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Application of Multiplex PCR Coupled with Matrix-Assisted Laser Desorption Ionization–Time of Flight Analysis for Simultaneous Detection of 21 Common Respiratory Viruses

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Respiratory infections continue to pose a significant threat to human health. It is important to accurately and rapidly detect respiratory viruses. To compensate for the limits of current respiratory virus detection methods, we developed a 24-plex analysis (common respiratory virus-mass spectrometry [CRV-MS]) that can simultaneously detect and identify 21 common respiratory viruses based on a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry system. To evaluate the efficacy of the CRV-MS method, we used 102 samples that were confirmed positive for these common respiratory viruses. All tests using the CRV-MS method were effective, with no cross-reactivity observed with other common respiratory viruses. To confirm the usefulness of the CRV-MS method, we screened 336 nasal and throat swabs that were collected from adults or children with suspected viral acute respiratory tract infections using the CRV-MS method and consensus PCR/reverse transcription-PCR (RT-PCR) methods. Excluding four RNase P-negative samples, the CRV-MS and consensus PCR/RT-PCR methods detected respiratory viruses in 92.5% (307/332) and 89.5% (297/332) of the samples, respectively. The two methods yielded identical results for 306 (92.2%) samples, including negative results for 25 samples (7.5%) and positive results for 281 samples (84.6%). Differences between the two methods may reflect their different sensitivities. The CRV-MS method proved to be sensitive and robust, and it can be used in large-scale epidemiological studies of common respiratory virus infections.

Respiratory infections continue to pose a threat to public health. A World Health Organization (WHO) report indicated that lower respiratory infections (LRIs) remain among the top five causes of death worldwide, killing 3.1 million people in 2012. In low-income countries, LRIs are the number one cause of death (http://www.who.int/mediacentre/factsheets/fs310/en/index.html). Many pathogens, especially respiratory viruses, are associated with respiratory infections. Different pathogens may cause similar clinical presentations, whereas their treatment differs greatly. Thus, it is important to accurately and rapidly detect respiratory pathogens.

For different respiratory viruses, the mean time to detection of conventional cell culture varies from 3 to 8 days. Because many respiratory viruses exhibit high mutation rates, traditional methods do not to provide complete and reliable etiological information. Pavia (1) indicated that better diagnostic tests can reduce antibiotic use and control nosocomial transmission. At present, molecular methods have been widely used to detect respiratory viruses. With respect to traditional methods, molecular methods have great sensitivity and a short turnaround time, and they can simultaneously detect multiple pathogens (2). This progress has enabled us to better understand the phenomenon of pathogen coinfection. For example, based on a number of different platforms, many multiplex methods have been applied in the detection of respiratory viruses, and they were shown to be sensitive and specific (3–9). However, there are still some disadvantages, such as limited detection range, high cost, complicated operation, and being labor-intensive. In this study, which is based on the MassARRAY matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry system (Sequenom, Inc., San Diego, CA, USA), we developed a common respiratory virus panel test (CRV-MS) that can simultaneously detect and identify 21 common respiratory virus types/subtypes, including several influenza A viruses (Flu-A H1N1, H1N1pdm09, and H3N2), influenza B viruses (Flu-B), parainfluenza virus types 1 to 4 (PIV1 to -4), adenovirus (AdV), four human coronaviruses (HCoV) (HCoV-OC43, 229E, NL63, and HKU1), respiratory syncytial viruses A and B (RSV-A and RSV-B, respectively), human metapneumoviruses A and B (HMPV-A and HMPV-B, respectively), human bocavirus 1 and 2 (HBoV1 and HBoV2, respectively), human rhinovirus (HRV), and human enterovirus (HEV). The MassARRAY MALDI-TOF MS system has successfully been used to detect multiple pathogens (10–16). Here, we also compare the performances of the CRV-MS and consensus PCR/reverse transcription-PCR (RT-PCR) methods.

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TABLE 1 Confirmed positive clinical samples used in this study

Virus name[a]	No.
Flu-A H1N1	6
Flu-A H1N1pdm09	4
Flu-A H3N2	4
Flu-B	2
PIV1	3
PIV2	3
PIV3	3
PIV4	3
HCoV-229E	4
HCoV-OC43	4
HCoV-NL63	4
HCoV-HKU1	4
HEV71	8
Coxsackievirus A16	8
HRV	6
AdV	10
HMPV-A	3
HMPV-B	2
RSV-A	5
RSV-B	5
HBoV1	9
HBoV2	2
Total	102

[a] Flu-A, influenza virus A; Flu-B, influenza virus B; PIV, parainfluenza virus; HCoV, human coronavirus; HEV, human enterovirus; HRV, human rhinovirus; AdV, adenovirus; HMPV, human metapneumovirus; RSV, respiratory syncytial virus; HBoV, human bocavirus.

