Journal of Medical Virology 87:1867–1871 (2015)

# Evaluation of a PCR/ESI-MS Platform to Identify Respiratory Viruses From Nasopharyngeal Aspirates

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Acute respiratory tract infection is a major cause of morbidity and mortality worldwide, particularly in infants and young children. High-throughput, accurate, broad-range tools for etiologic diagnosis are critical for effective epidemic control. In this study, the diagnostic capacities of an Ibis platform based on the PCR/ESI-MS assay were evaluated using clinical samples. Nasopharyngeal aspirates (NPAs) were collected from 120 children (<5 years old) who were hospitalized with lower respiratory tract infections between November 2010 and October 2011. The respiratory virus detection assay was performed using the PCR/ESI-MS assay and the DFA. The discordant PCR/ESI-MS and DFA results were resolved with RT-PCR plus sequencing. The overall agreement for PCR/ESI-MS and DFA was 98.3% (118/120). Compared with the results from DFA, the sensitivity and specificity of the PCR/ESI-MS assay were 100% and 97.5%, respectively. The PCR/ESI-MS assay also detected more multiple virus infections and revealed more detailed subtype information than DFA. Among the 12 original specimens with discordant results between PCR/ESI-MS and DFA, 11 had confirmed PCR/ESI-MS results. Thus, the PCR/ESI-MS assay is a high-throughput, sensitive, specific and promising method to detect and subtype conventional viruses in respiratory tract infections and allows rapid identification of mixed pathogens. ***J. Med. Virol. 87:1867–1871, 2015***. 

**KEY WORDS:** electrospray ionization mass spectrometry (ESI/MS); direct immunofluorescent assay (DFA); respiratory tract infections; virus; diagnosis

## INTRODUCTION

Acute respiratory tract infections (ARTIs) are a major cause of morbidity and mortality worldwide, particularly in infants and young children, who may experience multiple infections per year until they are 10 years of age. Costs attributable to viral lower respiratory tract infections in both outpatient and inpatient settings are a significant burden on healthcare budgets [Arnold et al., 2006]. Viruses are responsible for the majority of acute respiratory tract infections, with respiratory syncytial virus (RSV), influenza virus (INF), parainfluenza viruse (PIV), adenovirus, and human metapneumovirus (hMPV) considered the most common pathogens. Thus, high-throughput, accurate, broad-range tools for etiologic diagnosis are critical for maintaining reasonable use of antibiotics and effective epidemic control.

Traditionally, the diagnosis of respiratory infections relied on the isolation and identification of the viral agent by cell culture or detection of viral antigens by direct immunofluorescent assays (DFAs) [Arnold et al., 2006], which cannot satisfy adequately clinical needs due to their sub-optimal sensitivity. Recent advances in molecular biology, particularly the introduction of the real-time PCR assay, have

Grant sponsor: National Key Science & Technology Special Projects; Grant numbers: 2009ZX10004-104; 2012ZX10004-220.; Grant sponsor: National Natural Science Foundation of China; Grant number: NSFC81273204.

Conflict of interest: None.

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Accepted 4 May 2015

DOI 10.1002/jmv.24262
Published online 20 May 2015 in Wiley Online Library (wileyonlinelibrary.com).

improved greatly the detection of individual viral respiratory pathogens [Louie et al., 2005] and promoted the development of multiplex assays that can detect simultaneously multiple pathogens in a single test. Some of these multiplex technologies have potential applications in high-throughput testing, and others allow rapid, near-patient testing. However, these assays are still not used widely in clinical laboratories, and most are currently available only for research use. More studies are needed to elucidate their performance characteristics and to determine their ideal clinical applications [Caliendo, 2011].

Electrospray ionization mass spectrometry (ESI-MS) coupled with broad-range PCR (PCR/ESI-MS) is a high-throughput technology for the simultaneous, multiplex molecular detection of microbes on an Ibis platform. Compared with other methods that are dependent on indirect detection of fluorescent or radioactive reporter tags, PCR/ESI-MS can measure directly the intrinsic physical properties of molecules [Li et al., 2007; Benson et al., 2008; Ecker et al., 2009; Raymond et al., 2009]. The use of PCR/ESI-MS has been demonstrated for broad bacterial surveillance [Ecker et al., 2005] and the identification of virus families, including coronaviruses [Sampath et al., 2005], INFs [Sampath et al., 2007; Deyde et al., 2011; Jeng et al., 2012; Tang et al., 2013], adenoviruses [Blyn et al., 2008], alphaviruses [Eshoo et al., 2007], orthopoxviruses [Hofstadler et al., 2005], and enteroviruses [Piao et al., 2012].

