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Original Article

Comparison of two multiplex PCR tests for common pathogen detection in hospitalized children with acute respiratory infection

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Abstract

Introduction: Multiplex PCR methods have significantly improved the diagnosis of acute respiratory tract infections (ARTIs) in children. The ResP-CE System coupled with capillary electrophoresis is a highly specialized, automated, and expensive technology for detecting common pathogens in ARTIs. The XYRes-MCA System, a remarkably less expensive multiplex PCR instrument, employs hybridization for the detection of ARTI pathogens. Both methods detect 9 common microorganisms in ARTIs, i.e., RSV, FLUAV, FLUBV, ADV, PIV, HMPV, HBOV, HCOV, and MP. In this study, we aimed to compare the performance of these two methods in the detection of pathogens from sputum specimens collected from children with ARTIs.

Methodology: Sputum specimens were collected from 237 hospitalized children with ARTIs. Nucleic acid was extracted on an automated workstation. The ResP-CE and XYres-MCA systems were applied to detect pathogens from the samples, and the test result agreement between the two methods was evaluated using Kappa statistics.

Results: The ResP-CE and XYres-MCA identified pathogens, single or in combination, in 151 (63.7%) and 171 (72.1%) of 237 samples, respectively. Approximately 85% of positive samples identified by either method contained a single pathogen. Moderate to almost perfect concordance between the two methods was found in detecting the following 7 pathogens: RSV, FLUAV, FLUBV, PIV, HMPV, HBOV, and MP.

Conclusions: These two methods are comparable in detecting common pathogens of ARTIs in children. As XYres-MCA analysis is more cost-effective, it could play an important role in diagnosing ARTIs in children in less economically developed regions.

Key words: Pediatric acute respiratory infections; pathogens; multiplex PCR; virus.

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Introduction

Acute respiratory tract infections (ARTIs) are the leading cause of morbidity and mortality in children worldwide [1,2]. It has been shown that viruses are responsible for the majority of ARTIs in hospitalized children in both developing and developed countries [3-6]. The frequently detected viruses in ARTIs include respiratory syncytial virus (RSV), influenza virus types A and B (FLUAV and FLUBV), adenovirus (ADV), parainfluenza virus (PIV), humanmetapneumovirus (HMPV), human bocavirus (HBOV), and human coronavirus (HCOV) [7-9]. In China, Mycoplasma pneumoniae (MP) is also a common cause of pediatric ARTIs [10-12].

Multiplex PCR methods that detect multiple pathogens simultaneously have significantly improved the diagnosis of pediatric ARTIs, allowing timely and effective treatment, and avoiding unnecessary use of antibiotics [13-16]. The ResP-CE System (Health Gene Technologies, Ningbo, China) is a highly specialized, automated platform for the detection of multiple common pathogens of pediatric ARTIs, where genomic nucleotides of pathogens are amplified, and the PCR products are then differentiated by a built-in capillary electrophoresis (CE) instrument [17]. The XYres-MCA System (Geneworks Biotechnology, Jiangsu, China) is another multiplex PCR-based equipment developed to detect etiological agents of pediatric ARTIs. This method employs melting curve analysis (MCA) for the detection of pathogens: the PCR products are hybridized with different fluorescence-labeled probes and discriminated by the different fluorescence dyes appearing at different melting temperatures. Compared with the ResP-CE System, the XYres-MCA System is significantly less expensive, making it an affordable and valuable diagnostic tool in less economically developed regions. Both the ResP-CE and XYres-MCA methods detect the following nine pathogens: RSV,

FLUAV and FLUBV, ADV, PIV, HMPV, HBOV, HCOV, and MP. In this study, we aimed to compare the performance of these two methods in the detection of pathogens from sputum specimens collected from children with ARTIs.

Methodology

Ethics Statement

This study was approved by the Ethics Committee of Children's Hospital of Hebei Province, in compliance with the principles of the Declaration of Helsinki, the Code of Ethics of the World Medical Association.

