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Original Article

Application of MRT-qPCR for pathogen detection of lower respiratory tract infection

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Abstract: Objective: To analyze and clarify the application value of multiplex quantitative real-time PCR (MRT-PCR) assay in detecting pathogens involved in lower respiratory tract infection (LRTI), so as to realize accurate and rapid detection of respiratory pathogens. Methods: Bronchial alveolar lavage fluid (BALF) specimens from 186 patients with LRTI collected in the Cangzhou Central Hospital from June 2020 to September 2021 were analyzed retrospectively. Pathogen detection was performed by both MRT-PCR and direct immunofluorescence assay (DFA), and the results of different inspection methods were compared. Results: Among the seven pathogens detected by MRT-PCR, 140 positive specimens were identified out of the 186 patients, with the top three pathogens with the highest positive rates being influenza A virus (Flu A; 36 [19.35%]), respiratory syncytial virus (RSV; 30 [16.13%]) and human adenovirus (HAdV; 23 [12.37%]), and the pathogen with the lowest positive rate being parainfluenza virus type 3 (PIV3; 9 [4.84%]). DFA showed 110 pathogen-positive specimens, and the top three pathogens with the highest positive rates were Flu A (30 [16.13%]), HAdV (21 [11.29%]) and RSV (19 [10.22%]). The total sensitivity and accuracy of MRT-PCR assay were 93.01% and 98.69% respectively, which were statistically higher than those of 48.45% and 91.24% of DFA ($P<0.05$). The two inspection methods showed no significant difference in specificity (99.4% for MRT-PCR assay and 97.28% for DFA) ($P>0.05$). Conclusions: MRT-PCR is rapid, accurate and specific in detecting pathogens of LRTI, which significantly improves the detection rate, with reliable performance and it has high clinical application value.

Keywords: Multiplex quantitative real-time PCR, direct immunofluorescence, lower respiratory tract infection, pathogen, specificity

Introduction

Respiratory tract infection (RTI) is a common clinical condition that can cause mild pharyngolaryngitis, or develop into severe pneumonia or even cause death [1, 2]; with the characteristics of strong infectivity, rapid spread, short incubation period and acute onset. The long-term prognosis of patients with acute aggravation is poor, with a 5-year mortality rate of approximately 50% [3]. RTI can be divided into either upper respiratory tract infection (URTI) or lower respiratory tract infection (LRTI) [4, 5]. URTI mainly covers acute infections of the nose, sinuses, pharynx and throat, while LRTI includes trachea, bronchus and lung parenchyma [6]. LRTI is one of the leading causes of death from infectious disease worldwide, with high morbidity and mortality. Infectious diseases of the respiratory system ranked fourth in terms of mortality only after malignancies, cerebrovascular diseases and heart disease, as indicated by data from the National Bureau of Statistics in 2017. Among children under 5 years of age, respiratory infections are among the top three in terms of mortality [7]. From an epidemiological standpoint, most definitions of LRTI include pneumonia, influenza, bronchitis and bronchiolitis [8, 9]. RTI is mostly attributed to viral infections [10], with evidence indicating that viral pneumonia is responsible for 86% of community-acquired pneumonia among chil-

dren under the age of 2 and 37% among children aged above 5 [11].

Common respiratory pathogens include parainfluenza virus types 1 (PIV1), 2 (PIV2) and 3 (PIV3) [12], mycoplasma pneumoniae [13], chlamydia pneumoniae [14], respiratory syncytial virus (RSV) [15], human adenovirus (HAdV) [16], and influenza A [17] and B viruses [18]. However, the epidemiology of lower respiratory tract virus infection varies with age, season and region. On the other hand, the diseases, clinical manifestations and biological indicators caused by different viruses are also different [19, 20]. With the wide application of antibiotics in recent years, the pathogen spectrum of LRTI is constantly changing, which increases the difficulty of clinical diagnosis and treatment. Therefore, effective and accurate diagnosis of respiratory pathogen infections has always been a challenge, clinically.

