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Diagnostic Microbiology and Infectious Disease 88 (2017) 115–119

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Virology

Clinical evaluation of a new single-tube multiplex reverse transcription PCR assay for simultaneous detection of 11 respiratory viruses, *Mycoplasma pneumoniae* and *Chlamydia* in hospitalized children with acute respiratory infections

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ARTICLE INFO

Article history:
Received 3 November 2016
Received in revised form 13 March 2017
Accepted 16 March 2017
Available online 22 March 2017

Keywords:
Evaluation
Mycoplasma pneumoniae
Chlamydia
Respiratory virus
Co-infection
Multiplex reverse transcription-PCR

ABSTRACT

Respiratory Pathogen 13 Detection Kit (13× kit) is able to simultaneously detect 11 respiratory viruses, *Mycoplasma pneumoniae* (*MP*) and *Chlamydia* in a single reaction. Using 572 Nasopharyngeal aspirates collected from hospitalized children, the clinical performance of 13× kit for detecting 11 respiratory viruses was evaluated in comparison with a routinely used 2-tube multiplex reverse transcription PCR assay (2-tube assay) at provincial Centers for Disease Control and Prevention in China. The clinical performance of 13× kit for detecting *MP* and *Chlamydia* was evaluated by commercial real-time quantitative PCR (qPCR) kits or sequencing. For tested viruses, the assay concordance was 95.98% and the kappa coefficient was 0.89. All the *MP* and *Chlamydia* positive samples detected by 13× kit were confirmed as true positives. The utilization of the 13× kit in clinical settings will be helpful for doctors to assess clinical outcome according to virus type or multiple infections, and to limit the use of antibiotics.

1. Introduction

Respiratory virus infections are an important cause of hospitalization for young Children. Different respiratory virus infections often present similar influenza-like symptoms (Kelly and Birch, 2004), laboratory analysis is therefore essential for etiological diagnosis. Recently, molecular assays, especially in a multiplex format, have been accepted as an excellent choice for broad spectrum detection of respiratory viruses (Kim et al., 2009; Lee et al., 2007; Mahony et al., 2007; Raymond et al., 2009). However, these methods or kits are either lower throughput (real-time reverse transcription-PCR), labor intensive (microarray) or costly (Luminex xTAG RVP Fast kit, FilmArray Respiratory Panel and next generation sequencing), which limits their wide use in the clinical setting. Multiple reverse transcription-PCR (RT-PCR) assays (Hu et al., 2012; Nagel et al., 2009; Qin et al., 2010) are a good alternative with acceptable sensitivity, specificity and reasonable expense. Our previous study (Li et al., 2013) reported a 2-tube multiplex RT-PCR assay (2-tube assay) using automated electrophoresis system to detect 16 respiratory viruses. The 2-tube assay is now commercialized (ABT 9 + 7, Zhuochenhuisheng, Beijing, China) and has routinely used at most of provincial Centers for Disease Control and Prevention in China.

In addition to respiratory virus, *Mycoplasma pneumoniae* (*MP*) is an important pathogen of respiratory infections in children, especially plays a significant role in community-acquired pneumonia (CAP) in children (Ferwerda et al., 2001; Zhuo et al., 2015). *Chlamydia pneumoniae* (*CP*) and *Chlamydia trachomatis* (*CT*) are 2 of the most common members of the Chlamydiaceae family that infect humans. *CP* is now recognized worldwide as a common cause of respiratory infections in adults and children, *CT* can be found in respiratory tract of newborns and can lead to pneumonitis (Hammerschlag, 2003; Webley et al., 2009). It has long been known that *MP* pneumonia (MPP) is associated with preceding or concomitant viral or *Chlamydia* infections. These co-infections should be considered in refractory MPP, as more severe outcome was found in co-infections patients than singe infection, and also more hospitalization expenses of patients with co-infections were