MATERIALS AND METHODS

Clinical samples. This study was approved by the institutional review board of the Institute of Pathogen Biology, China. All nasal and throat swabs were obtained from our institute collections. These samples were collected from adults or children with suspected viral acute respiratory tract infections between January 2013 and November 2013. Nasal and throat swabs that were collected from the same patient were combined in one tube containing 3 ml of viral transport medium and stored at −80°C (17). Informed consent was obtained from all patients or from the parents of all of the children who provided specimens.

In an exploratory test, the target genes of each respiratory virus were amplified from confirmed clinical samples by RT-PCR/PCR and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), as described in previous reports (3, 17). To evaluate the specificity of the CRV-MS method, we used plasmids (500 copies per reaction) containing specific inserts of each target respiratory virus. To evaluate the sensitivity of the CRV-MS method, we used a standard 10-fold dilution series (1 to 10,000 plasmid copies/reaction) of these plasmids. Each plasmid concentration was tested in triplicate. Sterile water was used as a negative control. In this study, we also used 102 clinical samples, which were confirmed positive for 21 common respiratory viruses, to test the specificity of the CRV-MS method (Table 1). In a validation test, 336 nasal and throat swabs were used.

Total DNA and RNA were extracted from 200 μl of viral transport medium, according to a previous report (17). The Invitrogen One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) was used in the RT-PCR experiments.

The CRV-MS method. Representative respiratory virus strains used in the CRV-MS method design are shown in Table S1 in the supplemental material. The nucleotide sequences of these representative respiratory virus strains were downloaded from the GenBank database. The CRV-MS method was designed using MassARRAY assay design 4.0 software, according to the user's guide (Sequenom, Inc., San Diego, CA, USA). All primers and probes (see Table S2 in the supplemental material) were analyzed using BLASTn searches against the nonredundant nucleotide database of the National Center for Biotechnology Information.

The experimental procedures of the CRV-MS method are described briefly in Table S3 in the supplemental material. Primary PCR amplifications and iPLEX reactions were performed in 384-well plates using an S1000 thermal cycler (Bio-Rad, Hercules, CA, USA), according to a previous report (12).

Consensus PCR/RT-PCR assays. All target respiratory viruses were screened using consensus PCR/RT-PCR assays, according to previous reports (3, 4, 18–23). PCR was performed using the FastStart high-fidelity PCR system (Roche Molecular Systems, Inc., Pleasanton, CA, USA). RT-PCR was performed using the One-Step RT-PCR kit (Invitrogen). Amplified DNA was purified using a QIAquick gel extraction kit, according to the manufacturer's protocol (Qiagen, Valencia, CA, USA) and sequenced on an ABI3730 automated sequencer (Applied Biosciences, Foster City, CA, USA).

Statistical analysis. Differences between the detection rates of the two methods were tested using the McNemar's test. A P value of <0.01 was considered statistically significant.

RESULTS

The CRV-MS method. In this study, we developed a 24-plex analysis that targets 21 common respiratory viruses, using β-globin and RNase P as DNA and RNA extraction quality controls, respectively. The results of the BLASTn search showed that the CRV-MS method can detect 21 common respiratory viruses. For Flu-A virus, our new method can detect Flu-A H1N1, Flu-A H1N1pdm09, and Flu-A H3N2. To expand the detection spectrum, we also designed an assay that targets the Flu-A MP gene in the CRV-MS method, thereby enabling the detection of nearly all Flu-A viruses. For example, if the avian Flu-A H7N9 virus, which cannot be detected using the CRV-MS method, is present in a tested sample, we will obtain a Flu-A MP-positive result. Thus, we can confirm the results using the other method.

Performance of the CRV-MS method. We tested the specificity of the CRV-MS method using virus-specific plasmids. The results showed that the CRV-MS method can accurately identify 21 common respiratory viruses, and no positive results were obtained for the negative controls. The sensitivity test indicated that the detection limit of the CRV-MS method is approximately 10 to 100 DNA copies (data not shown).

We also evaluated the CRV-MS method using 102 samples that were confirmed positive for common respiratory viruses (Table 1). The results showed that all tested primers and probes worked well (see Fig. S1 to S5 in the supplemental material). For any tested respiratory virus, there was no cross-reactivity with other respiratory viruses.

Comparison with the consensus PCR/RT-PCR method. A total of 336 nasal and throat swabs were analyzed using the CRV-MS method, and the results were compared to those from the consensus PCR/RT-PCR analysis (Table 2). Four samples that were RNase P negative were excluded from further analysis.