In this study, the Ibis platform-based PCR/ESI-MS assay, also called PLEX-ID, was used to identify multiple respiratory viruses in nasopharyngeal aspirates (NPA). The diagnostic performance characteristics of this assay compared with conventional virological procedures were evaluated using clinical specimens.

## MATERIALS AND METHODS

### Sample Collection

NPA specimens are sent routinely to the clinical virology laboratory at the Children's Hospital of Fudan University for the identification of respiratory viruses. A total of 120 NPAs were collected from 120 children (<5 years old) hospitalized with lower respiratory infections (LRTI) between November 2010 and October 2011. The LRTI patients were diagnosed on the basis of the WHO criteria which include a history of fever, cough, fast respiratory rate for age, chest in-drawing, and ronchi or crepitations on auscultation. NPA samples were maintained at −80°C in the clinical virology laboratory after standard virological procedures were completed as part of the standard laboratory protocol. The specimens involved were collected in the normal course of patient care, and this study was approved by the Ethics Committee of the Children's Hospital of Fudan University.

### Reference Assay Used in the Clinical Virology Laboratory

A direct immunofluorescent assay (DFA) [Diagnostic HYBRIDS, Athens, OH] was used to screen for respiratory syncytial virus, adenovirus, influenza virus, parainfluenza virus, and human metapneumovirus in NPAs, according to a standardized protocol from this clinical virology laboratory.

### Identification of Respiratory Viruses on ESI-MS Platform

Total nucleic acids, including both DNA and RNA, were extracted from 200 μL of NPA by a QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The respiratory virus detection assay was performed with an Abbott PLEX-ID respiratory kit (Abbott, Carlsbad, CA) according to the manufacturer's instructions. The PLEX-ID respiratory virus assay was designed to use 17 primer pairs distributed into 16 wells to identify 67 respiratory viral species, including adenovirus, coronavirus, hMPV, INFs (A, B, and C), PIVs (1, 2, and 3), Mumps virus, Newcastle disease virus and RSV et al. Each plate had the capacity to test six patient samples. Briefly, after a 5 μL aliquot of DNA and RNA was added to each of 16 wells, assay plates were sealed and PCR amplification was initiated. The RT-PCR cycling conditions were 60°C for 5 min, 4°C for 10 min, 55°C for 45 min, and 95°C for 10 min, followed by eight cycles of 95°C for 30 sec, 48°C for 30 sec, and 72°C for 30 sec. The annealing temperature of 48°C was increased by 0.9°C during each successive cycle. RT-PCR was continued for 37 additional cycles of 95°C for 15 sec, 56°C for 20 sec, and 72°C for 20 sec and then ended with a final extension of 72°C for 2 min, followed by a 4°C hold. Subsequently, the plates were stored at 4°C until base composition analysis was conducted by MS.

An PLEX-ID platform (Abbott) was used to perform automated post-PCR desalting, ESI-MS signal acquisition, spectral analysis, and data reporting to analyze PCR products as previously described [Chen et al., 2011a,2011b; Forman et al., 2012]. Unambiguous base compositions were derived from the exact mass measurements of the complementary single-stranded oligonucleotides. Information on the pathogens was acquired by screening the pathogen database.

### Resolution of Discordant Test Results

All discordant PCR/ESI-MS and DFA results were resolved with RT-PCR plus sequencing. cDNA synthesis was performed using a PrimeScript RT-PCR Kit (PrimeScript™ RT kit, Takara, Dalian, China) according to the manufacturer's instructions. In brief, 8 μL of total nucleic acids was mixed with 1 μL of 10 mM dNTP and 1 μL of 20 μM six random primers. After incubation at 65°C for 5 min and cooling down

to 4°C, the RT reaction was conducted by adding 20 U RNase inhibitor and 100 U PrimeScript RTase. The reaction mixture was incubated at 42°C for 60 min and then heated to 85°C for 5 sec. cDNA was either used immediately for PCR or stored at −20°C.

Each nested PCR was performed with specific primers to detect INF, RSV, hMPV, PIV, and adenoviruses as previously described [Allard et al., 1992; Coiras et al., 2003, 2004; Peiris et al., 2003]. For the first round of PCR, 4 μL cDNA was amplified in 25 μL containing 0.2 mM dNTP, 0.2 μM first-round PCR primer, and 0.5 U Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The thermal cycling program was as follows: 94°C for 2 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min; and 68°C for 5 min. For the second round of PCR, 2 μL of the first PCR product was amplified with the second-round PCR primers using the same reaction mixture and PCR cycling conditions. PCR products were analyzed by electrophoresis in 2% (w/v) agarose gels and stained with 0.5 μg/ml ethidium bromide.

DNA fragments were purified from agarose gels using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The nucleotide sequence of each PCR product was determined using a BigDye sequencing kit on a 3730 DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequence analysis of the PCR product of each strain was analyzed with Seqscanner (Applied Biosystems), and genetic identity was determined by comparing the sequence with standard strains in GenBank.

