Evaluation of two commercial multiplex PCR tests for the diagnosis of acute respiratory infections in hospitalized children

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Abstract

Background

Acute respiratory tract infections (ARTI), including the common cold, pharyngitis, sinusitis, otitis media, tonsillitis, bronchiolitis and pneumonia are the most common diagnoses among pediatric patients and account for the majority of antibiotic prescriptions. A clear and rapid diagnosis is the key to preventing antibiotic abuse. Recently, based on different detection principles, many multi-target molecular analyses that can simultaneously detect dozens of pathogens have been developed, thereby greatly improving sensitivity and shortening turnaround time. In this work, we conducted a head-to-head comparative study between melting curve analysis (MCA) and capillary electrophoresis assay (CE) in the detection of nine respiratory pathogens in sputum samples collected from hospitalized ARTI children.

Methods

Through MCA and CE analysis, nine common respiratory pathogens were tested on hospitalized children under the age of 13 who met the ARTI criteria.

Results

A total of 237 children with sputum specimens were tested. For all the targets combined, the positive detection rate of XYRes-MCA was significantly higher than ResP-CE (72.2% vs. 63.7%, $p = .002$). Some pathogens were detected more often with MCA, such as parainfluenza virus, influenza B and coronavirus, and some pathogens do the opposite, such as adenovirus and influenza A (all $p < .01$). Very good kappa values for most of pathogens were observed, except for Influenza B and coronavirus (both $\kappa = .39$).

Conclusions

Multiplex melting curve and capillary electrophoresis assays performed similarly for the detection of common respiratory pathogens in hospitalized children, except for Influenza B and coronavirus. Higher sensitivity was observed in the melting curve assay. By using this sensitive and rapid test, it may improve patient prognosis and antimicrobial management.

Background

Acute respiratory tract infections (ARTI) is acknowledged as one of the greatest threats to pediatric health [1, 2]. Nearly 1.9 million children died from ARTI, most of them from developing country [3]. Accurate early etiologic diagnosis is crucial to the outcome of ARTI [4]. Multiplexed PCR methods enable high-throughput pathogen detection in a short time using limited amount of samples, and this detection strategy may have a significant impact on the management of infectious diseases [5–7]. Our previous

research report pointed out that multiplex-PCR assays applied on an automated capillary electrophoresis system can simultaneously detect human rhinovirus (HRV), influenza A (FluA), FluA-H1N1, FluA-H3N2, influenza B (FluB), adenovirus (ADV), human Bocavirus (HBoV), human metapneumovirus (HMPV), parainfluenza virus (HPIV), human coronavirus (HCoV), respiratory syncytial virus (RSV), *M. pneumoniae* and *Chlamydia*, all in one reaction [8, 9]. However, many clinical laboratories, especially lab in the primary care, are not equipped with such highly specialized laboratory equipment, which limits their widespread use in developing countries like China.

In this regard, melting curve analysis (MCA) was developed based on multicolor probe-based fluorescence. T_m is a highly reproducible physical parameter, combining it with fluorophore signature, MCA assay is feasible identifying certain pathogenic bacteria or viruses [10]. This simple measurement is performed in a closed tube, reducing the possibility of contamination. It takes about 50 minutes to complete, ensuring fast turn-around time. Recently, a new kit based on MCA analysis has been commercialized and can detect FluA, FluB, ADV, HBoV, HMPV, HPIV-1, HPIV-2, HPIV-3, HCoV-229E, HCoV-OC43, RSV and *M. pneumoniae*. To our knowledge, no studies have been conducted to evaluate the clinical performance of this new method [11–13].

This study describes the first evaluation of the clinical performance of the XYres-MCA kit, compared against the ResP-CE method in a head-to-head manner using 237 sputum specimens from children with ARTI.

Material And Methods

Ethics Statement

All sputum samples were collected for their original testing purposes and provided to the study without any patient identity. All aspects of the study were conducted according to the Institutional Review Boards of Children's Hospital of Hebei Province.

Study population and specimen collection

Between April to September 2019, patients who had developed ARTI signs and symptoms were enrolled in this study. Patients were asked to cough, and the expectorated sputum was collected. If the child is too young to cough, a sterile negative pressure suction catheter can be used to obtain the oropharyngeal suction into a transport tube containing VTM (Hopebio Technologies, Qingdao, China). On the same day, the samples were stored at 4 °C for nucleic acid extraction. Before extraction, a pipette tip was used to gently mix the sputum and VTM to liquefy it.

Nucleic acid extraction

DNA/RNA extraction is performed on an automated workstation (Smart LabAssist-16/32, Health Gene Technologies, China) [14]. A total of 200μL of liquefied sputum was used for nucleic acid extraction and eluted in 30μL, which was divided into two parts for ResP-CE and XYres-MCA analysis, respectively.

