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Diagnosis of human metapneumovirus in patients hospitalized with acute lower respiratory tract infection using a metal-enhanced fluorescence technique

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A B S T R A C T

Human metapneumovirus (hMPV) is a common respiratory tract infection in children. However, conventional immunofluorescence assays (IFAs) for detecting hMPV in respiratory samples have limited reliability with a sensitivity and false-negative predictive value of 58.1% and approximately 17.8%, respectively. In this study, hMPV was measured in 91 clinical respiratory samples (55 sputum and 36 nasopharyngeal aspirate samples), which were obtained from children under three years of age, utilizing our previously developed high-throughput metal-enhanced fluorescence (MEF)-based biosensor (HT-MEFB). The sensitivity of HT-MEFB for hMPV detection in the 91 samples was improved by up to 77.4% compared with that obtained with IFAs, and the specificity of HT-MEFB for hMPV detection was 91.7%. In addition, the specificity and accuracy obtained after the selection of 55 sputum samples as the analyzed specimen reached 92.3% and 90.9%, respectively. Thus, in terms of accuracy, high throughput, and sensitivity, HT-MEFB exhibits considerable potential for hMPV detection in clinical settings.

1. Introduction

Community-acquired pneumonia is a major cause of morbidity and mortality in children because, even with advances in diagnostic tools, the causative agent of 20–30% of upper respiratory tract infections in children is undiagnosed (Janssen et al., 2010). In 2001, Dr. Bernadette van den Hoogen and colleagues first reported the isolation of the paramyxovirus human metapneumovirus (hMPV) from nasopharyngeal aspirate samples from children who suffered from respiratory tract disease (van den Hoogen et al., 2001), and ensuing reports have showed that the clinical symptoms associated with hMPV infection appear to be quite similar to those associated with respiratory syncytial virus (RSV) (Ebihara et al., 2005; Lin et al., 2005; Wei et al., 2013). Although RSV is the most common pathogen and is identified in 43.4% of children with respiratory tract infections, many studies have shown that hMPV is becoming one of the leading causes of pediatric respiratory tract infections (Williams et al., 2004; Huang et al., 2010, 2014).

Currently, there are several methods for the detection of hMPV infections. For example, viral isolation by culture remains the predominant diagnostic method for viral respiratory infections. However, culturing viruses is a labor-intensive procedure and has a long total turnaround time (Kikuta et al., 2008; Kukavica-Ibrulj and Boivin, 2009; Janssen et al., 2010). Enzyme-based immunoassays are another diagnostic method, and commercial enzyme-based immunoassays typically show an acceptable sensitivity (~80%) for hMPV detection (Kukavica-Ibrulj and Boivin, 2009; Janssen et al., 2010; Okamoto et al., 2010). To date, RNA detection by reverse-transcription polymerase chain reaction (RT-PCR) or isothermal amplification technologies (IATs) are the most sensitive

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and specific procedures for hMPV detection (Ebihara et al., 2004; Landry et al., 2005; Kikuta et al., 2008; Klemenc et al., 2012; Wang et al., 2012). However, IATs are performed only in special laboratories, and RT-PCR requires several hours to obtain results (Landry et al., 2005; Kikuta et al., 2008). The immunofluorescence assay (IFA), which provides results within 2–4 h, is also commonly used in clinical virology laboratories for the diagnosis of respiratory viruses (Ishiguro et al., 2005; Landry et al., 2005, 2008; Ingram et al., 2006; Fenwick et al., 2007). However, IFA not only requires a highly trained technologist to interpret the staining results with the use of a fluorescence microscope but also shows a relatively poor sensitivity of approximately 58.06% in our experimental results. Recently, a gold nanoparticle (GNP)-based immunochromatography assay (ICA) for hMPV detection proposed by Dr. Kikuta et al. showed 70.6% sensitivity and 95.5% specificity, whereas Dr. Matsuzaki et al. showed sensitivity and specificity of 82.3% and 93.8%, respectively.

GNP has been used to improve the detection sensitivity of biosensors because GNP has a high surface-area-to-volume ratio and exhibits unique optical properties, such as localized surface plasmon resonance (LSPR) (Baptista et al., 2008; Chen et al., 2014). In addition, many studies have integrated GNP with a fluorescence technique because GNP can not only produce a localized field that is significantly amplified through LSPR but also make a fluorophore that generates a larger quantum yield of fluorescence emission due to a theoretical increase of at least 1000-fold in the radiative decay rate of the fluorophore (Geddes et al., 2003; Morton et al., 2011). These cause a significantly enhance of the emission of a fluorophore near a GNP compared with that obtained in free-space condition; the phenomenon is so-called metal-enhanced fluorescence (MEF). Based on this technique, we previously developed MEF biosensors to study protein-protein interactions at ultralow concentrations at the pg/mL level (Hsieh et al., 2007; Chang et al., 2009, 2010, 2011, 2013; Huang et al., 2009; Chao et al., 2012). Moreover, the mechanism underlying the fluorescence enhancement using metallic nanoparticles in this biosensor was studied based on the scattering theory (Chang et al., 2009). In this study, hMPV detection using the developed high-throughput MEF-based biosensor (HT-MEFB) was compared to that obtained with RT-PCR and IFA using 91 clinical respiratory samples, including 55 sputum (SP) and 36 nasopharyngeal aspirate samples, all of which were obtained from children under 3 years of age. The sensitivity of HT-MEFB for hMPV diagnosis in all of the specimens was improved up to 77.4% compared with that obtained with IFA, and the specificity of HT-MEFB for hMPV detection was found to be 91.7%. In addition, the selection of only 55 SP samples as the analyzed specimen in this study resulted in specificity and accuracy values of 92.3% and 90.9%, respectively. As a result, in terms of accuracy, high throughput, and detection sensitivity, the HT-MEFB exhibits considerable potential for hMPV diagnosis in clinical settings.