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http://dx.doi.org/10.1016/j.diagmicrobio.2017.03.010
0732-8893/

observed than patients with single infection in the same hospital stay days (Song et al., 2015). Thus, in clinical settings, especially in Children's hospital, there is therefore a high demand of simultaneous detection of respiratory viruses, *MP* and *Chlamydia*. Recently, a new Respiratory Pathogen 13 Detection Kit (13× kit, Health Gene Technologies, Ningbo, Zhejiang, China) based on multiplex RT-PCR assay and automatic capillary electrophoresis is commercialized, which enables simultaneous detection of 11 respiratory viruses including *human rhinovirus* (*HRV*), *influenza virus types A* (*FluA*), *FluA-H1N1*, *FluA-H3*, *influenza virus types B* (*FluB*), *adenovirus* (*Adv*), *human Bocavirus* (*HBoV*), *metapneumovirus* (*HMPV*), *parainfluenza virus* (*PIV*), *coronavirus* (*COV*), *respiratory syncytial virus* (*RSV*), and *MP* and *Chlamydia* (including *CP* and *CT*) in a single reaction. However, no study was conducted to evaluate the clinical performance of this kit.

In this study, the clinical performance of the 13× kit was evaluated for the first time in a head to head comparison against the 2-tube assay and commercial Real-time Quantitative PCR (qPCR) kits using 572 Nasopharyngeal aspirates (NPAs) from children with acute respiratory tract infections (ARTI). The utilization of 13× kit in clinical practice was also discussed.

2. Materials and methods

2.1. Specimen collection

A total of 572 children with ARTI hospitalized in Children's hospital of Hebei, China, from May to October 2015 and from May to July 2016 were included in the study, of those 201 (35.1%) were female and 371 (64.9%) were male. Ages ranged from 1 month to 13 years old, and 83.2% were under 3 years old. NPAs collected consecutively from those children were added to 3.5 ml of transport medium and stored at −80 °C. All aspects of the study were performed in accordance with national ethics regulations and approved by the Institutional Review Boards of National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China. Children's parents were apprised of the study's purpose and of their right to keep information confidential. Written informed consent was obtained from parents or caregivers.

2.2. Extraction and purification of RNA/DNA

Total RNA/DNA was extracted from 200 μL of clinical samples using EasyPure Viral DNA/RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Two microliter MS2-based pseudovirus particles as a RT-PCR internal control were mixed with clinical samples and extracted together. The extracts was eluted in 50 μL of DNase- and RNase-free water and stored at −80 °C.

2.3. Detection of 13 respiratory pathogens (13× kit)

Each RNA/DNA preparation was subjected to RT-PCR procedure according to the manufacturer's instructions. Thermal cycling was performed on an ABI 7500 apparatus (Applied BioSystems, USA). The condition of RT-PCR was as follows: 5 min at 25 °C, 15 min at 50 °C, 2 min at 95 °C, 6 cycles of 94 °C for 30 sec, 65–60 °C for 30 sec (1 °C touchdown PCR), 72 °C for 60 sec and 29 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 60 sec, followed by a single incubation of 10 min at 70 °C. An aliquot (1 μL) of the PCR product for each sample or reference standards (Health Gene Technologies, China) was prepared for capillary electrophoresis by adding 28.7 μL of CEQ Sample Loading Solution (Beckman Coulter, USA) and 0.3 μL of CEQ DNA Size Standard 400 (Beckman Coulter, USA) in a 96-well CEQ electrophoresis plate (Beckman Coulter, USA), and then were analyzed by a GeXP system (Beckman Coulter, USA). For all amplified products, the reaction was considered positive when the value of sample dye signal was over the high value of reference standards or negative when under the low value. If dye signal value of the clinical sample was between the high value and low value (gray area), the sample was re-detected.

2.4. Validation of 11 respiratory viruses using 2-tube multiplex RT- PCR assay (2-tube assay)

The 2-tube assay was performed with a One Step RT-PCR kit (Qiagen, Hilden, Germany) in a 25 μL volume according to the protocols as described (Li et al., 2013), and the products were analyzed on the QIAxcel automatic electrophoresis using QIAxcel DNA High-Resolution kit.

