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

Yong Lin,1 Yongfeng Fu,3 Menghua Xu,4 Liyun Su,4 Lingfeng Cao,4 Jin Xu,4* and Xunjia Cheng3
1Department of Laboratory Medicine, Huashan Hospital of Fudan University, Shanghai, China
2Department of Center Laboratory, Jingan District Center Hospital of Shanghai, Shanghai, China
3Department of Medical Microbiology and Parasitology, Institutes of Biomedical Sciences, Shanghai Medical College of Fudan University, Shanghai, China
4Department of Clinical Laboratory Center, Children’s Hospital of Fudan University, Shanghai, China

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. © 2015 Wiley Periodicals, Inc.

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 virus (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...
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).

**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-
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, 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 coronavirus and indicated that more specimens need to be collected for evaluating the diagnostic characteristic of PLEX-ID respiratory kit on coronavirus. 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.

REFERENCES

Allard A, Albinsson B, Wadell G. 1992. Detection of adenoviruses in stools from healthy persons and patients with diarrhea by two-step polymerase chain reaction. J Med Virol 37:149–157.

Arnold JC, Singh RK, Spector SA, Sawyer MH. 2006. Human bocavirus: Prevalence and clinical spectrum at a children’s hospital. Clin Infect Dis 43:283–288.

Benson R, Tondella ML, Bhatnagar J, Carvalho Mda G, Sampson JS, Talkington DF, Whitney AM, Mothershed E, McGee L, Carlbo G, McCle V, Gupta J, Zeki S, Dejuri S, Cronin K, Han J, Fields BS. 2008. Development and evaluation of a novel multiplex PCR technology for molecular differential detection of bacterial respiratory disease pathogens. J Clin Microbiol 46:644–651.

Caliendo AM. 2011. Multiplex PCR and emerging technologies for the detection of respiratory pathogens. Clin Infect Dis 52:S263–S300.

Chen KF, Blyn L, Rothman RE, Ramachandran P, Valsamakis A, Ecker DJ, Sampath R, Gaydos CA. 2011a. Reverse transcription polymerase chain reaction and electrospray ionization mass spectrometry for identifying acute viral upper respiratory tract infections. Diag Microbiol Infect Dis 69:179–186.

Chen KF, Rothman RE, Ramachandran F, Blyn L, Sampath R, Ecker DJ, Valsamakis A, Gaydos CA. 2011b. Rapid identification viruses from nasal pharyngeal aspirates in acute viral respiratory infections by RT-PCR and electrospray ionization mass spectrometry. J Virol Methods 173:60–66.

Coiras MT, Perez-Brena P, Garcia ML, Casas I. 2003. Simultaneous detection of influenza A, B, and C viruses, respiratory syncytial virus, and adenoviruses in clinical specimens by multiplex reverse transcription nested-PCR assay. J Med Virol 69:122–144.

Coiras MT, Aguilar JC, Garcia ML, Casas I, Perez-Brena P. 2004. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J Med Virol 72:484–495.

Dvey DM, Sampath R, Gubareva LV. 2011. RT-PCR/electrospray ionization mass spectrometry approach in detection and characterization of influenza viruses. Expert Rev Mol Diagn 11:41–52.

Ecker DJ, Sampath R, Blyn LB, Eshoo MW, Hall TA, Sampath R. 2009. Molecular genotyping of influenza A viruses by multiplex PCR and mass spectrometry: A new tool for hospital infection control and public health surveillance. Methods Mol Biol 551:71–87.

Eshoo MW, Whitehouse CA, Zoll ST, Massire C, Pennella TT, Blyn LB, Sampath R, Hall TA, Ecker JA, Desai A, Waseleoski LF, Li F, Turel MJ, Schlink A, Rudnick K, Otero G, Weaver SC, Ludwig GV, Hofstadler SA, Ecker DJ. 2007. Direct broad-range detection of alphaviruses in mosquito extracts. Virology 368:286–295.

Forman MS, Advani S, Newman C, Gaydos CA, Miletich AM, Valsamakis A. 2012. Diagnostic performance of two highly multiplexed respiratory virus assays in a pediatric cohort. J Clinical Virol 55:168–172.

