluation of clinical specificity and clinical sensitivity. Clinical specificity and sensitivity were assessed using (i) clinical samples identified by the NucliSENS ASR assay as hMPV positive ($n = 35$), (ii) a subset ($n = 100$) of randomly selected NucliSENS ASR hMPV-negative clinical samples, and (iii) additional randomly selected discord adult and pediatric respiratory samples ($n = 98$) DFA negative for hMPV. NucliSENS hMPV-positive and -negative results were evaluated with an in-house-validated RT-PCR assay using the Pro-hMPV ASRs (Prodesse, WI). Assays were performed using theSmartCycler real-time PCR instrument (Cepheid, Sunnyvale, CA).

(iii) Evaluation and comparison of the NucliSENS miniMAG and easyMAG extraction platforms. Factors assessed in this study that can be affected by the extraction method include the LOD, the reproducibility of clinical sample results, and the rates of inhibited clinical samples. The LOD results generated from miniMAG and easyMAG NA eluates derived from the same dilution series were used to compare the extraction efficiencies of the two methods. Reproducibility of results was assessed by a comparison of the 85 samples extracted by both methods and then tested for hMPV. The rate of inhibited samples, as indicated by a failed h-IC, was determined for each method individually and from the subset of 85 samples extracted by both methods.

Statistical analysis. Significant difference between the performances of the two extraction methods, as determined by the numbers of positive samples detected in the sensitivity studies, was determined by the McNemar’s test.

RESULTS

Establishment of hMPV assay positive cutoff value. Critical to the development and clinical use of diagnostic molecular assays is the ability to clearly differentiate positive and negative results. To establish an accurate cutoff value for the identification of a true positive sample, initially 20 hMPV-negative respiratory specimens and a low-level positive control were tested and results evaluated using generic guidelines for the NucliSENS EasyQ Director 2.5 software. The initial parameters were then modified appropriately for the interpretation of positive and negative results based upon the raw data obtained using negative controls, positive controls, hMPV-spiked samples (sensitivity studies), and samples spiked with other viruses (specificity studies). Parameters were adjusted to afford a high level of sensitivity of detection without a loss of specificity due to cross-reactivity or background fluorescent counts (no false-positive results). The final selection of the parameters (detection threshold, $\geq 1.100$; IC lambda threshold, $\geq 1.300$; growth threshold, $>50%$; maximum time to primer depletion, 60 min) was confirmed with the clinical sample results described in the clinical study section.

Assay analytical sensitivity. The LOD of the hMPV ASRs was determined using both miniMAG and easyMAG eluates extracted from replicate aliquots of single-dilution series derived from hMPV A1 and B1 strains and hMPV RNA standards. As shown in Table 1, easyMAG eluates yielded positive results for all replicates of hMPV A1 at a 0.25 TCID$_{50}$/amp and for hMPV B1 at a 0.125 TCID$_{50}$/amp, miniMAG eluates yielded positive results for all replicates of hMPV A1 at a 1.25 TCID$_{50}$/amp and for hMPV B1 at a 0.125 TCID$_{50}$/amp. hMPV RNA was detected 100% of the time at 10 and 50 cps/amp for easyMAG and miniMAG eluates, respectively (Table 2). Based on the LOD studies, the overall percentages of hMPV replicates detected using miniMAG and easyMAG eluates were 55.7% and 73.6%, respectively. The McNemar test for a paired-sample nominal scale using pairs of data with an alpha value of 0.05 determined that this difference was statistically significant ($P = 0.0015$). The higher rate of hMPV detection using the easyMAG eluates was significant for the detection of both hMPV RNA ($P = 0.0015$) and hMPV A1 and B1 viral isolates ($P = 0.0233$).