If results were discordant between 13× kit and 2-tube assay, both tests were repeated concurrently to evaluate any problems relating to sample degradation or potential hands-on error. Assignment of such samples as having concordant or discordant results was based on the results of duplicate testing by both methods. If results were still discordant, mono-RT-PCR was then performed followed by sequencing using a pair of universal tag primers (Table 1). The specific primers for each pathogen were designed by Health Gene Technologies and the primers information is showed in Table 1.

2.5. Validation of MP and Chlamydia using real-time quantitative PCR (qPCR)/sequencing

For *MP*, all samples were validated by qPCR using commercial diagnostic kits for *MP* (Daan Gene, Guangzhou, China) according to the

Table 1
Information of primers for sequence.

Pathogen	Primer	Sequence(5′-3′)	Amplicon size (bp)
FluA	Seq-F	GACCRATCCTGTCACCTYTGAC	144
	Seq-R	GGGCATTYTGGACAAAKCGTCTACG	
FluA-H1	Seq-F	TTGCTTGGTCAGCAAGTGC	654
	Seq-R	CAGTCACACCATTTGGATCC and CAGTCCATCCRTTTGGATCC	
FluA-H3	Seq-F	ATGGGACCTTTTTRTYGAAMGMAGCA	558
	Seq-R	CCCCKAGGAGCAATTAGATTCCCTGT	
Adv	Seq-F	GCCCCAATGGGCDTACATGCACATC	340
	Seq-R	CAGCACVCCSCKRATGTCAAA	
PIV1	Seq-F	TCTCATTATTACCyGGACCAA or TTCTGGAGATGTCCCrTAGG	283 or 332
	Seq-R	TCCTGTTGTCrTTGATGTCATA	
PIV2	Seq-F	GAGYATGGTYCARGGAGATAATCA	262
	Seq-R	CTGATGACCCAACCCATAATTATTT	
PIV3	Seq-F	TTGTCAATTATGATGGYTCAATCT	231
	Seq-R	GACACCCAGTTGTRTTRCAG	
PIV4	Seq-F	GGAGACAATCAAACAAywGCAATAACTAC	244
	Seq-R	CCCTCTCCAAAAAATTCTTTTACCATATAC	
HBoV	Seq-F	CCTGCGAGCTCTGTAAGTACTATTAC	403
	Seq-R	GGAAGCTCTGTGTTGACTRAATACAG	
HMPV	Seq-F	GTTCCCTTTGTTTCARGCYAA	480
	Seq-R	CTTATAGCAGCTTCAACRGTRGCTG	
	Seq-F	TTTCAGGCCAAYACACCACC	460
	Seq-R	CTTCAACAGTRGCTGACTCACTCTC	
FluB	Seq-F	TCCTCAACTCACTCTTCGAGCG	142
	Seq-R	CGGTRCTCYTGACCAAATTGG	
HRV	Seq-F	CCAAAGTAGTYGGTYCCRTCC	179
	Seq-R	GGGTGYGAAGASCCYCG	
MP	Seq-F	TGGCGCTTGACTGATACCTG	256
	Seq-R	ACCTGATTACGTGTTGCCGT	
COV-229E/NL63	Seq-F	GCATAGCATAGACCAAGTCCATCAT and GCAGAGCGAAGCACAAATCCATCAT	205
	Seq-R	AAGTCAGTTATGGAMCACGAGCA	
COV-OC43/HKU1	Seq-F	TCAAATCCCAATGACAATCAAAKGG	293
	Seq-R	GAATGTTGCTAAGTAYACYCARTTATG	
RSV	Seq-F	GGAGCCATTGTRTCATGYTA	245
	Seq-R	TCATAGAAATTTATTATWGGTTCA and TCATAGTAATTTATTATAGGTTCC	
Chlamydia	Seq-F	GATGATTTGAGCGTGTGTAGCG	263
	Seq-R	TACGAGCCAGCACTCCAATTTC	
Universal tag primers [a]	M13–47	AGGGTTTTCCCAGTCACG	
	M13–48	GAGCGGATAACAATTTCACAC	