Patients and specimen collection

Children diagnosed with ARTI and hospitalized during April-September of 2019 were enrolled in this study. Sputum specimens were collected into a tube containing a viral transportation medium (VTM, Hopebio Technologies, Qingdao, China) following a protocol established at our hospital. Immediately after collection, the samples were placed into a 4 °C refrigerator and stored for a maximum of 48 hours before analysis.

Nucleic acid extraction

A total of 200 μL of liquefied sputum was used for nucleic acid extraction. The sputum sample was gently mixed by repetitive pipetting, and nucleic acid extraction was then completed on an automated workstation (Smart LabAssist-16/32, Health Gene Technologies, Ningbo, China) [17]. Nucleic acid from each sample was eluted with 30 μL DNase- and RNase-free water.

ResP-CE and XYres-MCA assays

ResP-CE analysis was performed as previously described [17]. For XYres-MCA analysis, a kit from the manufacturer (Geneworks Biotechnology) was used for nucleotide amplification followed by MCA analysis according to the manufacturer's instructions. Probes were labeled with FAM, ROX and Cy5. The RT-PCR was completed on a thermocycler (Veriti Thermal Cycler, Applied Biosystems China, Beijing, China) as follows: 5 μL RNA/DNA extraction was mixed with 4.3 μL Res-reaction Mixture 1 and 0.7 μL Res-enzyme 1, and the thermal protocol was 25 °C for 10 minutes and 50 °C for 30 minutes followed by 95 °C for 15 minutes, 40 cycles of (95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds), and 72 °C for 10 minutes. Subsequently, MCA analysis was done by mixing 10 μL of the RT-PCR product 39.8 μL of Res-reaction mixture 2, and 0.2 μL Res-enzyme 2. The mixture was denatured at 95 °C for 2 minutes, and hybridization was done at 40 °C for 90 seconds followed by a stepwise temperature increase (1 °C per 20 seconds) from 40 °C to 90 °C. Fluorescent FAM, ROX, and Cy5 were observed and recorded for the differentiation of different pathogens.

Table 1. Patient diagnosis.

Diagnosis	n (%)
Pneumonia	148 (62.4)
Bronchitis/capillary bronchitis	52 (21.9)
Common cold	16 (6.8)
Otitis media	12 (5.1)
Laryngitis/tonsillitis	8 (3.4)
Pertussis syndrome	1 (0.4)

Validation of discordant results

Viruses in samples detected by the two methods with low consistency were further validated by single PCR or DNA sequencing. A real-time PCR detection kit (Puruikang Biotechnology, Shenzhen, China) was used for ADV detection. The amplification and data analysis was completed on a ABI 7500 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For HCOV validation, reverse transcription (RT) reaction was carried out with 1 μL of eluted RNA using a OneStep RT-PCR Kit (Qiagen China Co., Ltd., Shanghai, China) according to the protocol from Qiagen. Subsequently, routine PCR was done using 5 μL RT product with 40 cycles of (95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds). The PCR product was purified using a PCR product purification kit from Qiagen following the manufacturer's instructions and sequenced using the Sanger sequencing method.

Statistical analyses

The McNemar's test is used to compare the overall positive rates of pathogens between XYres-MCA and ResP-CE. The test result agreement between the two methods was evaluated using Kappa statistics (κ value 0.21-0.4 fair, 0.41-0.6 moderate, 0.61-0.8 substantial, and 0.81-1 almost perfect). Analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, USA). A p value < 0.05 was considered statistically significant.

Results

Patients and samples

Sputum specimens were collected from a total of 237 children (143 boys and 94 girls) with a median age of 1.8 years (interquartile range 2.5 years). Of these

patients, the vast majority had pneumonia and bronchitis/capillary bronchitis, with 148 (62.4%) cases of pneumonia and 52 (21.9%) cases of bronchitis/capillary bronchitis (Table 1).