Establishment of the IC cutoff value. (See the supplemental material.) To monitor the fidelity of the hMPV assay, an hMPV-specific IC-RNA was added to each sample prior to NA extraction. Amplification of the h-IC is one of several test
affects the sensitivity of the assay only at very low hMPV RNA with the wild-type hMPV RNA is minimal. This competition the inhibitory effect of coamplifying the h-IC simultaneously two of six and three of six replicates containing h-IC at 5 and 50, 20, and 10 RNA cps/amp. hMPV RNA was detected in replicates without h-IC were positive at 50, 20, 10, 5, and 2 RNA cps/amp (Table 3). All replicates with h-IC added were positive at 50, 20, 10 5, and 2 RNA cps/amp. hMPV RNA was detected in two of six and three of six replicates containing h-IC at 5 and 2 RNA cps/amp, respectively. These data indicate that the inhibitory effect of coamplifying the h-IC simultaneously with the wild-type hMPV RNA is minimal. This competition affects the sensitivity of the assay only at very low hMPV RNA copy numbers per reaction, and, based upon clinical study parameters that are used to indicate the presence of amplification inhibitors in the sample and/or poor amplification kinetics. Since the h-IC is amplified using the same primers as the wild-type target hMPV, it was important to first ensure that h-IC amplification will not competitively inhibit the amplification of any wild-type hMPV, which would cause false-negative results. Prior to testing patient samples, we evaluated the potential for competition between the h-IC and wild-type hMPV RNA amplification. Two test panels containing six replicates of the same five serial dilutions of hMPV RNA were tested simultaneously, with and without the addition of h-IC. All replicates without h-IC were positive at 50, 20, 10, 5, and 2 RNA cps/amp (Table 3). All replicates with h-IC added were positive at 50, 20, and 10 RNA cps/amp. hMPV RNA was detected in two of six and three of six replicates containing h-IC at 5 and 2 hMPV RNA cps/amp, respectively. This data indicate that the inhibitory effect of coamplifying the h-IC simultaneously with the wild-type hMPV RNA is minimal. This competition affects the sensitivity of the assay only at very low hMPV RNA copy numbers per reaction, and, based upon clinical study results described in the next section, this competition appears to be minimal when testing patient samples.

Initially, the IC lambda threshold for a valid clinical sample result was set at ≥1.100, and samples with IC lambda threshold values of <1.100 would be considered invalid. For samples with an invalid h-IC value, a new aliquot was processed, extracted, and amplified. In total, there were 755 assays performed on clinical samples, including both initial and repeat testing. Thirteen (13) clinical sample results were reported as invalid, with IC lambda thresholds of <1.100. There were four samples (0.58%) with valid negative results with an IC lambda threshold in the range of ≥1.10 to <1.30. Since so few samples gave results within this range, the IC lambda threshold was raised to ≥1.30 to ensure that clinical samples with partial inhibition are not missed. An additional seven samples had IC values of ≥1.100 (range, 1.133 to 5.071) but were reported as invalid due to the failure of another assay parameter (for example, poor amplification kinetics) or multiple established parameters. After repeat testing, only three samples remained invalid due to an h-IC value of <1.300 and two samples remained so due to invalid assay kinetics, indicating inhibitory substances and/or poor amplification that could lead to false-negative results. One sample, invalid on initial testing, was found to be hMPV positive after reprocessing and amplification. This positive finding highlights the importance of an internal control to reduce the rate of false negatives due to amplification and/or single-sample test failure not identified through the use of external run controls.

Once the interpretive criteria for the h-IC were validated, the abilities of the extraction methods to remove potential inhibitors from clinical samples were evaluated and the methods compared based upon the rate of invalid results due to poor amplification kinetics or to h-ICs with values below the established threshold (<1.3) (Table 4). Failed h-IC amplification was detected in 1.88% (5/266) of the samples (all NP washes) after easyMAG extraction. Inhibition was resolved for all easyMAG samples after repeat easyMAG extraction from a new DNase-treated sample aliquot, for a final inhibition rate of 0%. Initial inhibition rates for the samples (14 NP wash samples, 1 NP aspirate) extracted with miniMAG were 3.17% (15/473). Repeat miniMAG extraction from a new DNase-treated aliquot resolved the inhibition for 10/15 of the samples for a final inhibition rate of (1.06%). Three of five samples that remained inhibited with miniMAG extraction were efficiently amplified after easyMAG extraction. The sample types inhibited after initial extraction by either system were similar and

### Table 1. Summary of hMPV LOD studies using hMPV strains A1 and B1

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<th>Strain</th>
<th>iso input&lt;sup&gt;a&lt;/sup&gt; (TCID&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>amp input&lt;sup&gt;b&lt;/sup&gt; (TCID&lt;sub&gt;50&lt;/sub&gt;)</th>
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<sup>a</sup> hMPV concentration (TCID<sub>50</sub>) added to lysis buffer prior to extraction.<br /></sup>b<sup>b</sup> Theoretical hMPV concentration (TCID<sub>50</sub>) in amplification reaction.<br /></sup>c<sup>c</sup> Pos, positive.

