Prospective Evaluation of Rapid Antigen Tests for Diagnosis of Respiratory Syncytial
Virus and Human Metapneumovirus Infections

Jaber Aslanzadeh1, Xiaotian Zheng2, Haijing Li3, Janice Tetreault1, Irene Ratkiewicz1, Shufang Meng3, Pamela Hamilton1, and Yi-Wei Tang3, 4

1Hartford Hospital and Clinical Laboratory Partners, Hartford, CT 06102; 2Children’s Memorial Hospital, and Northwestern University, the Feinberg School of Medicine, Chicago, IL 60614; and Departments of Medicine3 and Pathology4, Vanderbilt University School of Medicine, Nashville, TN 37232

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* Corresponding author. Mailing address:

Jaber Aslanzadeh, PhD, D (ABMM)
Director, Division of Clinical Microbiology
Hartford Hospital and Clinical Lab. Partners
Hartford, CT 06102
Phone: 860-545-4128; 860-696-8033
Fax 860-545-2726
Email: Jaslanz@harthosp.org
Abstract

Respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) are two important viral pathogens causing respiratory tract infections in the pediatric population. Rapid detection of these agents allows for the prompt isolation and treatment of infected patients. In this prospective study, we evaluated the performances of four rapid antigen detection assays including a rapid chromatographic immunoassay (CIA) for RSV (Directigen™ EZ RSV, Becton Dickinson, Sparks, MD); a DFA test for RSV (Bartels, Trinity Biotech Carlsbad, CA) and two DFA tests for hMPV manufactured by DHI (Diagnostic Hybrids Inc. Athens, OH) and Imagen (Oxoid Ltd., Basingstoke, Hampshire, U.K.). Clinical specimens tested were 515 nasopharyngeal aspirates submitted to the Clinical Microbiology Laboratory at Hartford Hospital from November 1, 2006 to April 21, 2007. Compared to real-time reverse-transcription PCR (RT-PCR), the CIA had a sensitivity of 79.8% and a specificity of 89.5%. RSV DFA using Bartels reagents showed a sensitivity of 94.1% and a specificity of 96.8%. For hMPV, the sensitivity and specificity was 62.5% and 99.8% for DHI DFA, and 63.2% and 100% for Imagen DFA, respectively. The hands-on and test turnaround times for CIA were 10 and 30 to 60 minutes and the hands-on and test turnaround time for RSV and hMPV DFA were 30 and 105 minutes respectively. We conclude that while RSV CIA is user friendly, it lacks sensitivity and specificity especially during off peak months. In contrast, RSV DFA is more sensitive and specific but is subjective and it demands technical time and expertise. Similarly, both hMPV DFA are highly specific in comparison to RT-PCR, but sensitivity awaits further improvement.
Introduction

Respiratory syncytial virus (RSV) is the single most important virus causing respiratory tract infection in children. It is estimated each year in the United States, 100,000 hospitalization and 4,500 deaths are attributed to RSV infection (20). Similar to RSV, human metapneumovirus (hMPV), identified in Netherlands in 2001, is thought to cause upper and lower respiratory tract infection in children (23). Both RSV and hMPV are members of the *Paramyxoviridae* family (20). They are enveloped single-stranded negative-sense RNA viruses. Epidemiological studies indicate that, like RSV, hMPV is a significant human respiratory pathogen with worldwide distribution (6, 16, 23, 24). Indeed, hMPV appears to affect many of the same subpopulations and cause similar clinical manifestations, including upper respiratory tract infections, bronchiolitis, and pneumonia, although of lower severity compared to RSV (24). Both RSV and hMPV have been shown to infect the majority of children by the age of 5 years. Moreover, reinfections have been observed in all age groups (4).

Laboratory diagnosis of RSV and hMPV can be made by virus isolation, detection of viral antigens, amplification of viral RNA by molecular techniques, demonstration of a rise in serum antibodies, or a combination of these approaches (7, 9, 13, 15, 21, 26). The use of rapid tests in diagnosis of RSV and hMPV infections allows implementation of appropriate infection control measures, thus reducing nosocomial spread, and is useful for consideration of timely treatment with antiviral agents (8, 12). The clinical and financial benefits of rapid detection of RSV in respiratory specimens have been demonstrated in several studies, indicating a direct correlation between rapid turnaround time and decreased mortality, length of stay, overall costs, and better antibiotic stewardship (1, 8, 12, 25). On the other hand, few rapid antigen assays on hMPV
detection by using hMPV-specific monoclonal antibodies have been reported (3, 9, 18). While enzyme immunoassay, chromatographic immunoassay (CIA) and direct fluorescent assay (DFA) have been adapted in RSV rapid antigen testing (13, 26), DFA remains the only format being used in hMPV rapid antigen testing (5, 15).