Pathogens detected by the two methods

The ResP-CE and XYres-MCA detected pathogens, single or in combination, in 151 (63.7%) and 171 (72.1%) of the 237 samples, respectively ($p = 0.002$ by McNemar's test). Table 2 and Table 3 show the number of samples with single and double pathogens detected by the methods, respectively. One sample had triple pathogens (PIV/FLUAV/FLUBV) detected by both methods, while the other triple pathogen-positive sample (RSV/PIV/FLUBV) was identified only by the MAC System. Of the 151 samples detected positive by the ResP-CE method, 128 (84.8%) contained a single pathogen, and of the 171 samples shown positive by the XYres-MCA method, 141 (82.5%) had a single pathogen. This data indicated that the vast majority of positive samples contained a single microorganism. A total of 115 samples were detected positive by both methods, including 102 with a single pathogen, 12 with double and 1 with triple pathogens.

The detection agreement between the two methods

The detection agreement between the two methods was analyzed by the Kappa test and the results are shown in Table 4. There was moderate to almost perfect consistency between the two methods for the detection of 7 pathogens, while for ADV and HCOV, the detection agreement was low.

Validation results

As the lowest detection agreement was observed for ADV and HCOV between ResP-CE and XYres-MCA, single PCR or DNA sequencing was further performed to validate the discordant results. Of the 14 samples that tested positive for HCOV by XYres-MCA but negative by ResP-CE (Table 4), 12 (2 samples shown positive by

Table 2. Single pathogen detected by the two methods.

Pathogen	CE (+), n	MCA (+), n	CE (+) but MCA (-), n	MCA (+) but CE (-), n
FLUAV	8	6	3	1
FLUBV	29	36	0	7
HMPV	6	5	1	0
HBOV	1	2	0	1
ADV	8	6	5	3
RSV	12	11	1	0
MP	13	9	6	2
PIV	48	58	9	19
HCOV	3	8	1	6

(+) and (-) represent testing positive and negative, respectively. FLUAV: influenza A virus; FLUBV: influenza B virus; HMPV: human metapneumovirus; HBOV: human bocavirus; ADV: adenovirus; RSV: respiratory syncytial virus; MP: Mycoplasma pneumoniae; PIV: parainfluenza virus; HCOV: human coronavirus.

Table 3. Double pathogens detected by the two methods.

Pathogens	CE (+), n	MCA (+), n	CE (+) but MCA (-), n	MCA (+) but CE (-), n
ADV/FLUBV	2	0	2	0
ADV/FLUAV	1	1	0	0
ADV/MP	1	0	1	0
ADV/PIV	4	2	3	1
ADV/RSV	1	1	1	1
FLUAV/FLUBV	1	0	1	0
FLUBV/MP	2	2	0	0
MP/PIV	3	7	0	4
PIV/HCOV	3	4	1	2
PIV/FLUBV	2	2	1	1
PIV/RSV	2	3	0	1
RSV/HCOV	0	1	0	1
MP/HCOV	0	2	0	2
ADV/HCOV	0	1	0	1
HMPV/HCOV	0	1	0	1
FLUAV/HCOV	0	1	0	1

(+) and (-) represent testing positive and negative, respectively. ADV: adenovirus; FLUBV: influenza B virus; FLUAV: influenza A virus; MP: Mycoplasma pneumoniae; PIV: parainfluenza virus; RSV: respiratory syncytial virus; HCOV: human coronavirus; HMPV: human metapneumovirus.

XYres-MCA did not have enough RNA for further testing) were analyzed by DNA sequencing which showed that HCOV was identified in 8 samples. Two samples positive for HCOV by ResP-CE but negative by XYres-MCA were positive as shown by DNA sequencing. Of the 12 samples that tested positive for ADV by ResP-CE but negative by XYres-MCA (Table 4), 9 tested positive by single PCR. Of the 6 samples that tested positive for ADV by XYres-MCA but negative by ResP-CE, all were positive shown by single PCR.