XYres-MCA and ResP-CE assays

The XYres-MCA kit (Geneworks Biotechnology, JiangSu, China) was used for amplification and MCA analysis. Probes are labeled with FAM, ROX and Cy5. MCA was performed after PCR amplification; thus, each hybrid will show unique characteristics through the combination of T_m and its corresponding fluorescent probes. The RT-PCR was completed on a thermocycler (Veriti Thermal Cycler, Applied Biosystems China, Beijing, China). Step 1: RT-PCR amplification. Mix 5 μ L RNA/DNA extraction with 4.3 μ L Res-reaction mixture¹ and 0.7 μ L Res-enzyme¹. The thermal profile was 10 min at 25 °C; 30 min at 50 °C; 15 min at 95 °C; 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C; 10 min at 72 °C. Step 2: PCR Amplification and MCA. 10 μ L of the RT-PCR product was mixed with 40 μ L PCR-MCA mixture, containing 39.8 μ L Res-reaction mixture² and 0.2 μ L Res-enzyme². The thermal profile was 2 min at 95 °C; 10 cycles of 15 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C; 30 cycles of 15 s at 94 °C, 15 s at 55 °C, 15 s at 72 °C; and then, the MCA began with denaturation for 2 min at 95 °C, hybridization for 90 s at 40 °C, and a stepwise temperature increase (1 °C per 20 s) from 40 °C to 90 °C. The levels of FAM, ROX, and Cy5 fluorescence were collected and recorded during the MCA procedure.

ResP-CE analysis has been described in previous work, and we performed the experiments according to previously reported methods [14]. Table 1 illustrates the different detection targets of XYres-MCA and ResP-CE. 1) Targets not detected by the MCA assay include H1 and H3 influenza A, human rhinovirus and *Chlamydia*. 2) CE analysis can detect 1/2/3/4 subtypes of parainfluenza virus but cannot distinguish them. MCA assay can detect and distinguish 1/2/3. 3) CE can detect 229E/OC43/HKU1/NL63 subtypes of human coronavirus but cannot distinguish them. MCA can detect and distinguish between 229E and OC43.

Table 1
The spectrum of tested agents.

	XYres-MCA	ResP-CE
FluA	√	√
FluA-H1N1		√
FluA-H3N2		√
FluB	√	√
HPIV	√	√ ^a
HPIV1	√	
HPIV2	√	
HPIV3	√	
HMPV	√	√
HBoV	√	√
ADV	√	√
HCoV	√	√ ^b
HCoV-229E	√	
HCoV-OC43	√	
RSV	√	√
MP	√	√
Ch		√
HRV		√
FluA, influenza A virus; FluB, Influenza B virus; HPIV, human parainfluenza virus; HMPV, human metapneumovirus; HBoV, human bocavirus; ADV, adenovirus; HCoV, human coronavirus; RSV, respiratory syncytial virus; MP, <i>Mycoplasma pneumoniae</i> ; Ch, <i>Chlamydia</i> ; HRV, human rhinovirus.		
^a HPIV include 1–4 subtypes of HPIV.		
^b HCoV include hCoV 229E, OC43, NL63 and HKU1		

Statistical analyses

The McNemar's test is used to compare the overall positive rate of pathogens between XYres-MCA and ResP-CE. The consistency 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) [15]. Analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, USA). If $p < .05$, it is statistically significant.

Results

Patient demographics and diagnosis

A total of 237 patients were included in this study, and their sputum specimens were collected. The main diagnosis was pneumonia (62.4%, Table 2). Participants were more likely to be male (143; 60.3%) with a median age of 1.8 years (interquartile range 0.5-3.0 years).

Table 2
Number of ARTI children enrolled by diagnosis.

Diagnosis	Number	Percentage
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%

Summary of XYres-MCA findings

XYres-MCA detected one or more pathogens in 171 (72.1%) of the 237 test samples (Table 3), with a total of 203 positive results (Table 4).

Table 3
Total number of XYres-MCA positive samples according to number of detected pathogens per sample.

Detected pathogens by XYres-MCA	Numbers of samples	% of total samples (n = 237)
> 0	171	72.1%
1	141	59.5%
2	28	11.8%
3	2	0.8%

Table 4
Number of XYres-MCA detected pathogens allocated by co-detection and age.