The goal of this study was to prospectively evaluate the performances of four commercially available rapid diagnostic assays (one CIA and three DFA) for detecting these two viruses in respiratory samples during a respiratory virus season.
Materials and Methods

Clinical Specimens. From November 1, 2006 to April 21, 2007, nasopharyngeal samples (NPS) submitted to the Clinical Microbiology Laboratory at Hartford Hospital for RSV testing was included in this study. Nasopharyngeal aspirate or wash received in a cup or French feeding tube was suspended in 2 mL of sterile saline and was mixed with a sterile disposable pipette. A 0.5 mL aliquot from each sample suspension was placed in a Sarstedt screw cap microcentrifuge tube and stored at -70°C until testing for RSV and hMPV by reverse-transcription RT-PCR. The remainder of the specimen was used immediately to perform a CIA test for RSV and DFA tests for RSV and hMPV.

RSV CIA. The Directigen™ EZ RSV (BD, Sparks MD) test was performed following the manufacturer's recommendation (13, 26). Briefly, 250 µL of each sample was extracted and added to individual CIA device. RSV antigen, if present, was allowed to bind to the antibody colloidal gold conjugate in the test strip to form an antigen-antibody complex. The complex was allowed to migrate across the test strip to the reaction area, where it was captured by the line of a second RSV antibody on the membrane. Excess conjugate in the strip also migrates along the strip and binds to a second line consisting of inactivated RSV antigen, which serves as an internal control.

RSV/hMPV DFA. Samples were first centrifuged at 1,500 rpm (400 ×g) for 10 minutes at 4°C to remove excess mucus. The supernatant was aspirated and the pellet was washed in 4-6 mL of PBS twice to break up and to remove excess mucus. The final pellet was resuspended in PBS to attain a turbidity of about 1 McFarland standard. Two smears from the cell suspension were
made on a two-well fluorescence slide. Smears were allowed to air dry thoroughly at room

temperature and were fixed in cold acetone for 10 min. Twenty μL of RSV specific FITC-

antibody (Trinity biotech, Carlsbad, CA) (17) was placed in one well and 20 μL of hMPV

specific FTIC-antibody (Diagnostic Hybrid Inc. Athens OH) was added to the second well.

Beginning on February 1st, an additional slides were prepared on all specimens as described

above and stained with 20 μL of hMPV specific FTIC-antibody Imagen (Oxoid, UK). Slides

were incubated for 30 minutes at 35°C in a moist chamber. After the incubation, excess antibody

was washed away using PBS and smears were allowed to air dry at room temperature. One drop

of mounting fluid was added to the center of each well and a cover slip was placed over the

mounting fluid. The entire well area of the slide was scanned using a fluorescent microscope (9).

Both RSV and hMPV were detected by their characteristic granular bright apple-green

fluorescence within the cell, which contrasted with the red background staining of uninfected

cells.

RNA extraction. Total nucleic acids were extracted by using a NucliSens easyMAG system

(bioMerieux Inc., Durham, NC). Briefly, 0.9 mL of Lysis Buffer was added to 0.2 mL of thawed

NPS. After a thorough vortex, 200 μL of the mixture was placed in the instrument using the

default extraction protocol (22). Total nucleic acids were eluted in 55 μL of Elution Buffer

(bioMerieux Inc.) and 5μL of the extracts was used for nucleic acid amplification.