[a] Primer M13–48 was added at 5′-end of Seq-F and Primer M13–47 was added at 5′-end of Seq-R. Chimeric primers were used for mono-RT-PCR and universal tag primers were used for sequencing.

manufacture's instruction. For discordant *MP* results, mono-RT-PCR was then performed followed by sequencing. For *Chlamydia*, all samples were validated by sequencing. The primers for mono-RT-PCR and sequencing are shown in Table 1.

3. Results

3.1. Summary of results for all samples

A total of 503 out of 572 (87.94%) specimens were positive for at least one of the respiratory pathogens by either 13× kit or 2-tube assay, 230 (40.21%) specimens with co-infections were detected by either of the 2 methods. Of the 494 positives by 13× kit, 267 (46.68%), 202 (35.31%), and 25 (4.37%) specimens were positive for a single pathogen, 2 pathogens, and 3 pathogens, respectively. Of the 433 positives by 2-tube assay, 240 (41.96%), 173 (30.24%), and 20 (3.50%) specimens were positive for a single virus, 2 viruses, and 3 viruses, respectively.

Table 2 shows the sample positivity for each pathogen detected by 2 methods. A specimen was considered positive for a pathogen if it was tested positive by either of 2 methods. The most frequently detected pathogen was *HRV* (241/572, 42.13%). *PIV* was detected in 179 of 572 (31.29%) specimens including 14/572 (2.45%) type 1, 1/572 (0.17%) type 2 and 164/572 (28.67%) type 3. 83 of *MP* and 17 of *Chlamydia* were identified by 13× kit. No *PIV2* were detected by either of 2 methods. Discordant results were found in 4.02% (23/572) of specimens. As detection of *MP* and *Chlamydia* was not done with 2-tube assay, *MP* and *Chlamydia* were not counted in the discordant results.

3.2. Validation of 11 respiratory viruses

Overall, 413 of the 572 specimens were positive for at least one virus with both techniques, 136 specimens were negative for all viruses by both methods. A sensitivity of 97.41% and a specificity of 91.89% for 13× kit were achieved using 2-tube assay as the reference method. The positive predictive value (PPV) and the negative predictive value (NPV) for the 13× kit were 97.18% and 92.52%, respectively. In a head-to-head comparison for all viruses, the assay concordance was 95.98%, the kappa coefficient was 0.89. The sensitivity, specificity, Youden's index, PPV, NPV, accordance rate and kappa coefficient for every target (*MP* and *Chlamydia* was not includEd.) using the 2-tube assay as the reference method are shown in Table 3.

Of the 4.02% (23/572) of specimens with discordant results, 12 specimens including 6 of *PIV*, 3 of *HRV*, 1 of *FluB*, 1 of *COV*, 1 of *Adv*, and 1 of *HBoV* were tested positive by the 13× kit but missed by the 2-tube assay; while 11 specimens missed by the 13× kit were identified by the 2-tube assay including 6 for *HRV*, 2 for *RSVB*, 2 for *Adv*, 1 for *PIV1*, 1 for *RSVA*, 1 for *HMPV* and 1 for *HBoV* (Table 4). All the positive specimens with discordant results were confirmed by mono-PCR and sequencing as true positives, as shown in Table 4.

Table 2
Sample positivity by 13× kit and 2-tube assay for each pathogen.

