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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

Abbreviations: hMPV, human metapneumovirus; RVP-Plus, Respiratory Viral Panel Plus; RT-PCR, reverse transcription PCR.

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2 major subgroups, A and B, were identified. Subsequent genetic analysis led to a further subdivision of the hMPV A and B subgroups into the subtypes A1, A2, B1, and B2 (Biacchesi et al., 2003). Moreover, subgroup A2 was further subdivided into 2 minor subgroups, A2a and A2b (Huck et al., 2006). hMPV infection is seasonal, with peak infection in the winter and spring months (Ali et al., 2010; Al-Turab et al., 2011; Sugrue et al., 2008).

Our previously published data have shown that the prevalence of hMPV infection in hospitalized patients with respiratory symptoms is 5.4%, and it is more prevalent among infants and elderly patients with pneumonia (Al-Turab et al., 2011). The results emphasized the need to establish a rapid and sensitive assay for the detection of hMPV in the clinical samples. Recently, commercial multiplex PCR assays based on microarray technology for simultaneous detection of a panel of respiratory viruses had been introduced to the market. However, such multiplex approaches may compromise the ability of these assays to detect low viral loads. Therefore, this study was aimed to compare the sensitivity of a multiplex PCR assay, the INFINITI® Respiratory Viral Panel Plus Assay (RVP-Plus; AutoGenomics, California, USA), to that of in-house RT-PCR and commercial real-time RT-PCR assays (PrimerDesign™ genesig; PrimerDesign Ltd., Southampton, Hants, UK), and a standardized hMPV RNA was used in the 3 assays as a positive control.

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 males and 88 (48%) were females; of all age groups, from 1 day up to 80 years; and from different nationalities. The total number of patients who were in the intensive care unit was 65 (35%), while 120 (65%) were from different hospital wards. Of the 185 patients, 49 (26.5%) 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 degenerate primers were selected on the basis of sequences previously published by Mackay et al. (2003) (forward primer [hMPVF]: 5′-AAYMGWGTRYTAAGT-GATGCRCTC-3′; nucleotide position, 601 to 624; reverse primer [hMPVR]: 5′-CAKTGTYTGRCCRGCHCCRTAA-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 (Primer Design) 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 1×10^2 and 1×10^8 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 χ^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 that 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
hMPV RNA detection and genotyping in patients with respiratory tract infections ($n = 185$)

Patient	Age	Sex	Clinical diagnosis	Molecular assays[a]			
				In-house RT-PCR assay	PrimerDesign™ genesig real-time RT-PCR assay	INFINITI® respiratory viral panel plus assay	Direct sequencing, similarity %[b]
A	2 Months	Male	Respiratory distress	+	+ (8.83×10^6)[c]	+ (hMPV-A)	hMPV-B2 (98%)
B	3 Months	Male	Pneumonia	+	+ (1.34×10^8)	+ (hMPV-B)	hMPV-B2 (99%)
C	3 Months	Male	Pneumonia	+	+ (3.15×10^6)	+ (hMPV-A)	hMPV-B2 (93%)
D	4 Months	Male	Respiratory distress	+	+ (1.44×10^9)	+ (hMPV-A)	hMPV-A2b (98%)
E	5 Months	Male	Pneumonia	+	+ (4.35×10^5)	− (NA)[d]	hMPV-B2 (94%)
F	10 Months	Male	Pneumonia	+	−	+ (hMPV-A)	hMPV-A2b (95%)
G	11 Years	Female	Pneumonia	+	+ (7×10^8)	+ (hMPV-A)	hMPV-A2b (97%)
H	33 Years	Male	URTI[e]	+	−	+ (hMPV-A)	hMPV-A1 (80%)
I	38 Years	Female	Pneumonia	+	+ (7.94×10^5)	+ (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%)	
Total hMPV RNA genotypes detected						A = 8 (89%); B = 1 (11%)	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 milliliter.
[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.

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	INFINITI® respiratory viral panel plus assay	Direct sequencing
Detection				
Qualitative	hMPV RNA ±[a]	hMPV RNA ±	hMPV RNA ± (+23 common respiratory viruses)	Confirmatory test
Quantitative	NA[b]	hMPV RNA load	NA	NA
Sensitivity	NA[b,c]	70%	90%	Confirmatory test
Genotyping	NA	NA	hMPV− A/B	hMPV− A/B
Turnaround time (h)	5	3	24	24
Cost (US$)[d]	8	7	330[e]	22
Automation	Manual	Manual	Automated	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 INFINITI® RVP-Plus assay for the detection and genotyping of hMPV RNA plus 23 common respiratory viruses/samples.

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 of the molecular methods compared to conventional ones (Ali et al., 2011). Therefore, in this study, we compared the sensitivity of 2 molecular assays, the PrimerDesign™ genesig real-time RT-PCR and the INFINITI® RVP-Plus assays, with a reference assay, the in-house RT-PCR, using a set of degenerate primers targeting the N gene, for the detection of hMPV RNA in clinical specimens. The in-house RT-PCR was used as a gold standard test to confirm the presence or absence of hMPV RNA in cases of discrepancy between the 2 assays. All the 10 hMPV RNA-positive samples detected by the in-house RT-PCR were confirmed by direct sequencing. Although RT-PCR is still the method of choice for the detection of hMPV RNA in many clinical laboratories for its high sensitivity and specificity (Jin et al., 2012; Kroll & Weinberg, 2011; Zhu et al., 2011), it is not preferred due to the total turnaround time needed along with some other confirmatory tests such as sequencing which may take a total of 48 h.

The commercial real-time RT-PCR also targeted the N gene but showed lower sensitivity (70%) compared with both in-house RT-PCR and the INFINITI® 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 INFINITI® 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 INFINITI® RVP-Plus required around 24 h for 24 samples. hMPV RNA could not be detected in 1 sample by the INFINITI® 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). Our results indicated that both hMPV genotypes A and B were circulating with no predominance for certain genotypes. However, the INFINITI® 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 assay automated by the INFINITI analyzer and the real-time PCR assay for the detection of respiratory viruses has shown that both assays were able to detect a panel of respiratory viruses in clinical specimens with concordant result in 94.1% of specimens (Raymond et al., 2009). Recently, several data were published concerning the evaluation of different molecular-based techniques for the detection of respiratory viruses showing some discrepancies in the results between different molecular methods (Ali et al., 2011; Arens et al., 2010; Dare et al., 2007; Gharabaghi et al., 2011; Raymond et al., 2009). Although a multiplex PCR combined with microarray detection such as the INFINITI® RVP-Plus assay is sensitive, has high-throughput (24 samples/run), is time saving,

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 showing that hMPV infection occurs in adults of all ages and may account for a significant portion of persons hospitalized with respiratory infections (Falsey et al., 2003). In addition, in our previous study, rhinovirus was found to be the major cause of acute respiratory infections followed by hMPV, respiratory syncytial virus, and then adenoviruses, a finding that was also obtained in the present study (data not shown) (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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