Real-time TaqMan RT-PCR assays. Two real-time RT-PCR assays that detect RSV and

hMPV were performed using an ABI PRISM 7700 sequence detection system (Applied

Biosystems, Foster City, CA) as previously described (11). In brief, 25 μL reaction mixture

containing 5 μl extracted RNA, 0.5 μM each primer, and 0.2 μM TaqMan probe was mixed with

25 μL TaqMan One-Step RT-PCR 2× Master Mix (Applied Biosystems). Reaction conditions
were designed as follows: RT at 48°C for 30 minutes, initial denaturation at 95°C for 10 minutes, and 40 cycles of denaturation (95°C for 15 seconds) and annealing/extension (60°C for 1 minute). Probes were dual labeled with the reporter dye, FAM (6-carboxyfluorescein) at the 5’ end and TAMRA (6-carboxytetramethylrhodamine) quencher at the 3’ end (11).

**RT-PCR-EIA.** A microtiter RT-PCR-EIA assay was used to detect RSV as previously described (21). The PCR mixture (50 µL) contained the following: 1× EN buffer, 18% glycerol, 300 µM dATP, dCTP, and dGTP, 285µM dUTP, 15 µM digoxigenin-11-dUTP (Roche Diagnostics, Indianapolis, IN), 0.5 µM each primer, 0.01 units/µL uracil N-glycosylase (UNG) (Epicentre Technologies, Madison, WI), 0.15 units/µL *Tth* polymerase (Applied Biosystems, Foster City, CA), and 10 µL of specimen extract. The reaction mixture was placed in an ABI 9700 thermal cycler programmed for a one-step RT-PCR procedure. The procedure included (i) an initial UNG activation, RT, and UNG inactivation/denaturation of 5 min at 50°C, 30 min at 65°C, and 3 min at 94°C; (ii) 5 cycles of 15 sec at 94°C, 30 sec at 60°C; (iii) 45 cycles of 15 sec at 90°C, 30 sec at 60°C; and (iv) a 10 min extension at 72°C. Output signal was measured at an optical density of 450 (OD$_{450}$). A positive result was defined as an OD$_{450}$ value greater than or equal to 0.1.

**RT-PCR for testing hMPV with commercial reagents.** The assay was performed with the Pro hMPV™ Real Time Assay kit (Prodesse, Inc., Waukesha, WI). Reagents not included in the kit were Platinum® *Taq* DNA Polymerase (Invitrogen Cor., Carlsbad, CA) and MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA). The manufacturer recommended procedure was followed. The assay was performed on a SmartCycler instrument (Cepheid, Sunnyvale, CA).
Evaluation references. Results inconsistent between rapid DFA and TaqMan assays were repeated by RT-PCR-EIA for RSV and by Prodesse assay for hMPV. Results with majority matches ($\geq 2/3$) were considered as references.
Results

A total of 515 pediatric specimens had adequate volume or cells to perform all the tests. A specimen was considered adequate if there were at least one or more DFA positive cell or 20 DFA negative cells/slide. Specimens from ED, outpatient clinic, inpatient non-ICU and inpatient ICU floors accounted for 71%, 6%, 14% and 9% of all the submitted samples respectively. A total of 283 (55%) of the samples were collected from male patients and 232 (45%) from female patients. The median age for all children was 4 months. Overall, 67% of the specimens were from children 6 month or younger and 33% from children older than 6 month of age.

Seasonal distribution of RSV and hMPV detection is presented in Figure 1. As expected, November, December, January and February had the highest numbers of RSV positive samples; of those months, December had the most positives. While remaining in relative high detection rate throughout this period, RSV peaked in December. In contrast, few positive hMPVs were detected except during its peak in January.

Of 515 specimens 272 (53%) were positive for RSV with at least one assay. Overall 219 samples tested positive by CIA, 233 by DFA and 235 by TaqMan PCR. A second PCR assay, RT-PCR-EIA was used to retest specimens with discordant results between RSV DFA and TaqMan RT-PCR. There were 40 specimens that tested positive only by RSV CIA (29), by RSV DFA (4) and by both RT-PCR and RT-PCR-EIA (7). Three (3) specimens that had tested positive by RSV CIA and DFA were found to be negative by RT-PCR but tested positive by RT-PCR-EIA. Similarly, three (3) specimens that had tested positive by CIA and DFA were found to be negative by both PCR tests. These specimens were considered false CIA and DFA positive. However, we cannot rule out the possibility that both PCR assays failed to detect these specimens due to mutations in
When the majority results were used as the evaluation standard, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 79.8%, 89.5%, 86.8% and 83.8% for RSV CIA, and 94.1%, 96.8%, 96.1%, and 95.0% for RSV DFA, respectively (Table 1). RT-PCR TaqMan detected hMPV on 32 samples, of which 20 were also positive by DHI DFA. Tests on the 12 DFA-negative, TaqMan RT-PCR-positive samples were repeated using the Prodesse real-time PCR and they were all tested positive for hMPV. Using RT-PCR results as the gold standard, sensitivity, specificity, PPV, and NPV for the DHI hMPV DFA assay were 62.5%, 99.8%, 95.2% and 97.6%, respectively. The Imagen hMPV DFA assay entered the trial and detected hMPV in 12 of 118 specimens, giving sensitivity, specificity, PPV, and NPV of 63.2%, 100.0%, 100.0% and 93.4%, respectively (Table 1). One PCR negative sample tested positive for hMPV by the DHI DFA and tested negative by the Imagen DFA but was shown to be positive by CIA, DFA and both PCR assays for RSV.