Pathogen	No.(%) of specimens(n = 572) positive by		
	13× kit [a]	Two-tube assay	13× kit or 2-tube assay
FluA [c]	7 (1.22)	7 (1.22)	7 (1.22)
FluA-H1	1 (0.17)	NA	1 (0.17)
FluA-H3	6 (1.99)	NA	6 (1.99)
FluB	9 (1.57)	8 (1.40)	9 (1.57)
PIV	178 (31.12)		179 (31.29)
PIV1	NA	14 (2.45)	NA
PIV2	NA	0	NA
PIV3	NA	159 (27.80)	NA
HRV	235 (41.08)	238 (41.61)	241 (42.13)
HMPV	37 (6.47)	38 (6.64)	38 (6.64)
Adv	72 (12.59)	73 (12.76)	74 (12.94)
COV	36 (6.29)		36 (6.29)
COV-299E	NA	7 (1.22)	NA
COV-OC43	NA	22 (3.85)	NA
COV-HKU1	NA	4 (0.70)	NA
COV-NL63	NA	2 (0.35)	NA
RSV	21 (3.67)		24 (4.20)
RSVA	NA	12 (2.10)	NA
RSVB	NA	12 (2.10)	NA
HBoV	50 (8.74)	50 (8.74)	51 (8.92)
MP [b]	83 (14.51)	NA	83 (14.51)
Chlamydia [b]	17 (2.97)	NA	17 (2.97)
Mixed infection	**227 (39.68)**	**193 (33.74)**	**230 (40.21)**
Total	**494 (86.36)**	**433 (75.70)**	**503 (87.94)**

[a] Genotyping of PIV, COV and RSV was not done with the 13× kit method.
[b] Detection of MP and *Chlamydia* was not done with the 2-tube assay.
[c] A specimen was considered positive for FluA-H3/H1 if it was positive for both FluA and FluA-H3/H1. NA, not applicable.

3.3. Validation of MP and Chlamydia

The results of 13× kit were in accordance with qPCR or sequencing. Eighty-three positive samples for *MP* were detected and 26 of 83 samples were co-infected with viruses. Seventeen cases of *Chlamydia* were detected and 7 cases were co-infected with viruses. Seventeen cases of *Chlamydia* detected by 13× kit were confirmed by sequencing as true positives for *CT*.

4. Discussion

There are many reports on the multiple causative viruses of acute respiratory tract infections with different approaches using molecular detection techniques (Coiras et al., 2004; Li et al., 2013; Pabbaraju et al., 2011; Raymond et al., 2009). Nucleic acid amplification methods such as conventional PCR, nested PCR and real-time PCR have increasingly been explored for identification of *MP* and *Chlamydia* in respiratory diseases (Higgins et al., 2009; Kumar and Hammerschlag, 2007). However, no commercial assay for simultaneous detection of common respiratory viruses, *MP* and *Chlamydia* in respiratory specimens was available in China. Recently, the first commercial kit (13× kit, Health Gene Technologies, Ningbo, Zhejiang, China) was launched in China for the simultaneous detection of 11 common respiratory viruses, *MP* and *Chlamydia*. In the present study, the feasibility of potential clinical practice of this kit is evaluated in comparison with the 2-tube assay and qPCR using 572 nasopharyngeal specimens from hospitalized children with ARTI. The overall detection rate of 13× kit for each virus was comparable to that of 2-tube assay (kappa >0.75) revealing the high sensitivity (97.41%) and specificity (91.89%) of 13× kit in the analysis of clinical samples. We also found 100% diagnosis agreement for detection of *MP* and *Chlamydia* between 13× kit and qPCR/sequencing. We showed that the detection rate of co-infections was similar between 13× kit (227/572) and 2-tube assay (193/572). Moreover, 29 of 34 co-infections detected by 13× kit were *MP* or *CT* co-infections with viruses, demonstrating the ability of 13× kit to detect the mixed infections involved in *MP*/*CT* and respiratory viruses. Additionally, 13× kit is more sensitive than 2-tube assay in the detection of *PIV*, as *PIV4* was only identified by 13× kit (Table 3). In this study, *HRV* was the most frequently detected pathogen followed by *PIV, MP, Adv* and *HBoV*, and *FluA* and *FluB* were detected at a low prevalence. These results were similar to our previous study in 2016 about common respiratory viruses (Le et al., 2016) except that the prevalence of *RSV* was very low in this study. This difference might be caused by the following 2 aspects: 1. The detection of *RSV* increased during the winter (Cui et al., 2015; Jain et al., 2015), but the collection time of the samples in our study was not in winter (May to October). 2. Our previous study only referred to patients with community acquired pneumonia. In this study, the patients were hospitalized for both the upper respiratory tract and lower respiratory tract infections. In addition, the positive percentage of *CT* was 2.97% in our study which was consistent with the reported results of Chen et al. (2010).