The CRV-MS and consensus PCR/RT-PCR methods detected respiratory viruses in 92.5% (307/332) and 89.5% (297/332) of the samples, respectively. The respiratory virus detection rate differed significantly between the two methods ($P = 0.002$ by McNemar's test). All 297 positive samples detected by the consensus PCR/RT-PCR method were also detected by the CRV-MS method. Twenty-five samples tested negative by both methods. All of the 21 common respiratory viruses were detected. Multiple respiratory viruses were identified in 23.2% (77/332) and 20.8% (69/332) of

TABLE 2 Comparison of the results of CRV-MS and consensus PCR/RT-PCR[a]

ID[b]	CRV-MS		Consensus PCR/RT-PCR	
	No. of positive samples	%	No. of positive samples	%
AdV	54	16.3	49	14.8
Flu-A H1N1	8	2.4	8	2.4
Flu-A H1N1pdm09	8	2.4	8	2.4
Flu-A H3N2	13	3.9	13	3.9
Flu-B	6	1.8	8	2.4
HBoV1	33	9.9	31	9.3
HBoV2	2	0.6	2	0. 6
HCoV-229E	11	3.3	11	3.3
HCoV-NL63	9	2.7	9	2.7
HCoV-HKU1	9	2.7	9	2.7
HCoV-OC43	11	3.3	11	3.3
HEV	121	36.4	110	33.1
HMPV-A	7	2.1	7	0.2.1
HMPV-B	1	0.3	1	0.3
HRV	37	11.1	33	9.9
PIV1	13	3.9	13	3.9
PIV2	8	2.4	8	2.4
PIV3	15	4.5	14	4.2
PIV4	3	0.9	3	0. 9
RSV-A	17	5.1	17	5.1
RSV-B	16	4.8	14	4.2

[a] The total number of samples was 332.
[b] AdV, adenovirus; Flu-A, influenza virus A; Flu-B, influenza virus B; HBoV, human bocavirus; HCoV, human coronavirus; HEV, human enterovirus; HMPV, human metapneumovirus; HRV, human rhinovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

TABLE 3 Concordant multiple infections detected by two methods

Infection type[a]	No. of samples
Dual infections ($n = 51$)	
AdV/HBoV1	3
AdV/HEV	8
AdV/PIV2	1
AdV/PIV4	1
AdV/RSV-B	1
Flu-A H1N1pdm09/HCoV-NL63	1
Flu-A H3N2/HEV	1
Flu-A H3N2/HMPV-A	1
Flu-A H3N2/RSV-A	1
Flu-B/PIV1	1
Flu-B/RSV-B	1
HBoV1/HEV	2
HBoV1/PIV1	1
HCoV-229E/HEV	1
HCoV-229E/RSV-A	2
HCoV-229E/RSV-B	4
HCoV-HKU1/PIV3	2
HCoV-HKU1/RSV-A	1
HCoV-NL63/HRV	1
HCoV-NL63/PIV3	1
HCoV-OC43/RSV-A	3
HEV/HRV	10
HEV/HMPV-A	1
HRV/PIV2	1
HRV/RSV-B	1
Triple infections ($n = 12$)	
AdV/HBoV1/HEV	1
AdV/HEV/HRV	1
AdV/HRV/RSV-A	1
Flu-B/HEV/RSV-B	1
HBoV1/HEV/RSV-A	1
HCoV-HKU1/HRV/PIV3	1
HCoV-HKU1/HRV/RSV-B	2
HCoV-OC43/HRV/PIV1	1
HRV/HMPV-A/RSV-A	2
HRV/PIV3/RSV-A	1

[a] AdV, adenovirus; Flu-A, influenza virus A; Flu-B, influenza virus B; HBoV, human bocavirus; HCoV, human coronavirus; HEV, human enterovirus; HMPV, human metapneumovirus; HRV, human rhinovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

the samples by the CRV-MS and consensus PCR/RT-PCR methods, respectively. The multiple-respiratory virus detection rate did not differ significantly between the two methods ($P = 0.04$ by McNemar's test).

The two methods yielded identical positive results for 281 samples (84.6%), including 218 single-infection and 63 multiple-infection samples (Table 3). Ten discordant samples tested positive by the CRV-MS method and negative by the consensus PCR/RT-PCR method. The CRV-MS method identified eight of these samples as HEV and the other two as HRV and AdV. The results for 16 other discordant samples are shown in Table 4. The difference between the two methods may reflect their different sensitivities.

DISCUSSION

Thanks to the advances in multiplex PCR/RT-PCR technology, many good methods have been developed to detect respiratory viruses. The application of these methods demonstrated that multiplex PCR/RT-PCR is more sensitive and efficient in detecting a broad spectrum of respiratory viruses (2). For example, Lam et al. (24) developed five groups of multiplex nested-PCR assays that can detect 18 respiratory viruses and three kinds of bacteria. Bellau-Pujol et al. (18) designed three multiplex RT-PCR assays that can detect 12 respiratory viruses. These methods depend on agarose gel electrophoresis to differentiate amplification products corresponding to different pathogens. If multiple respiratory viruses are present in one sample, the sensitivity of these methods will be decreased.