Pathogen	Total number	Detection in co-infection	Co-detection rate (%)	< 1 year	1–3 years	3–5 years	> 5 years	<i>P</i>
HPIV	78	20	25.6	48	24	3	3	0.009
FluB	42	6	14.3	11	17	7	7	0.003
<i>MP</i>	20	11	55.0	7	4	3	6	0.031
ADV	11	6	54.5	8	2	0	1	0.279
RSV	17	6	35.3	15	2	0	0	0.004
HCoV	18	10	55.6	10	7	0	1	0.157
FluA	9	3	33.3	4	2	2	1	0.778
HMPV	6	1	16.7	4	1	1	0	0.597
HBoV	2	0	0.0	1	1	0	0	0.780
Total	203	63		108	60	16	19	

Multiple pathogens were detected in 17.5% (30/171) of positive samples, and three types of pathogens (RSV/HPIV3/FluB, HPIV3/FluB/FluA) could be detected in one specimen. Most mixed infections contain two types of pathogens, which is the case in 28/30 samples (93.3%).

Table 4 shows the number of each pathogen detected by XYres-MCA according to co-infections, diagnosis and age. The most frequently detected pathogen was human parainfluenza virus, which was detected in 78 samples, of which 48 (61.5%) were from infants under 1 year old. In addition, the positive detection rates of influenza B virus, RSV and *M. pneumoniae* were also related with age.

Performance comparison of XYres-MCA and ResP-CE

Overall, the positive detection rate of XYRes-MCA was significantly higher than ResP-CE (72.2% vs. 63.7%, Table 5, $p = .002$).

Table 5
Total positive detection rates between XYres-MCA and ResP-CE analyses.

		No. (%) of ResP-CE result	
		Positive	Negative
No. (%) of XYres-MCA result	Positive	142 (60.0)	29 (12.2)
	Negative	9 (3.8)	57 (24.0)
<i>P</i> = 0.002 by McNemar's test.			

XYRes-MCA and ResP-CE agreed on the detection of 114 pathogens in the 237 samples (Table 6). Though either assay, at least one pathogen was detected in 124 cases (52.3%): 101 with one pathogen and 13 with two or three pathogens. There were 57 (24.1%) ARTIs with no pathogens detected by either assay. In consistent cases, HPIV was the most common single virus (16.9%). Of the 66 (27.8%) inconsistent cases, 7 (3%) were completely inconsistent, and 59 (24.9%) were partially inconsistent. Among the co-infected cases, most differences were MCA positive / CE negative (17.3% vs 7.2%) (Table 6).

Table 6
Performance comparison between XYres-MCA and ResP-CE according to per sample.

Items	Number	Percentage
Concordant specimens	171	72.2%
No pathogen detected	57	24.1%
Single virus detected	101	42.6%
HPIV	40	16.9%
FluB	29	12.2%
RSV	9	3.8%
<i>MP</i>	7	3.0%
HMPV	5	2.1%
FluA	5	2.1%
ADV	3	1.3%
HCoV	2	0.8%
HBoV	1	0.4%
two or three pathogens detected	13	5.5%
Discordant specimens	66	27.8%
completely inconsistent	7	3.0%
partially consistent: more types of pathogens by MCA	41	17.3%
partially consistent: more types of pathogens by CE	17	7.2%
partially consistent: 1 pathogen inconsistent, others are consistent	1	0.4%

The performance for individual targets is presented in Table 7. For most organisms, the agreement between XYRes-MCA and ResP-CE was excellent ($\kappa > 0.61$). However, for certain organisms, including FluB and HCoV, XYRes-MCA results seem to be more sensitive than ResP-CE, as the positive detection rate of FluB was 100% vs. 88%, HCoV was 95% vs. 32%.

Table 7
Detection sensitivity of XYres-MCA versus ResP-CE among individual targets.

Pathogens	Positive cases	XYres-MCA (%)	ResP-CE (%)	kappa value	<i>P</i> value
HPIV	81	78 (96)	64 (79)	0.800	< .001
FluB	42	42 (100)	37 (88)	0.394	< .001
<i>MP</i>	26	20 (77)	19 (73)	0.637	< .001
ADV	22	11 (50)	17 (77)	0.924	< .001
RSV	19	17 (89)	16 (84)	0.837	< .001
HCoV	19	18 (95)	6 (32)	0.394	< .001
FluA	12	9 (75)	11 (92)	0.791	< .001
HMPV	7	6 (86)	6 (86)	0.829	< .001
HBoV	2	2 (100)	1 (50)	0.665	< .001

Discussion

In China, the abuse of antibiotics is recognized as one of the greatest threats to human health, and it is also the major contributor to rising healthcare costs [16]. Due to the clinical similarity between bacterial and viral symptoms, rapid and comprehensive pathogen diagnostic tests should be used to prevent empirical or unnecessary antimicrobial treatment [17]. This study demonstrates for the first time the clinical performance of the new XYres-MCA analysis in detection of 12 types/subtypes respiratory pathogens. Compared with a CFDA approval multiplex-PCR assay, XYres-MCA can detect a higher percentage of positive results. Except for coronavirus and influenza B virus, the two were very consistent for all pathogens (κ value > 0.61),

