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Virology

Evaluation of the PrimerDesign™ genesig real-time reverse transcription–polymerase chain reaction assay and the INFINITI® Respiratory Viral Panel Plus assay for the detection of human metapneumovirus in Kuwait

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Abstract

Human metapneumovirus (hMPV) is a respiratory pathogen that was discovered in 2001 and is considered a major cause of both upper and lower respiratory tract infections. A sensitive, fast, and high-throughput diagnostic test is needed for the detection of hMPV that may assist in the clinical management as well as in the reduction of inappropriate therapy. Therefore, a comparison assessment was performed in this study between the PrimerDesign™ genesig real-time reverse transcription–polymerase chain reaction (RT-PCR) Assay and the INFINITI® Respiratory Viral Panel Plus Assay (RVP-Plus) for the detection of hMPV infection in patients with respiratory tract infections. A total of 200 respiratory samples were collected from 185 hospitalized patients, during the winter season in Kuwait. Of 185 patients, 10 (5.4%) were positive for hMPV RNA by the in-house RT-PCR assay, while 7 (4%) were positive for hMPV RNA by the real-time RT-PCR assay and 9 (5%) were positive for hMPV RNA by the INFINITI® RVP-Plus assay. The high incidence rate (60%) of hMPV infection was in January 2011. The sensitivity of the real-time RT-PCR and INFINITI® RVP-Plus assays was 70% and 90%, respectively, with specificity of 100% for both assays. hMPV types A and B could be identified in this study; however, discordant genotyping results were found between the direct sequencing method and the INFINITI® RVP-Plus assay in 33% of hMPV-positive patients.

Keywords: Human metapneumovirus (hMPV); Respiratory virus; Molecular; RT-PCR; Real-time PCR; Microarray

1. Introduction

The most common cause of respiratory tract infections is viruses, although the etiologic agents of viral respiratory infections are identified in only half of the cases (Dong et al., 2008; Snell, 2001). Nucleic acid amplification tests are the gold standard approach for the diagnosis of viral respiratory infections (Lee et al., 2006), compared to old conventional approaches such as culture methods. In spite of the difficulties in the diagnosis of a wide range of viral respiratory infections due to the similarity in the clinical presentations, symptoms, and signs, nucleic acid amplification tests can assist in this process (Fox, 2007). Recently, nucleic acid amplification tests such as polymerase chain reaction method (PCR), reverse transcription–polymerase chain reaction (RT-PCR), real-time PCR, and multiplex PCR combined with microarray detection are being used widely in the diagnosis of respiratory viruses for their enhanced sensitivity and specificity above the conventional methods.

Human metapneumovirus (hMPV) was first identified by Van den Hoogen et al. (2001) from nasopharyngeal aspirates of children with respiratory tract infection in the Netherlands. Since then, it has been implicated as a common cause of upper and lower respiratory tract infections in children and adults of all ages (Boivin et al., 2002). hMPV is a single negative-stranded RNA enveloped virus that is classified in the Pneumovirinae subfamily of the Paramyxoviridae family. Genetic analysis revealed a relatively high degree of sequence variability between different hMPV isolates, and...
2. Materials and methods

2.1. Sample collection

A total of 200 respiratory samples were collected from 185 hospitalized patients in the Mubarak Al-Kabir Hospital with either upper or lower respiratory tract infections, during the winter season in the state of Kuwait, from December 2010 till February 2011. Ninety-seven (52%) of them were infants less than 2 years, 38 (20.5%) were children from 2 to 17 years, 65 (35%) were adults from 18 to 60 years, and 33 (18%) were from elderly patients above 60 years old. Respiratory samples included 141 (70.5%) nasopharyngeal swabs, 27 (13.5%) endotracheal tube secretion, 9 (4.5%) tracheal aspirate, 9 (4.5%) bronchoalveolar lavage, 7 (3.5%) throat swabs, 6 (3%) nasal swabs, and 1 (0.5%) nasopharyngeal aspirate. The total number of respiratory samples received in December 2010, January 2011, and February 2011 was 108, 57, and 35, respectively. All respiratory samples were collected after obtaining written informed consents from patients. The ethical permission on this research study was granted by the Ethical Decision Committee of the Research Administration, Faculty of Medicine, Kuwait University.

2.2. Viral RNA extraction

hMPV RNA was extracted from 140-μL respiratory specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA was then eluted in 60 μL of buffer provided in the kit and was stored at −70 °C until processing.