### Table 2. Summary of miniMAG and easyMAG LOD studies for hMPV in vitro-transcribed RNAs

<table>
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<th>hMPV iso input&lt;sup&gt;a&lt;/sup&gt;</th>
<th>amp input&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
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<td>10</td>
<td>2/10</td>
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</tr>
<tr>
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<td>20</td>
<td>8/20</td>
<td>6/8</td>
<td>100</td>
</tr>
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<td>50</td>
<td>8/50</td>
<td>8/8</td>
<td>100</td>
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</table>

<sup>a</sup> Data are from testing replicates derived from a single dilution series extracted with both the miniMAG and easyMAG.<br /></sup>b<sup>b</sup> Data are combined results from both miniMAG and easyMAG extractions tested in multiple runs performed over multiple days.<br /></sup>c<sup>c</sup> RNA copies added to lysis buffer prior to extraction.<br /></sup>d<sup>d</sup> Theoretical RNA copies present in amplification reaction.<br /></sup>e<sup>e</sup> det, detected.<br /></sup>f<sup>f</sup> Detection rate (%).
and h-ICs values were wild-type fluorescent signals below the positive cutoff value. Samples (listed in Materials and Methods) was tested with the assay, a variety of viruses isolated from clinical respiratory samples (23). Using spiked samples, this study demonstrated similar clinical sensitivities and provided reproducible results. Overall, both extraction methods demonstrated similar clinical sensitivities and provided reproducible results.

**Assay specificity.** To determine the specificity of the hMPV assay, a variety of viruses isolated from clinical respiratory samples (listed in Materials and Methods) was tested with the hMPV assay. All non-hMPV viral isolates generated hMPV wild-type fluorescent signals below the positive cutoff value (1.10) and h-ICs values were ≥1.30, indicating no amplification inhibition and a valid result. All four strains of hMPV (A1, A2, B1, B2) were detected by the assay. The specificity of the assay was also evaluated by testing a set of adult and pediatric samples (n = 54) positive for hMPV by DFA and by R-Mix culture. hMPV was detected by the NucliSENS ASRs in all original samples and from their R-Mix culture supernatants. All positive samples were confirmed to be true positives by testing with the Prodesse Pro-hMPV ASRs.

**Detection of hMPV in clinical study samples.** In total, 653 pediatric respiratory samples were tested for hMPV as described above. For all initial and repeat testing, the mean wild-type target detection maximum ratio for negative samples (n = 688) was 1.023 (range, 1.000 to 1.073). Three hMPV-negative samples with signal ratios (1.052, 1.059, 1.073) close to the positive cutoff value (>1.05 but <1.10) were tested with the Prodesse Pro-hMPV ASRs and yielded negative results with this assay also. For all initial and repeat testing (n = 49) for hMPV-positive samples (n = 35), the mean wild-type target detection maximum ratio was 3.954 (range, 1.144 to 6.157). All 35 NucliSENS hMPV-negative samples were confirmed as true positive using the Prodesse Pro-hMPV ASRs. Finally, chart review of all children with positive results indicated the presence of respiratory disease (data not shown), indicating a clinical specificity of 100%.

To further evaluate the sensitivity and specificity of the assay, a randomly selected set (n = 100) of the NucliSENS hMPV-negative samples was tested with the Pro-hMPV ASRs. Ninety-nine samples were confirmed negative with the Pro-hMPV ASRs, and 1 sample tested positive. Repeat testing of the sample with the NucliSENS assay was positive for hMPV. Finally, 98 randomly selected adult and pediatric DFA hMPV-negative samples were tested and 7 were found to be hMPV positive by the NucliSENS hMPV ASRs. All positive and negative results were confirmed with the Prodesse Pro-hMPV ASRs.

The overall detection rate of hMPV in the pediatric study group was 5.36%. hMPV prevalence in children aged <2 years was 5.77%, and that for children aged from 2 to <6 years was 4.55%. During the winter months, in comparison to other respiratory viruses identified by DFA and/or R-Mix culture, hMPV was the third most commonly detected virus in children aged <6 years, after RSV (22.51%) and influenza virus A (5.97%). hMPV was detected in three samples (0.46%) also containing RSV. The prevalence of hMPV in our study was similar to rates determined by other investigators (1, 7, 10–13, 21, 28, 30).

