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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A R T I C L E I N F O

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

A B S T R A C T

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.