2.3. In-house RT-PCR for hMPV RNA detection

A set of degenerative primers were selected on the basis of sequences previously published by Mackay et al. (2003) (forward primer [hMPVF]: 5′-AAYMGWTRYTAAGTGATGCRCTC-3′; nucleotide position, 601 to 624; reverse primer [hMPVR]: 5′-CAKTGTYGTRGCRGCHCRCRTAA-3′; nucleotide position, 792 to 813) that amplify the N gene (hMPV isolate 00-1; GenBank accession number AF371337). The RT-PCR was performed using the Qiagen One-Step RT-PCR Kit (Qiagen) as previously described (Al-Turab et al., 2011).

2.4. PrimerDesign™ genesig real-time RT-PCR assay for hMPV RNA detection and quantification

The hMPV RNA was detected using the commercial genesig real-time RT-PCR assay for hMPV detection and quantification, using primers that amplify the hMPV nucleoprotein gene (PrimerDesign™). Ten microliters of Precision™ OneStep 2× q RT-PCR MasterMix (PrimerDesign) was added to 1 μL of hMPV-specific primer/probe mix, 3 μL of nuclease-free water, and 6 μL of the extracted RNA sample. The Applied Biosystem 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used for hMPV detection and quantification according to the following cycles: first, 45 °C for 10 min; second, 95 °C for 10 min; third, 50 cycles of 95 °C for 15 s; and 60 °C for 60 s. According to the manufacturer’s instructions, the PrimerDesign™ genesig real-time RT-PCR Quantification kit was designed for the detection and quantification of all hMPV subtypes. Under optimal PCR conditions, the assay can detect between 1 × 102 and 1 × 108 copies of the target template.

2.5. Detection of hMPV RNA by the INFINITI™ Respiratory Virus Panel Plus assay

The INFINITI™ Respiratory Virus Panel Plus (RVP-Plus) assay is automated by the INFINITI® Analyzer (AutoGenomics) and designed to detect 25 common respiratory viruses including subtypes (influenza A; swine H1N1 and B; human parainfluenza virus 1, 2, 3, and 4; rhinovirus A and B; enterovirus A, B, C, and D; coronavirus [HKU1, OC43, NL63, 229E], hMPV A and B; human respiratory syncytial virus A and B; and adenovirus A, B, C, and E). First-strand cDNA synthesis was performed using SuperScript III RT SuperMix (Invitrogen, California, USA). A PCR master mix was prepared by adding 17.4 μL of the RVP Plus amplification mix (AutoGenomics) and 0.1 μL of Platinum Taq Polymerase (Invitrogen). The PCR mix was then gently mixed and dispensed into each well of 24-well plates. Then,
2.5 μL of the template cDNA was added to the appropriate well. The PCR conditions were as follows: 2 min at 94 °C, 39 cycles of 94 °C for 30 s, 55 °C for 30 min, 72 °C for 1 min, and then 3 min at 72 °C. Amplification products were cleaned by enzymatic reactions and were then subjected to primer extension with the INFINITI® analyzer (AutoGenomics) as indicated by the manufacturer’s instructions. Samples were considered positive if the ratio between the signal for the virus and the background signal was greater than the threshold predetermined by the manufacturer’s software.

2.6. Genotyping of hMPV RNA by direct sequencing

The PCR products obtained from the in-house RT-PCR assay were purified and directly sequenced using the ABI PRISM®BigDye® TerminatorCycle Sequencing v. 3.1 Ready Reaction kit (Applied Biosystems) using both the hMPVF and hMPVR primers as described earlier (Al-Turab et al., 2011).

2.7. Statistical analysis

The significance of the difference in categorical variables among various groups of patients was tested using the $\chi^2$ test and the Fisher exact test, as appropriate. The statistical analysis was performed using the SPSS software v. 17.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Detection of hMPV RNA in hospitalized patients with respiratory illness

All patients involved in this study had either upper (82/185, 44%) or lower respiratory tract infections (103/185, 56%). Among 103 patients having lower respiratory tract infections, 58 (31%) had pneumonia, 14 (8%) had respiratory distress, 9 (5%) had bronchiolitis, 8 (4%) had bronchial asthma, 8 (4%) had chronic obstructive pulmonary disease, 3 (2%) had bronchopneumonia, and 3 (2%) had pulmonary edema. Ten (5%) of 200 samples were found positive for hMPV RNA by the in-house RT-PCR, which corresponds to 10 (5.4%) of 185 patients; the turnaround time for this test was 5 h. However, only 7 patients (4%) were positive for hMPV RNA by the genesig real-time RT-PCR assay (sensitivity, 70%), with a total turnaround time of 3 h. Nine (5%) were positive by the INFINITI® RVP-Plus assay (sensitivity, 90%) with a turnaround time of 24 h. The specificity of both assays, the real-time RT-PCR and the INFINITI® RVP-Plus, was 100%.