**DISCUSSION**

Multiple factors can affect the overall performance of a molecular diagnostic assay, including specimen collection, storage, transport, NA extraction, and assay design. Optimization of each step is important and limited recovery or poor quality of NAs after extraction can significantly affect the performance of even the best-designed molecular assays. Particularly when dealing with difficult sample types that may contain amplification inhibitors (i.e., stool, urine, and respiratory samples), it is imperative to use an NA extraction method that provides highly concentrated and purified NAs (3, 14–17, 20, 22, 23, 25–27, 33). Coupled with robust amplification and detection methods, efficient NA extraction facilitates the detection of low numbers of target NAs, allows for multianalyte identification, and removes inhibitory substances that could lead to inconclusive or false-negative results.

Overall, the miniMAG and easyMAG extraction systems provided highly purified NAs and efficiently removed inhibitory substances from a variety of respiratory samples. A slightly better performance (recovery of low-copy-number RNA as determined by sensitivity studies and the identification of fewer inhibited samples) seen with easyMAG may relate to the greater extent of the automation and the low extent of operator error compared to what was seen for the semiautomated miniMAG instrument. Other benefits of easyMAG were better throughput per run (24 samples in 45 min versus 12 samples in 50 min for miniMAG) and less hands-on time (15 min versus 45 min for miniMAG). In addition, better assay sensitivity was achieved with easyMAG extraction.

Several recent papers have also demonstrated the robustness of both miniMAG and easyMAG extraction methods (16, 20, 22, 23, 25, 26, 27). Tang et al. compared three commercial systems, BioRobot EZ1 (Qiagen, Inc., Valencia, CA), MagnaPure Compact (Roche Diagnostic Corp., Indianapolis, IN), and miniMAG, for NA extraction from urine samples and found that miniMAG produced the highest quality of NAs and the best precision of the three systems (27). A study by Petrich et al. compared extraction methods for the optimal detection of severe acute respiratory syndrome coronavirus from stool specimens (23). Using spiked samples, this study demonstrated that miniMAG had a sensitivity of 100% for the detection of severe acute respiratory syndrome coronavirus, gave the highest overall numbers of copies per sample tested, and demon-

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Extraction</th>
<th>No. inhibited/no. tested</th>
<th>% Inhibited</th>
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</thead>
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<tr>
<td>miniMAG</td>
<td>Initial</td>
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<td>5/473</td>
<td>1.06</td>
</tr>
<tr>
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<td>Initial</td>
<td>5/266</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>Repeat</td>
<td>0/266</td>
<td>0</td>
</tr>
</tbody>
</table>

a Results after first extraction and testing.
b Results after repeat sample processing, extraction, and testing.
c Number of inhibited samples/total no. of samples tested.
strated a lack of inhibitors in the purified extraction eluates. Loens et al. compared easyMAG to miniMAG and Qiagen extraction by testing throat swabs for the detection of \textit{Mycoplasma pneumoniae} and \textit{Chlamydia pneumoniae} and EDTA anticoagulated blood specimens for the detection of cytomegalovirus (16). They found easyMAG extraction resulted in a higher recovery of both RNA and DNA and less inhibition than Qiagen extraction. No samples demonstrated amplification inhibition after easyMAG extraction. Results were comparable to those obtained with miniMAG, but easyMAG was more user-friendly. Additional studies from our laboratory have demonstrated the robustness of miniMAG (F. Zhang, D. Barth, B. Jacobs, J. Watz, M. Vossinas, R. Manji, and C. C. Ginocchio, presented at the Clinical Virology Symposium, Clearwater Beach, FL, April 2005) and easyMAG (20; M. Vossinas, F. Zhang, R. Manji, and C. C. Ginocchio, presented at the Clinical Virology Symposium, Clearwater Beach, FL, May 2006; F. Zhang and C. C. Ginocchio, presented at the Clinical Virology Symposium, Clearwater Beach, FL, April 2007) for the extraction of different RNA viruses (influenza virus types A and B, parainfluenza virus types 1, 2, and 3, hepatitis C virus, human immunodeficiency virus), DNA viruses (herpes simplex virus 1 and 2, varicella-zoster virus, human herpes viruses 6, 7, and 8, Epstein-Barr virus, cytomegalovirus, bacteria \textit{Bordetella pertussis} and \textit{B. parapertussis}), and parasites \textit{(Plasmodium spp.)} from a variety of sample types (whole blood, plasma, cerebrospinal fluid, NP swabs, NP aspirates, bronchial alveolar lavage fluids, sputa) by use of a variety of amplification methods (NASBA, PCR, RT-PCR, target-specific primer extension) and detection technologies (molecular beacons, TaqMan probes, fluorescent resonance energy transfer probes, bead arrays).