A substantial number of false positive and false negative results were given by the RSV CIA assay when the combination results from DFA and two RT-PCR assays were used as the evaluation standard (Table 1). The total false rates, ranged from 12.5-24.4%, distributed relatively evenly through the season. False negatives by the RSV CIA happened mainly in the early stage of the season. In contrast, false positives by the RSV CIA happened mainly in the late stage of season. (Table 2)
Discussion

RSV and hMPV are the two most common causes of bronchiolitis and pneumonia among infants and children under 1 year of age. Babies, especially those born prematurely, people with immune system problems, people with heart or lung problems, and older adults have an increased risk of developing complications from RSV infection. Clinical symptoms and laboratory findings associated with hMPV infection exhibit a spectrum virtually indistinguishable from those associated with respiratory syncytial virus disease. With one possible exception: RSV peaks from December to February while hMPV is increasingly detected from January to April (14, 24).

However, it is generally accepted that during the respiratory season laboratories must take into account the existence of both viruses.

We received 515 clinical samples with adequate volume to perform all of the tests. While, the overall sensitivity and specificity of the CIA for RSV was 79.8% and 89.5% respectively, CIA was more specific during the peak months of November, December and January and far less specific during off-peak months of February, March and April. Our data indicated that RSV CIA false negatives happened mainly in the early stage of the season and false positives in the late stage of the season. Because RSV is seen sporadically throughout the year we concur with other investigators that CIA alone should not be used to detect RSV during off peak months (20). However, considering the ease of performance and, hands on and TAT of 10 and 30 to 60 minutes respectively, CIA is very useful for rapid detection of most positive samples during the peak respiratory months. In contrast to CIA, RSV DFA test had a sensitivity and specificity of 94.1% and 96.8% respectively, which remained consistent throughout the season. This is consistent with the manufacturer’s performance claim of sensitivity of 88-100% but we did not
attain 100% specificity as claimed by this manufacturer (20). The relative high sensitivity of this assay in our study may be because only specimens with adequate cells were included in this study.

In recent years, the possibility of hMPV and RSV coinfection has received considerable attention. Semple et al. in 2005 reported that children dually infected with hMPV and RSV present with severe bronchiolitis and increased risk of admission to a pediatric intensive care unit for mechanical ventilation (19). Others have shown no change in disease severity of the coinfected patients and significant variation in the frequency of coinfection based on geographic location and patient population. For example, Luis et al., studying 111 children with acute respiratory infections attending clinics and hospitals, with acute respiratory infections in Aracaju, Brazil, reported that 7% of all of the patients were coinfected RSV/hMPV (2). In contrast, Lazar et al 2004, studying 46 subjects in 2004, did not see any coinfection at the time that both viruses were circulating in their community in southern Connecticut (10). In this study we detected eight hMPV-positive cases, which were tested positive for RSV by CIA, but all tested negative for RSV by DFA and RT-PCR. These specimens were collected in the late study stage of the season and were considered false positive for RSV. Overall, there was evidence of coinfection in specimens from four patients (0.78%) of which only a single specimen was RT-PCR confirmed for both viruses. Unlike the study from Brazil, with its reported coinfection rate of 7%, the low rate of coinfection (0.78%) among our patient population may explain the lack of coinfection observed on the limited samples (46 subjects) tested by Lazar et al. during the 2004 respiratory season in Connecticut (10). We conclude that, on rare occasions, RSV and hMPV coinfection does occur among our patient population in northern Connecticut.
There were 32 specimens that were positive for hMPV by 2 RT-PCR assays performed in two separate laboratories. In addition, one specimen tested strongly positive for RSV by RT-PCR (Ct value=19), DFA and CIA was shown to be positive for hMPV by DHI DFA. The RT-PCRs for this specimen tested negative and the result of the DHI test was considered a false positive.