The incidence of hMPV infection was high in January ($n = 6$, 60%) and February ($n = 3$, 30%), and low in December ($n = 1$, 10%). The genotypes of hMPV RNA detected by the INFINITI® RVP-Plus assay were compared to those detected by the direct sequencing. Concordant results were obtained for only 6 (67%) of 9 samples (Table 1). A comparison between the different molecular assays for the detection and genotyping of hMPV RNA in terms of cost, turnaround time, and automation is shown in Table 2.

Most of the hMPV RNA–positive patients had pneumonia ($n = 7$, 70%), while 2 (20%) had respiratory distress and 1 (10%) had upper respiratory tract infection. The overall proportion of hMPV infection in females was 30% ($n = 3/10$) and in males was 70% ($n = 7/10$) ($P = 0.179$). Sixty percent of hMPV-positive patients were infants having less than 2 years, 10% were children from 2 to 17 years, and 30% were adults from 18 to 60 years. hMPV was not detected among elderly patients above 60 years. Moreover, 40% of hMPV-positive patients were admitted to the intensive care unit and 60% were from the hospitals wards. The hMPV was detected in the following types of respiratory samples: nasopharyngeal.

Table 1

| Patient | Age     | Sex   | Clinical diagnosis | Molecular assays | PrimerDesign™ gene| INFINITI™ respiratory | Direct sequencing, similarity %b |
|---------|---------|-------|-------------------|------------------|-------------------|------------------------|---------------------------------|
| Patient |         |       |                   | In-house RT-PCR assay | real-time RT-PCR assay | viral panel plus assay |                                      |
| A       | 2 Months| Male  | Respiratory distress | +                 | + (8.83 × 10⁶)c | + (hMPV-A)           | hMPV-B2 (98%)                   |
| B       | 3 Months| Male  | Pneumonia          | +                 | + (1.34 × 10⁶)   | + (hMPV-B)           | hMPV-B2 (99%)                   |
| C       | 3 Months| Male  | Pneumonia          | +                 | + (3.15 × 10⁶)   | + (hMPV-A)           | hMPV-B2 (93%)                   |
| D       | 4 Months| Male  | Respiratory distress | +                 | + (1.44 × 10⁶)   | + (hMPV-A)           | hMPV-A2b (98%)                  |
| E       | 5 Months| Male  | Pneumonia          | +                 | + (4.35 × 10⁶)   | – (NA)d              | hMPV-B2 (94%)                   |
| F       | 10 Months| Male | Pneumonia        | –                 | –                 | + (hMPV-A)           | hMPV-A2b (95%)                  |
| G       | 11 Years| Female| Pneumonia          | +                 | + (7 × 10⁵)      | + (hMPV-A)           | hMPV-A2b (97%)                  |
| H       | 33 Years| Male  | URTI               | +                 | –                 | + (hMPV-A)           | hMPV-A1 (80%)                   |
| I       | 38 Years| Female| Pneumonia          | +                 | + (7.94 × 10⁵)   | + (hMPV-A)           | hMPV-A2b (94%)                  |
| J       | 49 Years| Female| Pneumonia          | +                 | –                 | + (hMPV-A)           | hMPV-B2 (92%)                   |
| Total hMPV RNA detected | 10/185 (5.4%) | 7/185 (4%) | 9/185 (5%) | A = 8 (89%); B = 1 (11%); C = 5 (50%) | A = 5 (50%); B = 5 (50%) |

a Results of the hMPV RNA detection by the assigned test: (+) positive/(−) negative.
b Similarity with the reference genome from the GenBank database.
c hMPV RNA load copies per milliliters.
d NA – Not available since it was not detected by the INFINITI® RVP-Plus assay.
e URTI = Upper respiratory tract infection, which includes cough, fever, and tonsillitis.
swabs (30%), endotracheal tube secretions (30%), throat swabs (20%), tracheal aspirates (10%), and bronchoalveolar lavage (10%).

4. Discussion

The detection of hMPV by molecular methods is preferred to the conventional methods since the virus replicates poorly in most conventional cell cultures (Van den Hoogen et al., 2001). A sensitive, rapid, automated, and high-throughput diagnostic test is needed for the detection of hMPV and other respiratory viruses, which may assist in the clinical management as well as in the reduction of the implementation of inappropriate therapy and hospitalization (Barenfanger et al., 2000; Fox, 2007).