Table 3
Detection of 9 respiratory viruses in 572 specimens[a].

Virus [b]	No. of specimens with indicated 13× kit/2-tube assay results				Performance of 13× kit compared with 2-tube assay						
	+/+	+/−	−/+	−/−	Sensitivity %	Specificity %	Youden's index	PPV %	NPV %	Accordance rate	kappa
FluA	7	0	0	565	100.00	100.00	1.000	100.00	100.00	100.00	1.00
FluB	8	1	0	563	100.00	99.82	0.998	88.89	100.00	99.83	0.94
PIV	172	6	1	393	99.42	98.50	0.979	96.63	99.75	98.78	0.97
HRV	232	3	6	331	97.48	99.10	0.966	98.72	98.22	98.43	0.97
HMPV	37	0	1	534	97.37	100.00	0.974	100.00	99.81	99.83	0.99
Adv	71	1	2	498	97.26	99.80	0.971	98.61	99.60	99.48	0.98
COV	35	1	0	536	100.00	99.81	0.998	97.22	100.00	99.83	0.98
RSV	21	0	3	548	87.50	100.00	0.875	100.00	99.46	99.48	0.93
HBoV	49	1	1	521	98.00	99.81	0.978	98.00	99.81	99.65	0.98
Total	**413**	**12**	**11**	**136**	**97.41**	**91.89**	**0.893**	**97.18**	**92.52**	**95.98**	**0.89**

[a] The numbers of positives and negatives detected by both testing methods are shown. The sensitivity(true positives, TP), specificity(true negatives, TN), Youden's index, PPV(TP/TP + false positives), NPV(TN/TN + false negatives), accordance rate and Kappa coefficient for every target using the 2-tube assay as the reference method are shown.FluA-H3 and H1N1 was included in FluA.
[b] Detection of MP and *Chlamydia* was not done with 2-tube assay, so they were not included in this table.

Common atypical bacteria such as *MP* and *Chlamydia* are listed as common agents of CAP in Chinese Community-acquired Pneumonia Management Guidelines in Children (trial, 2007). Recent epidemiological studies (Liu et al., 2015a, 2015b; Wu et al., 2014) of pathogens leading to respiratory infection among hospitalized children in China showed that *MP* was the most frequently detected pathogen. These studies also reported that co-infections with multiple pathogens are common in children with respiratory infection and the most commonly seen was the combination of *MP* and viral pathogen. In this study, MP was detected in 83 children (68 cases ≥3 years), composed of 50 males and 33 females, and the co-infection rate was 31.3% (26/83), which was lower than the detection rates reported in other studies mentioned above. These differences may be associated with various factors such as sample size, types of samples, the timing of specimen collection and methodology. *CP* often mixes infections with *MP*, especially in children older than 3 years (Chen et al., 2012; Song et al., 2015). *CT* is mainly prevalent in infants less than 6 months hospitalized with pneumonia and severe pneumonia cases had a higher proportion of viral co-infection compared to mild pneumonia cases (Li et al., 2015; Liu et al., 2015b). In our study, 17 of *CT* were detected in 11 males and 6 females and 7 of them (41.2%) were co-infections with virus, suggesting a high proportion of viral co-infection in patients infected with *CT*. Furthermore, 16 of them were infants less than 6 months of age and the onset age was consistent with above studies. So, the diagnosis of atypical bacterial infection, especially co-infection with viruses, should be paid more attention.