Among the current detection methods for pathogens of LRTI, the traditional ones are virus isolation and culture [21], as well as direct and indirect immunofluorescence assay (DFA/IFA) [22, 23]. However, these methods are cumbersome and time-consuming, with relatively low diagnostic sensitivity and specificity, and have a high false positive rate. In recent years, the multiplex quantitative real-time PCR (MRT-PCR) technology [24] developed from the common PCR technology has become a novel direction of respiratory pathogens identification, as it can simultaneously detect a variety of pathogens, realizing high-throughput, high-sensitivity and high-specificity detection. In this paper, seven common respiratory pathogens such as PIV1, PIV2, and PIV3 in the bronchial alveolar lavage fluid (BALF) of hospitalized LRTI patients were detected by MRT-PCR and DFA respectively to compare their detection rates, so as to provide a rapid and accurate detection method for early screening and diagnosis of respiratory virus.

Materials and methods

Research participants and specimen collection

The study population was comprised of 186 patients (male-to-female ratio: 98:78, age range: 3-81 years old) with LRTI admitted to the Cangzhou Central Hospital from June 2020 to September 2021. Inclusion criteria: (1) Patients diagnosed with community-acquired LRTI [25, 26]; (2) Patients with complete medical records. Exclusion criteria: (1) Pregnant or breastfeeding women; (2) Patients with active pulmonary tuberculosis; (3) Patients with aspiration or obstructive pneumonia; (4) Patients with a history of hospitalization 2 weeks prior to disease onset that cannot exclude hospital acquired infections; (5) Patients with human immunodeficiency virus (HIV) infection; (6) Patients with incomplete clinical data. Ethical approval was obtained from the institutional committee of Cangzhou Central Hospital.

BALF specimen collection: All patients underwent bronchoscopy. The bronchoscope was inserted through the nasal cavity, and the tip of the bronchoscope was embedded at the opening of the segmental or subsegmental bronchus where the lesion was located. BALF specimen was obtained by bronchial lavage with sterile normal saline during rapid administration through the biopsy hole, with a recovery rate of about 60%.

All BALF samples were sent for microbial etiological diagnosis by Shanghai B&C Biological Technology Co., Ltd. before the conduction of this study. Bacterial or fungal cultures of BALF were negative in all patients, and these detection results served as the gold standard. The specimens for this study were the remaining lavage fluid specimens after routine tests in our department, and the test results generated in this study were only used for scientific research.

Sample viral nucleic acid (NA) extraction

Viral NA was extracted from specimens with the MagMAX Viral/Pathogen NA Isolation Kit (Dynabeads; Thermo Fisher) and the ABI MagMAX Express 96 Auto-NA Extractor according to the kit instructions and instrument operation manual.

MRT-PCR

All NA specimens were examined by MRT-PCR for respiratory viruses, including RSV, PIV1, PIV2, PIV3, HAdV, and influenza A (Flu A) and B virus (Flu B), all of which were supplied by Fast-track diagnostics (Cat. No. FTD 2-96/12, Fast-track Diagnostics, Luxembourg). After extracting NA from the specimens, 5 μL of NA was taken from each tube of reaction solution for detection. Takara reverse transcriptase

Table 1. Results of multiplex quantitative real-time PCR in patients with lower respiratory tract infection

Pathogen	Positive [n (%)]			Negative [n (%)]		
	True positive	False positive	Positive rate	True negative	False negative	Negative rate
RSV	28 (15.05)	2 (1.08)	30 (16.13)	153 (82.26)	3 (1.61)	156 (83.87)
PIV1	16 (8.60)	0 (0.00)	16 (8.60)	168 (90.32)	2 (1.08)	170 (91.40)
PIV2	11 (5.91)	0 (0.00)	11 (5.91)	174 (93.55)	1 (0.54)	175 (94.09)
PIV3	9 (4.84)	0 (0.00)	9 (4.84)	177 (95.16)	0 (0.00)	177 (95.16)
HAdV	21 (11.29)	2 (1.08)	23 (12.37)	161 (86.56)	2 (1.08)	163 (87.63)
Flu A	33 (17.74)	3 (1.61)	36 (19.35)	149 (80.11)	1 (0.54)	150 (80.65)
Flu B	15 (8.06)	0 (0.00)	15 (8.06)	170 (91.40)	1 (0.54)	171 (91.94)

Note: RSV: respiratory syncytial virus; PIV1: parainfluenza virus 1; PIV2: parainfluenza virus 2; PIV3: parainfluenza virus 3; HAdV: human adenovirus; Flu A: influenza A virus; Flu B: influenza B virus.

(Takara, Japan) was used for reverse transcription, and ABI7500 was used for amplification. The amplification procedure consisted of the following steps: pre-denaturation (95°C for 1 minute); denaturation (95°C for 15 seconds), annealing and extension (60°C for 30 seconds), for 45 cycles; fluorescence signals were collected simultaneously. The instrument was cooled at 25°C for 10 seconds.