Some recent studies suggested the importance of comparing the molecular diagnostic methods with other molecular techniques to assess performance, because of the superior sensitivity and specificity (Jin et al., 2012; Kroll & Weinberg, 2011; Zhu et al., 2011), it is not preferred due to the difference in the sensitivity of the 2 assays to detect low viral load as reported earlier (Ali et al., 2011). Our results indicated that both hMPV genotypes A and B were circulating with no predominance for certain genotypes. However, the INFINIT™ RVP-Plus assay showed discrepancies in genotyping results with the direct sequencing method. Although genotyping is required in some viral infection cases, some studies showed that, in case of hMPV infection, there is no such correlation between different hMPV genotypes and the severity or the outcome of the disease (Agapov et al., 2006; Debur et al., 2010).

A previous study conducted to compare between the microarray detection such as the INFINIT™ RVP-Plus assay is sensitive, has high-throughput (24 samples/run), is time-saving, house RT-PCR and the INFINIT™ RVP-Plus assays. The turnaround time to accomplish the real-time RT-PCR was 3 h for hMPV RNA detection as well as quantification. The INFINIT™ RVP-Plus assay showed higher sensitivity (90%) than the genesig real-time RT-PCR, and all specimen results were positive for the internal control, ensuring the validity of results. The turnaround time for the detection and genotyping of hMPV RNA and other respiratory viruses using the INFINIT™ RVP-Plus required around 24 h for 24 samples. hMPV RNA could not be detected in 1 sample by the INFINIT™ RVP-Plus assay and was also hardly detected by the real-time RT-PCR, showing very low viral load suggesting that the discordance is due to the difference in the sensitivity of the 2 assays to detect low viral load as reported earlier (Ali et al., 2011).

Table 2: Comparison between the different molecular assays used for hMPV RNA detection and genotyping

| Molecular assays | In-house RT-PCR assay | PrimerDesign™ genesig real-time RT-PCR assay | INFINIT™ respiratory viral panel plus assay | Direct sequencing |
|------------------|-----------------------|----------------------------------------------|------------------------------------------|------------------|
| Detection        | hMPV RNA ± a           | hMPV RNA ±                                   | hMPV RNA ± (+23 common respiratory viruses) | Confirmatory test |
| Qualitative      | NA b                  | hMPV RNA load                                | NA                                       | NA               |
| Quantitative     | NA b,c                | 70%                                          | 90%                                      | Confirmatory test |
| Sensitivity      |                      |                                               | 24                                       |                  |
| Genotyping       |                      | hMPV – A/B                                   | hMPV – A/B                               |                  |
| Turnaround time (h) | 5                    | 3                                            | 24                                       | 24               |
| Cost (US$) d     | 8                     | 7                                            | 330 e                                    | 22               |
| Automation       | Manual                | Manual                                       | Manual                                   |                  |

a ± = Positive or negative result of hMPV RNA detection.
b NA = Not available for the test.
c Reference assay.
d Cost/sample of kits used for hMPV RNA detection and genotyping by each method; devices and machines were not included.
e Price of the INFINIT™ RVP-Plus assay for the detection and genotyping of hMPV RNA plus 23 common respiratory viruses/samples.
and has expert techniques, it is not commonly used in clinical laboratories owing to its cost and complexity.

hMPV is known as a common disease in young children (Al-Turab et al., 2011; Falsey et al., 2003; Principi et al., 2006; Williams et al., 2004; Van den Hoogen et al., 2004), and our data have shown that most hMPV-positive patients were infants less than 2 years (60%). In our previous work, hMPV infection was not detected among children and adults from 2 years up to 60 years (Al-Turab et al., 2011). However, in this study, hMPV was detected in 10% of children aged from 2 to 17 years and in 30% of adults aged from 18 up to 60 years. Three of 4 in these 2 age groups (75%) had pneumonia, 2 of whom were admitted to the intensive care unit. This emphasizes the importance of hMPV infection not only in young children and elderly people, but also in middle-age groups, which supports the finding of a previous study that hMPV infection was not detected among children and adults from 2 years up to 60 years (Al-Turab et al., 2011).

In conclusion, the current study shows the importance of the detection of hMPV as a respiratory pathogen using advanced molecular assay, such as the microarray-based assay for its higher sensitivity, rapidity, and throughput compared to other molecular assays.

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