Among the 12 RT-PCR positive samples tested by both Imagen and DHI, DHI detected six positive samples compared to 7 by Imagen. Similarly, the retrospective retesting of all of the specimens that were RT-PCR positive but DHI DFA negative by Imagen DFA results in two additional DFA positive samples. The overall sensitivity and specificity were 62.5% and 99.8% for DHI and 63.2% and 100% for Imagen, respectively.

We conclude and concur with previous studies that RSV CIA lacks sensitivity and specificity especially during off peak months. In contrast, RSV DFA is more sensitive and specific throughout the year. Similarly, both hMPV DFA are highly specific in comparison to RT-PCR, but sensitivity awaits further improvement.
Acknowledgment

We wish to thank Ms. Carol Latter for her editorial assistance in preparing this manuscript.
1 **Figure Legends**

2 Seasonal distribution of RSV and hMPV antigens detected in nasopharyngeal specimens.
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antigen detection assays for respiratory syncytial virus with another assay and shell vial
Table 1. Sensitivity, specificity and predictive values of the four rapid antigen tests

<table>
<thead>
<tr>
<th>Test</th>
<th>Numbers</th>
<th>S+, T+</th>
<th>S+, T−</th>
<th>S−, T+</th>
<th>S−, T−</th>
<th>Sen (%)</th>
<th>Spe (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV/CIA</td>
<td>515</td>
<td>190</td>
<td>48</td>
<td>29</td>
<td>248</td>
<td>79.8</td>
<td>89.5</td>
<td>86.8</td>
<td>83.8</td>
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<tr>
<td>RSV/DFA</td>
<td>515</td>
<td>224</td>
<td>14</td>
<td>9</td>
<td>268</td>
<td>94.1</td>
<td>96.8</td>
<td>96.1</td>
<td>95.0</td>
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<tr>
<td>hMPV(DHI)</td>
<td>515</td>
<td>20</td>
<td>12</td>
<td>1</td>
<td>482</td>
<td>62.5</td>
<td>99.8</td>
<td>95.2</td>
<td>97.6</td>
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<tr>
<td>hMPV(Imagen)</td>
<td>118*</td>
<td>12</td>
<td>7</td>
<td>0</td>
<td>99</td>
<td>63.2</td>
<td>100.0</td>
<td>100.0</td>
<td>93.4</td>
</tr>
</tbody>
</table>

* Validation of Imagen DFA kit was initiated in the middle of the study period.

Sen, sensitivity; Spe, specificity; PPV, positive predictive value; NPV, negative predictive value; S, standard; T, test; +, positive; −, negative.
Table 2. Seasonal distribution of false negative and positive RSV CIA results

<table>
<thead>
<tr>
<th>RSV CIA false results</th>
<th>Nov. (n=96)</th>
<th>Dec. (n=152)</th>
<th>Jan. (n=114)</th>
<th>Feb. (n=79)</th>
<th>March (n=45)</th>
<th>April (n=29)</th>
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<tbody>
<tr>
<td>False positive</td>
<td>0 (0.0%)</td>
<td>3 (2.0%)</td>
<td>2 (1.8%)</td>
<td>10 (12.7%)</td>
<td>9 (20.0%)</td>
<td>5 (17.2%)</td>
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<tr>
<td>(n=29)</td>
<td></td>
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<tr>
<td>False negative</td>
<td>12 (12.5%)</td>
<td>16 (10.5%)</td>
<td>14 (12.3%)</td>
<td>4 (5.1%)</td>
<td>2 (4.4%)</td>
<td>0 (0.0%)</td>
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<tr>
<td>(n=48)</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total false</td>
<td>12 (12.5%)</td>
<td>19 (12.5%)</td>
<td>16 (14.0%)</td>
<td>14 (17.7%)</td>
<td>11 (24.4%)</td>
<td>5 (17.2%)</td>
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<td>(n=77)</td>
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