### Statistical Methods

The sensitivity and specificity of PCR/ESI-MS were calculated using the DFA results as the reference assay.

## RESULTS

### Diagnostic Performance Characteristics of RT-PCR/ESI-MS Using Clinical Specimens

Of the 120 NPA samples tested, respiratory viruses were identified in 43 samples by PCR/ESI-MS assay, 41 of which were found positive by DFA. The overall agreement for the accuracy of PCR/ESI-MS and DFA was 98.3% (118/120). Compared with the DFA results, the sensitivity and specificity of PCR/ESI-MS were 100% and 97.5%, respectively (Table I). Compared with DFA, PCR/ESI-MS successfully verified more pathogens, including 30 RSVs, 8 adenoviruses, 1 PIV1, 7 PIV3, 4 INF A, 1 INF B, and 3 hMPVs (Table II).

### Detection of Multiple Viral Infections by PCR/ESI-MS

In addition to the detection of single viruses in the clinical samples, PCR/ESI-MS also detected more multiple viral infections, primarily co-infections, than did DFA. The presence of multiple pathogens was verified in a subset of nine clinical samples using PCR/ESI-MS. Out of the nine co-infections, seven involved two viruses and two involved three viruses. RSV was found to be the most common viral co-infection, followed by adenovirus and PIV3 (Table III).

TABLE I. Diagnostic Performance Characteristics of PCR/ESI-MS Compared With DFA Using 120 Clinical Specimens

| | | Direct immunofluorescent assay | | |
|---|---|---|---|---|
| | | + | − | Total |
| PCR/ESI-MS | + | 41 | 2 | 43 |
| | − | 0 | 77 | 77 |
| | Total | 41 | 79 | 120 |

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### Data From PCR/ESI-MS

More detailed data were obtained directly from PCR/ESI-MS than from DFA. Of the 30 RSV strains, 24 were type A and six were type B. The eight adenoviruses belonged to type 1 (1), type 3 (3), type 7 (2), and group B (2). All four INF A strains were identified as the 2009 pandemic H1N1 strain.

### Sequence Analysis of Discordant Specimens

Among the 12 original specimens with disagreement between the PCR/ESI-MS and DFA results, eleven were confirmed by sequencing, of which eight were multiple virus infections. One sample identified as INF B-positive by PCR/ESI-MS, was not confirmed by conventional PCR and sequence analysis (Table III).

## DISCUSSION

To the best of our knowledge, this study is the first to describe the performance characteristics of a novel PCR/ESI-MS platform using clinical specimens in China. This assay uses the principle of measuring the mass of the PCR amplicon based on the nucleo-

TABLE II. Total Virus Numbers Detected by PCR/ESI-MS or DFA

| Viruses | PCR/ESI-MS | DFA |
|---|---|---|
| RSV | 30 | 28 |
| Adenovirus | 8 | 7 |
| PIV1 | 1 | 1 |
| PIV2 | NA | NA |
| PIV3 | 7 | 3 |
| INFA | 4 | 1 |
| INFB | 1 | 1 |
| hMPV | 3 | 2 |
| Total | 54 | 43 |

RSV, Respiratory syncytial virus; PIV1, Parainfluenza virus 1; PIV2, Parainfluenza virus 2; PIV3, Parainfluenza virus 3; INFA, Influenza virus A; INFB, Influenza virus B; hMPV, human Metapneumovirus.

TABLE III. Sequence Analysis From 12 Subjects With Discordant PCR/ESI-MS and DFA Results

| Subject ID | PCR/ESI-MS | DFA | PCR/sequencing |
|---|---|---|---|
| #1 (10–744) | RSV, PIV3 | RSV | RSV, PIV3 |
| #2 (10–1103) | INFB, Adenovirus, PIV1 | PIV1 | Adenovirus, PIV1 |
| #3 (10–1134) | RSV, PIV3 | RSV | RSV, PIV3 |
| #4 (10–1184) | PIV3 | Adenovirus | PIV3 |
| #5 (10–1200) | hMPV | hMPV, Adenovirus | hMPV |
| #6 (11–84) | RSV, IFNA, Adenovirus | Adenovirus | RSV, INFA, Adenovirus |
| #7 (11–150) | RSV, PIV3 | RSV | RSV, PIV3 |
| #8 (11–156) | RSV, hMPV | RSV | RSV, hMPV |
| #9 (11–246) | Adenovirus, INFA | Adenovirus | Adenovirus, INFA |
| #10 (11–552) | RSV, Adenovirus | RSV | RSV, Adenovirus |
| #11 (11–72) | INFA | (−) | INFA |
| #12 (11–78) | RSV | (−) | RSV |