Table 4
Summary of the discrepant result (highlighted in bold type) between the 13× kit and 2-tube assay.

Sample#	13× kit result	Two-tube assay result	Validation result
23	**FluB**, RSV	RSVA	FluB
105	**PIV**	None	PIV3
110	**PIV**,HRV	HRV	PIV3
153	**PIV**,**HRV**,COV	COV-OC43	PIV3,HRV
156	**PIV**,HRV	HRV	PIV3
190	**PIV**,HRV	HRV	PIV3
10\108	**PIV**	None	PIV4[a]
148	**HRV**,PIV	PIV3	HRV
378	**HRV**,COV	COV-OC43	HRV
270	**Adv**, PIV, HMPV	PIV3,HMPV	Adv
306	**COV**,HRV	HRV	COV-OC43
22	**HBoV**	None	HBoV
385	None	**PIV1**,**Adv**	PIV1,Adv
79	None	**HRV**	HRV
209	None	**HRV**	HRV
246	None	**HRV**	HRV
10\90	COV	**HRV**,**RSVB**,COV-OC43	HRV,RSVB
10\91	None	**HRV**,**RSVB**	HRV,RSVB
10\106	None	**HRV**	HRV
217	None	**HMPV**	HMPV
164	HRV,HMPV	HRV, HMPV, **Adv**	Adv
239	None	**RSVA**	RSVA
10\77	None	**HBoV**	HBoV

[a] Detection of PIV4 was not done with 2-tube assay.

The distinctive feature of 13× kit is that it integrates multiplex RT-PCR with automatic capillary electrophoresis. A total of 13 sets of chimeric primers (comprising of universal tag at the 5′-end and pathogen specific primer at 3′ end) labeled with one fluorescence dye at 5′-end were designed. One-step multiplex RT-PCR was performed in a single tube, followed by capillary electrophoresis separation of different length of amplification products for different pathogens. DNA and RNA targets of samples were thus tested at the same time. Taking the advantages of its one-step multiplex procedure and high-throughput sample loading, 13× kit assays is labor-saving and time-saving. The detection of 96 samples can be completed in 10–16 hours, including extraction of RNA/DNA (40 minutes), the whole RT-PCR (2 hours), and the detection on the automatic capillary electrophoresis (8 tests/30 minutes on 3500Dx/3500xL Dx and 3130/3130xl genetic analyzer or 8 tests/1 hour on GeXP Genetic Analysis system). Moreover, an RT-PCR internal control was added to each specimen prior to extraction to monitor the whole process including nucleic acid extraction, RT-PCR and capillary electrophoresis.

A limitation of 13× kit is that an automated capillary electrophoresis system is needed and many clinical laboratories do not have this highly specialized laboratory equipment. Besides, 13× kit is unable to distinguish the infection between *CP* and *CT* on the etiology, and the etiological significance of *MP*, *CP* or *CT* in the co-infection or the severity of patients remains unclear due to the limited data in this study.

In conclusion, our present study highlights the important role of respiratory viruses and atypical pathogens in hospitalized children with acute respiratory infections. The utilization of the 13× kit in clinical settings will be helpful for doctors to assess clinical outcome according to virus type or multiple infections, and to limit the use of antibiotics.

Conflict of interests

None.

Funding

This work was supported by the National key research and development plan (2016TFC1202700, 2016YFC120090); Beijing Municipal Science & Technology Commission project [grant numbers D151100002115003] and Guangzhou Municipal Science & Technology Commission project (grant numbers 2015B2150820).

Ethical approval

All aspects of the study were performed in accordance with national ethics regulations and approved by the Institutional Review Boards of National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China.

Acknowledgments

The authors would like to thank staff in the department of respiration and Xiao-tong Yan in the Pediatric Research Institute at Children's Hospital of Hebei Province for collecting samples.

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