Discussion

Multiplex PCR methods are recommended as first-line tests for the detection of respiratory pathogens [18], due to their advantages over conventional singleplex assays, which include automation, cost-effectiveness, high repeatability, and high sensitivity. The multiplex-PCR kits vary from manufacturer to manufacturer in the assay principles and the scope of pathogens tested [19,20]. In China, the ResP-CE System is the only platform that has been used in clinical diagnosis. This system costs several millions of Chinese Yuan. Another multiplex PCR-based diagnostic tool for ARTIs, namely, the XYres-MCA system, is pending the approval of the National Medical Products Administration (NMPA) of China. The MCA technology only requires a PCR instrument with four fluorescent channels, representing a cost-effective alternative to the ResP-CE System for less economically developed regions. In the present study, we found that the XYres-MCA System is not inferior to the CE System in detecting 9 common pathogens in samples collected from children with ARTIs.

The MCA method identified a significantly higher number of positive samples than the CE method. The reason, we speculated, is that the XYres-MCA assay contains only one internal control (IC) for detection of 12 pathogens, fewer than one IC, one human DNA and one human RNA sequence control for the detection of 13 pathogens in the CE system. Fewer controls and targets in one reaction result in less competition for primers, nucleotides or enzymes, which may lead to increased sensitivity [20, 21].

We observed fair agreement between the two methods in the detection of ADV and HCOV, with the CE and MCA method identifying more ADV and HCOV-positive samples, respectively. It has been shown that the sensitivity varies between a test designed for detecting the entire family of virus without differentiation of subtypes and a test specifically picking up every single subtype, with the latter being more sensitive [20]. As the MCA method detects each subtype of HCOV while the CE method does not, this may be the reason that more HCOV-positive samples were identified by the MCA System. With regard to the discrepancy in ADV positivity, the reason remains unknown.

The XYres-MCA System employs fluorophore and the melting temperature for the differentiation of pathogens. It has the following advantages: flexibility in probe design and across-platform compatibility; the assay can be readily modified to accommodate more probes to detect new viruses or their subtypes.

Conclusions

In conclusion, this is the first comparison of the XYres-MCA and ResP-CE methods in detecting 9 common ARTI pathogens in samples from children with ARTIs. Moderate to almost perfect concordance between the XYres-MCA and ResP-CE systems was found in the detection of 7 pathogens, suggesting comparable performance of these two methods. As XYres-MCA analysis is more cost-effective, it could play an important role in diagnosing ARTIs in children, especially in less economically developed regions.

Table 4. Testing agreement between the two methods.

Pathogen	CE+ (n)	MCA+ (n)	CE+ but MCA- (n)	MCA+ but CE- (n)	Kappa value
FLUAV	11	9	4	2	0.687
FLUBV	35	40	4	9	0.794
HMPV	6	5	1	0	0.907
HBOV	1	2	0	1	0.665
ADV	17	11	12	6	0.319
RSV	15	17	2	4	0.799
MP	19	20	7	8	0.581
PIV	63	78	14	29	0.568
HCOV	6	18	2	14	0.307

(+) and (-) represent testing positive and negative, respectively. Comparison was done for every pathogen detected in single- and co-infection. FLUAV: influenza A virus; FLUBV: influenza B virus; HMPV: human metapneumovirus; HBOV: human bocavirus; ADV: adenovirus; RSV: respiratory syncytial virus; MP: Mycoplasma pneumoniae; PIV: parainfluenza virus; HCOV: human coronavirus.

Author Contributions

Conceptualization: Le Wang; Methodology: Le Wang, Shuo Yang, Yinghui Guo; Data curation: Fang Chen, Xianping Zeng, Weijian Wang; Formal analysis: Le Wang, Suzhen Sun, Dianping You, Shuo Yang; Writing-original draft: Le Wang; Writing-review & editing: Le Wang, Shuo Yang. All authors have read and agreed to the final version of the manuscript for submission.

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Institutional Review Board Statement

This study was approved by the Ethics Committee of Children's Hospital of Hebei Province (approval number: 2017016), in compliance with the principles of the Declaration of Helsinki, the Code of Ethics of the World Medical Association.

Informed Consent Statement

As all specimens were allocated from those used for clinical diagnosis, and this study presents no risk of harm to subjects, and no privacy of individuals was exposed, informed consent was waived.

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