RSV, Respiratory syncytial virus; PIV1, Parainfluenza virus 1; PIV2, Parainfluenza virus 2; PIV3, Parainfluenza virus 3; INFA, Influenza virus A; INFB, Influenza virus B; hMPV, human Metapneumovirus.
Ten subjects from No.1–10 were the samples with multiple viruses detected by RT-PCR/ESI-MS or DFA.

tide base composition (i.e., the number of A, G, C, and T nucleotides for that DNA molecule). Compared with conventional antigen detection by DFA, PCR/ESI-MS showed high-throughput capacity. It can also simultaneously detect and type multiple clinically relevant respiratory pathogens in nasopharyngeal aspirates from patients with lower respiratory tract infections. The sensitivity and specificity of PCR/ESI-MS were found to be 100% and 97.5%, respectively.

Consistent with other clinical trial data [Chen et al., 2011a, 2011b], PCR/ESI-MS demonstrated several advantages over regular DFA in detecting respiratory viruses. First, the time to first result from sample preparation to detection of PCR/ESI-MS was 6 hr: 0.5 hr of RNA extraction, 3 hr of RT-PCR, and 2.5 hr of processing in ESI-MS. The estimated throughput of PCR/ESI-MS was 90 samples with automatic nucleic acid extraction, using one technician working for 8 hr, which represented 1,530 PCR reactions. However, DFA may require at least two persons to complete the same number of samples within one working day. Second, more viruses were detected using PCR/ESI-MS, especially PIV3, INF A, and RSV. These viruses are the major viral agents that cause lower respiratory tract infections, but their prevalence may be underestimated because of the low sensitivity of DFA [Sloots et al., 2008]. Third, nine viral co-infection samples were verified by PCR/ESI-MS, whereas only two were detected by DFA. Dual viral infections were predominant, but co-infections with three different viruses were observed in two samples. As of this writing, the clinical importance of such multiple viral infections remains uncertain [Paranhos-Baccala et al., 2008]. Our findings indicated that PCR/ESI-MS can provide more information than DFA on viral co-infections in patients. The additional data will help in clarifying the association of the simultaneous presence of more than one viral pathogen with epidemiological and clinical features that differ from those in single infections, especially with respect to disease type and severity. Fourth, a more detailed pathogen characterization, especially type and subtype information from RSV, adenovirus, and INF, was obtained directly and rapidly using PCR/ESI-MS. Such detailed information was not obtained using DFA or conventional PCR. The key feature of this new technology is the use of MS to quickly and accurately detect the amplicons and "weigh" them with sufficient mass accuracy to determine the nucleotide base compositions that are associated with sequence information to identify and genotype the virus in the sample.

No more species of virus was found by PCR/ESI-MS than that by DFA, however, compared with the low detection sensitivity of adenovirus and MPV previously reported in two other studies [Chen et al., 2011a, 2011b; Forman et al., 2014], a high degree of agreement was found between PCR/ESI-MS and DFA. Moreover, the fact that no coronavirus was detected by PCR/ESI-MS and DFA in this study which was similar to that in another report [Chen et al., 2011b] showed the low detection rate of conronavirus and indicated that more specimens need to be collected for evaluating the diagnostic characteristic of PLEX-ID respiratory kit on conronavirus. Unlike other assays based on antigen detection or nucleic acid amplification, which are limited to the detection of known microbes, PCR/ESI-MS is suitable for discovery of pathogens and evaluation of emerging pathogens. In PCR/ESI-MS, the base compositions are compared with a database of calculated base compositions from the sequences of known organisms to determine the identities of the microorganisms present. However, when no match is found between the measured base composition and the database, the nearest neighbor organism is identified. Thus, PCR/ESI-MS provides information about pathogens in a sample without having to anticipate the pathogens that might be present [Caliendo, 2011]. This unique technology will be helpful in the rapid identification of pathogens with regard to emerging or reemerging infectious diseases and outbreaks.

Regarding the practical use of this new platform for clinical diagnosis, some limitations need to be addressed. Although the testing is highly informative,

has high-throughput capacity, can be completed within 6 hr, and is designed for use in clinical laboratories, the high cost of the instrument and the kit limits their use to research studies only. The PLEX-ID respiratory virus kit used in this study was not designed to detect rhinoviruses, bocaviruses, and PIV4, which are significant viral agents for respiratory tract infections. A modified respiratory virus surveillance kit should be developed to include more emerging and vital viral agents in the future.

In conclusion, a new PCR/ESI-MS assay was reported to detect respiratory viruses in clinical specimens in China. According to the methodological assessment, this assay is a high-throughput, sensitive, specific, and promising method for detecting and subtyping the common respiratory viruses that cause respiratory tract infections; it also allows rapid identification of mixed pathogens.

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