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 in pediatric patients, and account for most antibiotic prescriptions. A confirmed and rapid ARTI diagnosis is key to preventing antibiotic abuse. Recently, based on different detection principles, many multi-target molecular analyses that can detect dozens of pathogens at the same time have been developed, greatly improving sensitivity and shortening turnaround time. In this work, we performed 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 children with ARTI.

Methods By MCA and CE analysis, nine common respiratory pathogens were tested in hospitalized children < 13 years of age who met the ARTI criteria respectively.

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 that of 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. A higher sensitivity was observed in the melting curve assay. By using this sensitive and rapid test, it may be possible to achieve improved patient prognosis and antimicrobial management.

Background

Acute respiratory tract infections (ARTI) is acknowledged as one of greatest threats to pediatric health [1, 2], and nearly 1.9 million children died from ARTI and the majority were from developing country [3]. Accurate early etiologic diagnosis is crucial to the outcome of ARTI [4]. Multiplexed PCR methods enables high-throughput pathogen detection in a short time using limited amount of samples, and this detection strategy may have a significant impact on infectious disease management [5–7]. Our previous research reports that multiplex-PCR assays applied on 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 named capillary electrophoresis system, which limits their widespread use in developing country like China.

In this regard, detection kit based on multicolor probe-based fluorescence melting curve analysis (MCA) was developed. T_m is a highly reproducible physical parameter, combining it with fluorophore signature, MCA assay is feasible for 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, which enables detection of 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 assess 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 purpose and provided to this study without any patient identity. All aspects of the study were performed according to the Institutional Review Boards of Children's Hospital of Hebei Province.

Study population and specimen collection

During April to September 2019, patients had presented with signs and symptoms of ARTI were enrolled into this study. The sputum collected consecutively from these patients was liquefied with a transport medium and stored at -4 °C for nucleic acid extraction.

Nucleic acid extraction

The DNA/RNA extraction was performed on an automated workstation (Smart LabAssist-16/32, Health Gene Technologies, China) [14]. A total of 200 mL of liquefied sputum was used for nucleic acid extraction and eluted in 30μL, which was divided into two 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. Step 1: RT-PCR amplification. Mix 5μL RNA/DNA extraction with 4.3μL Res-reaction mixture1 and 0.7μL 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. 10μL of the RT-PCR product was mixed with 40μL PCR-MCA mixture, containing 39.8μL Res-reaction mixture2 and 0.2μL 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.

The ResP-CE analysis has been described in previous work, and we performed the experiment based on previously reported methods [14]. Table 1 illustrated the different detection targets of XYres-MCA and ResP-CE, respectively. 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, Mycoplasma pneumoniae; Ch, Chlamydia; 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 was used to compare the overall positive rates of pathogens between XYres-MCA and ResP-CE. Agreement between the two methods was assessed 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 the study, their sputum specimens were collected, and the main diagnosis was bronchopneumonia (46.8%, 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
Bronchopneumonia/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) for a total of 203 pathogens (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	P
HPIV	78	20	25.6	48	24	3	3	0.009
FluB	42	6	14.3	11	17	7	7	0.003
MP	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 for 28/30 samples (93.3%).

Grouped by co-infections, diagnosis and age, Table 4 shows the number of each pathogen detected by XYres-MCA. The most frequently detected pathogen was human parainfluenza virus, which was detected in 78 samples, 48 of which (61.5%) came from infants less than 1 year of age. 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 that of ResP-CE (72.2% vs. 63.7%, Table 5, $p = .002$).

Table 5
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)
P = 0.002 by McNemar's test.			

XYRes-MCA and ResP-CE agreed on the detection of 114 pathogens in the 237 samples (Table 6). By 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. Among the consistent cases, HPIV was the most common single virus (16.9%). Among 66 (27.8%) inconsistent cases, 7 (3%) were completely inconsistent, and 59 (24.9%) were partially inconsistent. Most of the discrepancies were MCA positive/CE negative results in the coinfection cases (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%
MP	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 all organisms except Influenza B and coronavirus, the agreement was excellent.