External negative and positive controls are good overall indicators of assay run performance but do not address the performance of individual samples within a run. The incorporation of an h-IC that utilized the same primer set as the wild-type hMPV target was important to ensure that wild-type target amplification in each individual sample would be successful if the target was present at levels above the LOD. However, any IC that utilizes the same primer set as the wild-type target can also result in partial or substantial competition of wild-type amplification. Studies that evaluated the LOD with and without h-IC demonstrated that the h-IC minimally affected the sensitivity of detection of wild-type hMPV. The h-IC also served as an excellent monitor of potential amplification inhibitors that might lead to false-negative results. This was demonstrated by a sample that had a negative wild-type result and an h-IC value below the established h-IC threshold but upon reextraction and reamplification was found to be a true positive sample. Without the h-IC result, this sample would have been reported out as falsely negative.

The NucliSENS hMPV ASR assay was very sensitive and was able to detect as few as 2 RNA cps/amp in the presence of a potentially competitive h-IC. Differences in the LODs of the hMPV A1 and B1 strains most probably related to sequence variation present in the assay target region of the matrix gene (29, 31). The NucliSENS ASR detected more positive samples than did DFA and was comparable to the Prodesse Pro-hMPV ASR, indicating that h-IC competition did not reduce the clinical sensitivity of the assay. The assay was also highly specific, showing no cross-reactivity to other viral agents that may be present in respiratory samples. Specificity was also demonstrated by the confirmation of all hMPV-positive clinical samples by testing with the Prodesse Pro-hMPV ASRs. In addition, the hMPV ASRs also detected hMPV in clinical samples that were hMPV DFA and/or R-Mix culture positive. The assay was able to detect the four lineages of hMPV tested. Our results were in accordance with studies by Bufflier et al. which demonstrated that the NucliSENS hMPV ASRs were able to detect the A1, A2, B1, and B2 subtypes of hMPV and that no cross-reactivity to the common respiratory viruses was found (E. Bufflier, H. Savelli, F. Jacobs, C. Moore, and P. van de Weil, presented at the Fourth European Meeting on Molecular Diagnostics, October 2005). These data and the reactivity with the local clinical isolates suggest that the hMPV ASRs should be able to detect most hMPV strains circulating in the region. However, as new lineages are detected, strains must be tested to ensure a broad depth of cross-reactivity (29, 31).

The prevalence of hMPV in the pediatric samples tested in the clinical sample study was within the range reported by other researchers (1, 7, 10–13, 21, 28, 30). Subsequent studies, using all respiratory samples (adults and pediatric) tested during the respiratory infection season of November 2006 through April 2007, determined that hMPV is present in all age groups, and the majority of the hMPV-positive samples (70.8%) were detected in the months of February through April. This is in contrast to RSV, which was predominantly detected (82.5%) in the months of November through January. The routine detection of hMPV in all age groups indicates that testing for hMPV should not be limited to pediatric, geriatric, or immunocompromised patients. Several prevalence studies have confirmed the role of hMPV in adults with respiratory illnesses (1, 2, 8, 10–13).

A recent study by Dare et al. evaluated the NucliSENS ASRs for the detection of hMPV in immunosuppressed lung transplant recipients and children evaluated for pertussis (8). NucliSENS results were compared to an in-house-developed RT-PCR assay. For the lung transplant group, the hMPV detection rate was 1.7% using NucliSENS ASRs and 1.2% using the RT-PCR assay. For the pediatric samples tested, the hMPV detection rate was 6.4% using the NucliSENS ASRs and 9.4% using the RT-PCR assay.

In summary, the NucliSENS hMPV ASR-based assay is very sensitive and has excellent specificity, and there is a clear delineation of positive samples. The extraction method resulted in highly purified concentrated total NAs with minimal inhibitory substances. The assay is easy to perform, requires limited hands-on time (45 min), and can be completed in approximately 3 to 3.5 h, including sample preparation and NA extraction.

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REFERENCES