Hofstadler SA, Sampath R, Blyn LB, Eshoo MW, Hall TA, Jiang Y, Drader JJ, Hansis JC, Sannes-Lowery KA, Cummins LL, Libby B, Walcott DJ, Schink A, Massire C, Ranken R, Gutierrez J, Manalili S, Ivey C, Melton R, Levene H, Barrett-Wilt G, Li F, Zapp V, White N, Sampath R, McNeil JA, Knize D, Robbins D, Rudnick K, Desai A, Moradi E, Ecker DJ. 2005. TIGER: The universal biosensor. Int J Mass Spectrometry 242:22–29.

Jeng K, Massire C, Zembower TR, Deyde VM, Gubareva LV, Hsieh YH, Rothman RE, Sampath R, Penugonda S, Metzgar D, Blyn LB, Hardick J, Gaydos CA. 2012. Monitoring seasonal influenza A virus evolution: Rapid 2009 pandemic H1N1 virus detection with an reverse transcription-polymerase chain reaction/electro-spray ionization mass spectrometry assay. J Clin Virol 54:332–336.

Li H, McCormac MA, Estes RW, Sefera SE, Dare RK, Chappell JD, Erdman DD, Wright PF, Tang YW. 2007. Simultaneous detection and high-throughput identification of a panel of RNA viruses causing respiratory tract infections. J Clin Microbiol 45:2105–2109.

Louie JK, Hacker JK, Gonzales R, Mark J, Maselli JH, Yagi S, Drew WL. 2005. Characterization of viral agents causing acute respiratory infection in a San Francisco University Medical Center Clinic during the influenza season. Clin Infect Dis 41:822–828.

Panaro-Baccala G, Komurian-Pradel F, Richard N, Vernet G, Lina B, Floret D. 2008. Mixed respiratory virus infections. J Clin Virol 43:407–410.

Peiris JS, Tang WH, Chan KH, Khong PL, Guan Y, Lau YL, Chiu SS. 2003. Children with respiratory disease associated with metapneumovirus in Hong Kong. Emerg Infect Dis 9:628–633.

Piao J, Jiang J, Xu B, Wang X, Guan Y, Wu LU, Lii Y, Zhang H, Li X, Wang P, Fan N, Zeng H, Jiang J, Li Y, Yang Y, Chen W. 2012. Simultaneous detection and identification of enteric viruses by PCR-mass assay. PLoS ONE 7:e42251.

Roberst F, Carbonneau J, Boucher N, Robitaille L, Boisvert S, Wu WK, De Serres G, Boivin G, Corbeil J. 2009. Comparison of automated microarray detection with real-time PCR assays for detection of respiratory viruses in specimens obtained from children. J Clin Microbiol 47:745–750.

Sampath R, Hofstadler SA, Blyn LB, Eshoo MW, Hall TA, Massire C, Levene HM, Hansis JC, Harrell PM, Neuman B, Buchmeier MJ, Jiang Y, Ranken R, Drader JJ, Sampath R, Griffey RH, McNeil JA, Crooke ST, Ecker DJ. 2005. Rapid identification of emerging pathogens: Coronavirus. Emerg Infect Dis 11:373–379.

Sampath R, Russell KL, Massire C, Eshoo MW, Harpin V, Blyn LB, Melton R, Ivey C, Pennella T, Li F, Levene H, Hall TA, Libby B, Fan N, Walcott DJ, Ranken R, Pear M, Schink A, Gutierrez J, Drader J, Moore D, Metzgar D, Additiong L, Rothman R, Gaydos CA, Yang S, St George R, Fuchsho ME, Deen AB, Stahlknecht DE, Goekjian G, Yingst S, Monteville M, Saad MD, Whitehouse CA, Baldwin C, Rudnick KH, Hofstadler SA, Lemon SM, Ecker DJ. 2007. Global surveillance of emerging influenza virus genotypes by mass spectrometry. PLoS ONE 2:e489.

Sloots TP, Whiley DM, Lambert SB, Nissen MD. 2008. Emerging respiratory viruses by PCR/ESI-MS. J. Med. Virol. DOI 10.1002/jmv