DFA

After centrifugation and precipitation, the specimen was sliced and placed at room temperature for natural drying. The slices were then fixed with cold acetone (10 min) and taken out to let the cold acetone volatilize completely. Fluoresce-labeled monoclonal antibodies (Thermo Scientific, USA) against respiratory virus were used to detect the pathogens of the specimens, and the specific steps were operated following the instructions of the kit. Respiratory virus screen kit (K6120112) was purchased from Thermo Scientific™ IMAGEN™. Positive was determined by the presence of bright yellow-green fluorescence in 2 or more intact cells.

Statistical analysis

The negative and positive results of each pathogen were used to establish a database with Excel, and descriptive statistical analysis was performed. After data verification, SPSS22.0 was used for statistical processing. The comparison of counting data was performed by χ^2 test or Fisher exact probability method. For measurement data expressed by mean ± standard deviation, t test was used, and one-way ANOVA was used for comparison among groups. For all analyses, $P<0.05$ was used to indicate significant differences.

Results

Overall distribution of positive rates of seven respiratory pathogens detected by MRT-PCR

Through MRT-PCR assay of seven respiratory pathogens, 114 (61.29%) of the 186 patients were found to be pathogen positive, in which 12 samples were detected with two or more pathogens, accounting for 6.45%. Among the seven pathogens monitored, there were 140 positive specimens in total in 114 patients, with the first three pathogens with the highest positive rates being Flu A (36 [19.35%]), RSV (30 [16.13%]) and HAdV (23 [12.37%]) and the pathogen with the lowest positive rate being PIV3 (9 [4.84%]). **Table 1**.

DFA detection results of patients with LRTI

DFA analysis of seven respiratory pathogens in the 186 patients showed that 75 cases (40.32%) were positive for pathogens, among which three samples were detected with two or more pathogens, accounting for 1.61%. Among the seven pathogens monitored, there were 110 positive specimens in total, and the first three pathogens with the highest positive rates were Flu A (30 [16.13%]), HAdV (21 [11.29%]) and RSV (19 [10.22%]), while the pathogen with the lowest positive rate was PIV3 (7 [3.76%]). **Table 2**.

Detection of infection by MRT-PCR in different age groups

Among the 186 specimens, 162 (87.10%) were children aged five or under, 11 were aged 5 to

Table 2. Direct immunofluorescence assay results of patients with lower respiratory tract infection

Pathogen	Positive [n (%)]			Negative [n (%)]		
	True positive	False positive	Positive rate	True negative	False negative	Negative rate
RSV	13 (6.99)	6 (3.23)	19 (10.22)	152 (81.72)	15 (8.06)	167 (89.78)
PIV1	9 (4.84)	3 (1.61)	12 (6.45)	167 (89.78)	7 (3.76)	174 (93.55)
PIV2	7 (3.76)	2 (1.08)	9 (4.84)	169 (90.86)	8 (4.30)	177 (95.16)
PIV3	6 (3.23)	1 (0.54)	7 (3.76)	172 (92.47)	7 (3.76)	179 (96.24)
HAdV	13 (6.99)	8 (4.30)	21 (11.29)	148 (79.57)	17 (9.14)	165 (88.71)
Flu A	21 (11.29)	9 (4.84)	30 (16.13)	132 (70.97)	24 (12.90)	156 (83.87)
Flu B	9 (4.84)	2 (1.08)	11 (5.91)	170 (91.40)	5 (2.69)	175 (94.09)

Note: RSV: respiratory syncytial virus; PIV1: parainfluenza virus 1; PIV2: parainfluenza virus 2; PIV3: parainfluenza virus 3; HAdV: human adenovirus; Flu A: influenza A virus; Flu B: influenza B virus.