An increasing number of recently published studies focus on the development of molecular methods to solve multiple detection in one reaction [5, 13, 14, 18, 19]. The multiplex-PCR methods has been previously evaluated versus conventional techniques [1, 6, 8, 20] or single-plex PCR [21]. Due to their advantages of automation, high repeatability, cost-effectiveness and high sensitivity, the use of multiplex PCR are recommended as first-line tests for detection of respiratory pathogens [7]. The comparative study is worthy of performed as the multiplex-PCR kits vary widely from a manufactory to another, on the scope of tested pathogens, detection principles and the equipment used [6, 22–24]. The CFDA has approved only one multiplex PCR kit, which requires highly specialized equipment worth millions of RMB, namely an automated capillary electrophoresis system, which exceeds the limit of purchasing equipment in most primary hospitals. MCA technology only requires a PCR instrument with four fluorescent channels. It is hoped that by comparative research data and low equipment price, it is possible to promote the application of multiple PCR technologies in primary hospitals.

An important aspect of this study is the observation that a higher clinical sensitivity of the MCA in detecting viral infections in clinical samples than CE. The reason for the inconsistent results of influenza B virus is unclear, but it may be explained by different target gene regions of the virus. In addition, the following two observations may explain why XYres-MCA showed advanced detection of parainfluenza virus and coronavirus, whereas a drop in sensitivity for the detection of Influenza A virus. First, when the test is applicable to the entire family or specific to a single type, the sensitivity may vary [25]. In this study, the subtypes of parainfluenza and coronavirus can be distinguished by XYres-MCA, while the subtypes of influenza A can be distinguished by ResP-CE. Second, 12 pathogens and one internal control (IC) were set in the same reaction in XYres-MCA, while CE detected 13 pathogens, one IC, one human DNA and one human RNA sequences. There are fewer targets in one reaction, resulting in less competition for primers, nucleotides or enzymes, which may lead to increased sensitivity [25, 26]. In addition, by using fluorophore and T_m value as dual labels, XYres-MCA has the following advantages: it can increase flexibility of probe design and can expand cross-platform compatibility, and if required, the assay can be changed to accommodate more probes to detect new viruses or their subtypes.

In terms of co-detection, the core question is how to interpret it in a clinical sense. Though molecular methods, a positive result may indicate the true pathogen causing the disease or a harmless colonizer. It is well known that most common respiratory pathogens can exist in asymptomatic carriers [27], and high co-detection rates have been observed in our previous work and other studies using PCR methods [18, 28]. In the routine clinical settings, children with mixed infections of two or more respiratory viruses are common due to the undeveloped immune system, but conventional methods are not easy to find. Whether the viral co-infection will cause the illness to become more serious is still controversial [29, 30]. Therefore, results of additional detected viruses must be interpreted with caution, and their clinical relevance needs to be correlated in further studies.

Limitations

Our research has several limitations. First, due to budget restrictions, the study lasted 6 months. This may weaken the ability to capture epidemic pathogens during seasons. Second, as several pathogens (eg, Rhinovirus, *Chlamydia*) were not included in the target of XYres-MCA, so the performance of XYres-MCA assay to detect these pathogens cannot be determined. Third, compared to ResP-CE, the MCA panel does not contain a human DNA or RNA for monitoring the quality of respiratory specimens.

Conclusions

In conclusion, in this first clinical study of the new XYres-MCA analysis of 237 samples, we observed excellent sensitivity of this assay compared to another multiplex-PCR assay based on capillary electrophoresis. XYres-MCA analysis may be a new tool for respiratory virus testing in clinical laboratories and may have a positive impact on antimicrobial stewardship and isolation facilities. The flexibility of MCA technology in primer design and the characteristics that do not require highly specialized laboratory equipment are expected to play an active role in the emergence of new viruses.

Abbreviation

HRV, human rhinovirus; RSV, respiratory syncytial virus; FluA, influenza A virus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; FluB, Influenza B virus; ADV, adenovirus; MP, *Mycoplasma pneumoniae*; HCoV, human coronavirus; HBoV, human bocavirus; Ch, *Chlamydia*.

Declarations

Funding

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the [Figshare] repository, [<https://figshare.com/articles/MCAvsCE/12037701>].

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Authors' contributions

LW and GXL designed the study and take responsibility for the entire process; LW conducted literature search, data extraction, quality assessment and draft writing; SY, XTY, TL and MCZ collected and analyzed the data, DPY edited the paper. All authors have read and approved the final paper.

Ethics approval and consent to participate

The study was approved by the Children's hospital Hebei Province Ethics Committee. Because there was no need to collect new specimens and the clinical data was de-identified, so the consent was waived by ethics committee of Children's Hospital of Hebei Province. After obtaining the permission, we can review patient records and use these data.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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