The multiplex real-time PCR/RT-PCR assay is a good alternative to the conventional PCR/RT-PCR assay, as it can increase the specificity and sensitivity of respiratory virus detection (25–27). However, the ability of multiplex real-time PCR/RT-PCR to identify multiple respiratory viruses in one reaction is limited to the number of fluorescent dyes that can be used. Based on liquid chip technology, several commercial methods for multiple respiratory virus detection have been developed, including xTAG (Luminex), MultiCode-PLx (EraGen Biosciences), and ResPlex II panel version 2.0 (Qiagen). Balada-Llasat et al. (28) performed an evaluation study that showed that these methods work better than traditional methods. Some other multiplex methods have also been developed (5, 6, 29, 30).

Several novel common respiratory viruses have been identified over the past 15 years, including HCoV-NL63, HCoV-HKU1, and HBoV, which have been associated with respiratory infections (22, 31, 32). We need to update our routine detection method to deal with newly discovered respiratory viruses. In this study, we devel-

TABLE 4 Discordant results of two methods

Sample	CRV-MS result[a]	Consensus PCR/RT-PCR result
S1	AdV	−
S2	HRV	−
S3	HEV	−
S4	HEV	−
S5	HEV	−
S6	HEV	−
S7	HEV	−
S8	HEV	−
S9	HEV	−
S10	HEV	−
S11	HCoV-229E/HRV/PIV3/HEV	HCoV-229E/HRV/PIV3
S12	HCoV-229E/RSV-A/HEV	HCoV-229E/RSV-A
S13	AdV/HBoV1	AdV
S14	HEV/HBoV1	HEV
S15	HEV	HEV/Flu-B
S16	HBoV1/AdV	HBoV1
S17	HCoV-HKU1/PIV3	HCoV-HKU1
S18	HCoV-HKU1	HCoV-HKU1/Flu-B
S19	HCoV-NL63/AdV	HCoV-NL63
S20	HCoV-OC43/RSV-A/HRV	HCoV-OC43/RSV-A
S21	HCoV-OC43/RSV-B	HCoV-OC43
S22	PIV1/HEV	PIV1
S23	PIV1/HRV/RSV-B	PIV1/HRV
S24	RSV-A/AdV/HRV	RSV-A
S25	RSV-B/HRV	RSV-B
S26	RSV-B/AdV	RSV-B

[a] AdV, adenovirus; Flu-B, influenza virus B; HBoV, human bocavirus; HCoV, human coronavirus; HEV, human enterovirus; HRV, human rhinovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

oped a 24-plex assay that can detect and identify 21 common respiratory viruses simultaneously in one reaction, which can help us to delineate the clinical role played by these respiratory viruses. Therefore, the CRV-MS method can be a useful alternative for those samples that are suspected to contain multiple respiratory viruses.

Respiratory virus coinfections have been frequently detected in clinical samples (33–41). Pretorius et al. (8) found that 17% of 8,173 patients with a severe acute respiratory illness in South Africa had respiratory virus coinfections. Several studies indicated that respiratory virus coinfections were associated with increased disease severity, such as HMPV-RSV (39, 40), RSV-HCoV (42), RSV-influenza virus, RSV-HRV, and PIV-HRV (43). Two studies indicated that coinfection with avian and human viral strains is a potential source for influenza virus reassortment (41, 44). Kouni and colleagues (43) also indicated that respiratory virus coinfections may increase the risk of hospitalization. In this study, we found that 19.0% of patients had respiratory coinfections, including HEV-HRV and RSV-HCoV coinfections.

Several important respiratory viruses have been identified in recent years, including severe acute respiratory syndrome coronavirus, Middle East respiratory syndrome coronavirus, avian Flu-A H5N1, H7N9, H10N8, etc. However, these viruses are not common in humans (45–49). We will develop specific methods that can be used to complement the CRV-MS method designed in this study to target these viruses in the future.

Compared to other multiplex methods, the CRV-MS method demonstrates high throughput. Following nucleic acid extraction and RT-PCR for RNA respiratory viruses, one technologist in a clinical laboratory can use the CRV-MS method to analyze two 384-format plates, which are sufficient for 760 clinical specimens, within one working day. Moreover, the results can be automatically determined by the software. However, the turnaround time of CRV-MS method is longer than FilmArray and ResPlex II. The CRV-MS method does not employ fluorescent dyes, which will help to improve repeatability and stability. Because of the high degree of multiplexing, the cost for analyzing all 21 common respiratory viruses using the CRV-MS method is <$4, excluding the cost of nucleic acid extraction and RT-PCR for RNA viruses. Thus, the CRV-MS method can be used in large-scale epidemiological studies of common respiratory virus infections, and it can provide more comprehensive information for disease control and vaccine research.

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