2. Materials and methods

2.1. Clinical samples

This study was approved by the institutional review board of Chang Gung Memorial Hospital (IRB 99-2855B) at Taoyuan, Taiwan. Written consent was obtained from all of the participants. All of the clinical samples were independently obtained from 91 patients under 3 years of age with acute lower respiratory tract infection. A total of 91 patients were recruited from February 2011 to August 2011 in Linkou Chang Gung Memorial Hospital. The 91 specimens included 36 nasopharyngeal aspirate and 55 SP samples, and these were prepared by the Department of Laboratory Medicine, Linkou Chang Gung Memorial Hospital, Taoyuan, Taiwan. Each specimen was collected from a different patient.

Table 1
Primers and probe sequence used for the detection of hMPV.

Primer	Sequence (5′ to 3′)	Size	Position
NLN-forward	5′-CATAYAARCATGCTATATT AAAAGAGTCTC-3′	162 bp	2478–2497
NLN-reverse	5′-CCTATYTCWGCAGCATAT TTGTARTCAG-3′		2558–2537
NLN-probe	5′-FAM-AATGATGARGGTGT CACTG-MGBNFQ-3′		2500–2528

2.2. hMPV detection using the PCR-based assay

The viral RNA from 140 μL of the specimens was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA was eluted into 30 μL of elution buffer. The resulting RNA (8 μL) was subjected to cDNA synthesis using 50 ng of a random hexamer primer and 500 nM dNTPs. This step was performed at 65 °C for 10 min followed by 4 °C for at least 1 min. An RT mixture containing 1X RT buffer, 10 mM DTT, 40 units of RNaseOut and 200 units of SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was applied and cycled at 25 °C for 10 min, 50 °C for 60 min, and 85 °C for 5 min on a BioMetra Thermocycler (Biometra, Goettingen, Germany).

The real-time PCR detection of hMPV was performed using the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with iTaq™ Supermix (Bio-Rad, Hercules, CA, USA). The primers and probe sequence for the detection of hMPV are shown in Table 1. Amplification was performed in a total volume of 20 μL containing 10 μL of 2X iTaq™ Supermix RT-PCR Master mix, 400 nM of each NLN primer, 1 μM NLN probe, 3.6 μL of DEPC H_2O and 4 μL of cDNA template. The RT-PCR program was performed under the following conditions: 95 °C for 3 min followed by 45 cycles of 95 °C for 3 s and 60 °C for 30 s.

2.3. hMPV detection using IFA

The specimens from clinical patients were washed three times with phosphate-buffered saline (PBS) solution. The respiratory epithelial cells were then centrifuged at 2000 rpm for 10 min, and the supernatant was discarded. An appropriate volume of PBS solution was added, and the sample solution was mixed. The epithelial cells were spotted onto slides, and the cell smears were air-dried and fixed in cold acetone for 10 min. The cell smears were incubated for 30 min at 37 °C with a FITC-labeled D^3 DFA Metapneumovirus Identification Kit (Diagnostic Hybrids, Athens, OH, USA). After incubation, the cells were washed three times in PBS solution for 10 min, air-dried and mounted with mounting media. The slides were viewed and evaluated using a fluorescence microscope (Olympus Optical, Hamburg, Germany), and the IFA data were used to assess the detection of hMPV-infected cells in the specimens.

2.4. Optical setup of HT-MEFB

In HT-MEFB, a He–Ne laser (17 mW, 632.8 nm, Newport) was used as the excitation light source. The intensity of the laser beam was modulated at a frequency of 1 kHz using a chopper. A beam splitter was applied to split the laser beam into a signal beam and a reference beam. The intensity-modulated signal beam was directed incident to each well of a 96-well immunoplate to measure the antigen–antibody interaction. The immunocomplexes were placed on the bottom of the immunoplate wells. Two identical long-pass filters (cutoff wavelength = 640 nm) were introduced into the instrument to detect the intensity-modulated fluorescence signal via a photomultiplier tube (PMT). Two lock-in amplifiers were used