Table 3. Analysis of infection status of samples detected by multiplex real-time quantitative PCR in different age groups

	Minors				Adults			
	≤5 years old		5-18 (inclusive) years old		18-60 (inclusive) years old		>60 years old	
	Number of positives (cases)	Positive rate (%)	Number of positives (cases)	Positive rate (%)	Number of positives (cases)	Positive rate (%)	Number of positives (cases)	Positive rate (%)
RSV	18	19.35	5	20.00	5	27.78	2	50.00
PIV1	11	11.83	3	12.00	2	11.11	0	-
PIV2	8	8.60	2	8.00	1	5.56	0	-
PIV3	6	6.45	2	8.00	1	5.56	0	-
HAdV	15	16.13	5	20.00	3	16.67	0	-
Flu A	27	29.03	5	20.00	2	11.11	2	50.00
Flu B	8	8.60	3	12.00	4	22.22	0	-
Total	93		25		18		4	

Note: RSV: respiratory syncytial virus; PIV1: parainfluenza virus 1; PIV2: parainfluenza virus 2; PIV3: parainfluenza virus 3; HAdV: human adenovirus; Flu A: influenza A virus; Flu B: influenza B virus.

18 (inclusive), 10 were aged 18 to 60 (inclusive), and 3 were older than 60. Among children aged five and under, the top three with the highest detection rates were Flu A (29.03%), RSV (19.35%) and HAdV (16.13%). **Table 3**.

DFA detection of infection in different age groups

Among children aged five and under, Flu A (27.16%), RSV (20.99%) and HAdV (18.52%) were the top three with the highest detection rates. **Table 4**.

Comparison of detection value between MRT-PCR and DFA

MRT-PCR assay showed obviously higher total sensitivity (χ^2=48.6810, P<0.0001) and accuracy (χ^2=4.7141, P=0.0299) than DFA; While there was no significant difference in specificity between the two inspection methods (χ^2= 1.0201, P=0.3124) **Tables 5** and **6**.

Discussion

RTI is one of the most common diseases, affecting adults about 2-4 times a year and children about 6-8 times a year [27]. The pathogens of RTI are extremely diverse, resulting in different disease outcomes. Recently, new pathogenic viruses are constantly being discovered, such as human metapneumovirus, SARS virus, highly pathogenic avian influenza virus, H7N9 Flu A, Middle East respiratory syndrome (MERS), as well as Coronavirus disease 2019 (COVID-19) [28-30], causing great challenges to clinical disease diagnosis and treatment. The virus detection methods available today mainly include virus isolation and culture, immunological specific antigen & serum antibody detection, and molecular biology detec-

Table 4. Analysis of direct immunofluorescence assay of infection in different age groups

	Minors				Adults			
	≤5 years old		5-18 (inclusive) years old		18-60 (inclusive) years old		>60 years old	
	Number of positives (cases)	Positive rate (%)	Number of positives (cases)	Positive rate (%)	Number of positives (cases)	Positive rate (%)	Number of positives (cases)	Positive rate (%)
RSV	17	20.99	2	13.33	2	20.00	0	-
PIV1	8	9.88	2	13.33	1	10.00	1	33.33
PIV2	7	8.64	1	6.67	1	10.00	0	-
PIV3	5	6.17	1	6.67	1	10.00	0	-
HAdV	15	18.52	2	13.33	2	20.00	0	-
Flu A	22	27.16	4	26.67	2	20.00	2	66.67
Flu B	7	8.64	3	20.00	1	10.00	0	-
Total	81		15		10		3	

Note: RSV: respiratory syncytial virus; PIV1: parainfluenza virus 1; PIV2: parainfluenza virus 2; PIV3: parainfluenza virus 3; HAdV: human adenovirus; Flu A: influenza A virus; Flu B: influenza B virus.

Table 5. Detection value of multiplex real-time quantitative PCR

	Sensitivity (%)	Specificity (%)	Accuracy (%)
RSV	90.32	98.71	97.31
PIV1	88.89	100.00	98.92
PIV2	91.67	100.00	99.46
PIV3	100.00	100.00	100.00
HAdV	91.30	98.77	98.85
Flu A	97.06	98.03	97.85
Flu B	93.75	100.00	99.46
Total	93.01	99.40	98.69

Note: RSV: respiratory syncytial virus; PIV1: parainfluenza virus 1; PIV2: parainfluenza virus 2; PIV3: parainfluenza virus 3; HAdV: human adenovirus; Flu A: influenza A virus; Flu B: influenza B virus.