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

Pathogens	Positive cases	XYres-MCA (%)	ResP-CE (%)	kappa value	P value
HPIV	81	78 (96)	64 (79)	0.800	< .001
FluB	42	42 (100)	37 (88)	0.394	< .001
MP	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 misuse of antibiotics is acknowledged as one of the greatest threats to people health, as well as a major contributor to rising healthcare costs [16]. Due to the clinical similarity between bacterial and viral symptoms, the rapid and comprehensive pathogen diagnostic testing should be used to prevent empiric or unnecessary antimicrobial treatment [17]. This study demonstrates for the first time the clinical performance of a new XYres-MCA analysis on the 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. The agreement between the two tests was strong for all pathogens (kappa test > 0.60), except for coronavirus and influenza B virus.

An increasing number of recently published studies have focused on the development of molecular methods to solve multiple detection in one reaction [5, 13, 14, 18, 19]. The multiplex-PCR methods had been previously evaluated versus conventional techniques [1, 6, 8, 20] or with mono-plex PCR [21]. Because of their advantage of automated, highly reproducible, cost-effective and excellent sensitivity, the use of multiplex PCR testing is 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]. Only one multiplex PCR kit has been approved by CFDA, and this kit needs to be used on a highly specialized equipment, worth millions of RMB, namely automated capillary electrophoresis system, which exceeds the limit for equipment purchasing in most primary hospitals. MCA technology requires only one PCR instrument with four fluorescent channels. It is hoped that the comparative research data and the

low price of equipment can help promote the application of multiple PCR technologies in primary hospitals.

An important aspect of this study was the observation that a higher clinical sensitivity of the MCA in the detection of virus infections in clinical samples in contrast to the CE. The reason for the inconsistent results of influenza B virus is unclear but may potentially be explained by different targeted gene regions of the virus. Furthermore, the following two aspects of observation may explain why the XYres-MCA showed an advanced detection of parainfluenza virus and coronavirus, whereas a drop in sensitivity for the detection of Influenza A virus. First, the sensitivity may vary when the test is applicable to the entire family or is specific to a single type [25]. In the present study, subtypes of parainfluenza and coronavirus can be distinguished by XYres-MCA, and subtypes of influenza A can be distinguished by ResP-CE. Second, 12 types of pathogens and one internal control (IC) are set in the same reaction in XYres-MCA, whereas CE detects 13 pathogens, one IC, one human DNA and one human RNA sequences. Fewer targets in one reaction results in reduced competition for primers, nucleotides or enzymes, which may lead to the increased sensitivity [25, 26]. In addition, By using the fluorophore and T_m value as dual labels, XYres-MCA has the combined advantages of improved flexibility in probe design and expanded 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. By molecular methods, a positive result may indicate the true pathogen causing the disease or a harmless colonizer. It is known that most common respiratory pathogens can be existed in asymptomatic carriers [27] and a high co-detection rate was observed in our previous study and other studies using PCR methods [18, 28]. In routine clinical settings, children with mixed infections of two or more respiratory viruses are common due to undeveloped immune system but are not easily detected by conventional methods. Whether viral co-infection causes the illness to be more severe is still under debate [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

There were several limitations in our study. First, due to the budget restrictions the study lasted 6 months. This may weaken the ability to capture epidemic pathogens during seasons not covered by this study. Second, as several pathogens, eg, Rhinovirus, Chlamydia, were not included into the target of XYres-MCA, the performance of XYres-MCA assay for detection of these pathogens is uncertain. Third, compared with ResP-CE, the MCA panel does not contain a human DNA or RNA to monitor the quality of respiratory specimens.

Conclusions

In conclusion, in this first clinical study of the new XYres-MCA analysis of 237 clinical samples, we observed excellent sensitivity of this assay compared to another multiplex-PCR assay based on the capillary electrophoresis. The XYres-MCA assay may be a new tool in clinical laboratories for respiratory virus testing, may potentially impact positively 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 virus.

Abbreviations

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, ZSF 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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