Table 6. Detection value of direct immunofluorescence assay

	Sensitivity (%)	Specificity (%)	Accuracy (%)
RSV	46.43	96.20	88.71
PIV1	56.25	98.24	94.62
PIV2	46.67	98.83	94.62
PIV3	46.15	99.42	95.70
HAdV	43.33	94.87	86.56
Flu A	46.67	93.62	82.26
Flu B	64.29	98.84	96.24
Total	48.45	97.28	91.24

Note: RSV: respiratory syncytial virus; PIV1: parainfluenza virus 1; PIV2: parainfluenza virus 2; PIV3: parainfluenza virus 3; HAdV: human adenovirus; Flu A: influenza A virus; Flu B: influenza B virus.

tion [31, 32]. Of them, virus culture and isolation are considered the "golden standard" for virus detection. However, the slow virus growth, demanding experimental conditions, and complicated operation, together with the presence of a certain biological risk and the infeasibility of early diagnosis all makes it unsuitable for the detection of large quantities of clinical specimens. Antigen detection methods such as immunofluorescence, enzyme immunoassay, gold-labelled antigen detection method, on the other hand, are easy to operate, with high specificity and feasibility for early diagnosis; but they need monoclonal antibodies that specifically bind to known antigens and can cover multiple non-cross-reactive serotypes, which require high quality of antibodies and are technically difficult to develop [33]. Therefore, antigen detection methods also have certain limitations in clinical application. With the rapid development of molecular biology detection technology, MRT-PCR, which applies fluorescent labeling to target fragment analysis, has played an increasingly critical role in clinical practice, especially in the detection of respiratory viruses.

In this research, 7 viruses associated with LRTI were detected by MRT-PCR and DFA, and the application value of MRT-PCR was analyzed. The results showed that among the 7 pathogens detected by MRT-PCR, 140 positive specimens were detected in 114 patients, with the top three pathogens with the highest positive rates being Flu A (36 [19.35%]), RSV (30 [16.13%]) and HAdV (23 [12.37%]), and the pathogen with the lowest positive rate being PIV3 (9 cases [4.84%]). The total sensitivity and

accuracy of MRT-PCR assay, which were 93.01% and 98.69% respectively, were statistically higher than those of 48.45% and 91.24% of DFA, which is consistent with previous findings. Choudhary et al. [34] used MRT-PCR to detect three groups of 18 respiratory viruses and compared its sensitivity and specificity with RT-PCR. The results showed that MRT-PCR was a rapid, economical, specific and highly sensitive detection method for respiratory viruses. The essence of MRT-PCR is to add primers for multiple genes and probes labeled with different markers in the same reaction system to realize simultaneous amplification and detection of multiple genes. There is also co-infection of multiple viruses in the tested specimens, with early studies indicating a co-infection rate of 2-82% [35-37], but the mechanism of co-infection has not been fully clarified. In addition, the results of this study showed that the infection rates of various viruses were higher among children. The reason may be that 90% of URTI is caused by viruses, which can be easily transmitted by virus-containing droplets and fog droplets. Moreover, children are more susceptible to infection due to their weaker immune resistance. Quantification of genomic viral load might improve specificity of virus detection, with higher organism burden being associated with higher risk of complications and severe disease in adults and children [38, 39]. Children, especially those under 5 years old, have an increased risk of co-infections. Multiple detection methods containing multiple viral gene targets in one test tube have the advantage of rapid detection of multiple potential viral pathogens at the same time [40]. MRT-PCR has allowed simultaneous detection of multiple respiratory viruses in a short time. Compared with the single approach, the multiplex diagnostic approach has higher sample throughput, shorter turnaround time, and a smaller amount of sample requirement [41].

It is worth mentioning that in the MRT-PCR detection, some false positive samples appeared, which may be related to the interpretation method of the results. Since the method is manually interpreted according to the results of the amplification curve, some atypical amplification curves may be generated in the experiment, resulting in misjudgment and false positive results.

All in all, compared with the traditional DFA, MRT-PCR has the advantages of simple detection, high sensitivity, rapidity and low cost, with greater clinical value than the immunological detection methods and single respiratory pathogen NA detection methods currently on the market. However, this study still shows some limitations. In terms of detection methods, although MRT-PCR detection has high sensitivity and specificity, it is limited by the number of fluorescence types in each reaction. Generally, one reaction can only detect 3-4 pathogens, and detecting more pathogens means that more reactions are required, which greatly reduces detection convenience and throughput [42]. From the perspective of research samples, recruiting larger research samples across all seasons can provide a clearer picture of various etiologies. Judging from the content of detection, the types of pathogens detected in this study are limited. Other pathogens such as mycoplasma pneumoniae which is also one of the pathogenic factors, can be investigated in future research.

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Disclosure of conflict of interest

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