

Rapid diagnosis of human adenovirus B, C and E in the respiratory tract using multiplex quantitative polymerase chain reaction

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Abstract. Human adenovirus (HAdV) is increasingly recognized as a major cause of human respiratory tract viral infections. Its outbreaks and epidemics in various populations resulted in considerable morbidity and mortality. Therefore, a rapid and specific assay for HAdV in clinical samples is of crucial importance to diagnosing HAdV infections. The present study aimed to develop and evaluate a multiplex quantitative polymerase chain reaction (qPCR) assay for the rapid detection and accurate quantification of HAdV B, C and E. The lower limit of detection for this assay was two genomic copies per reaction, and quantitative linearity ranged from 2 to 2×10^6 copies per reaction of the input viral DNA. Furthermore, 3,160 throat swab samples that tested HAdV negative by the immunofluorescence assay were collected and retested using the multiplex qPCR assay. The results showed that 2,906 samples were HAdV negative and the other 254 samples were HAdV positive. The HAdV species identified included B (184 samples), C (51 samples), and E (39 samples). Among the three HAdV species, HAdV B and E were detected from 8 samples, and HAdV C and E were detected from other 12 samples. The overall results demonstrated that the sensitivity and specificity of the proposed assay

were 100% (254/254) and 99.6% (2894/2906), respectively. From the perspective of routine clinical diagnosis, this assay represented a rapid (≤ 1.5 h) and economic strategy, and had the potential to be used for the rapid and accurate diagnosis of human respiratory infections caused by HAdV B, C and E.

Introduction

Human adenoviruses (HAdVs), also known as nonenveloped double-stranded DNA viruses, are members of the Adenoviridae family with a size ranging from 70 to 100 nm (1). HAdV infections are now recognized as a significant source of human morbidity and mortality, and affect patients worldwide and across all age groups. HAdV infections are readily transmissible and, in some cases, highly contagious. Currently, more than 84 genotypes, including all previously characterized serotypes, have been identified and grouped into seven different species (A-G) (<http://hadv.wg.gmu.edu/>), based on their immunochemical responses, nucleic acid characteristics, hexon and fiber protein characteristics, biological properties, and phylogenetic relationships (2-6). This subdivision also has some clinical relevance, as distinct adenovirus species show a preference for specific organs: C, E, and some B species typically infect the respiratory tract; other B species infect the urinary tract; species A and F target the gastrointestinal tract; and species D target the eyes (7,8). Among the HAdV-associated respiratory diseases, viruses in species HAdV B (HAdV-3, -7, -11, -14, -16, -21, -34, -35, -50, -55, and -66), species HAdV C (HAdV-1, -2, -5, and -6), and species HAdV E (HAdV-4) are recognized as the main pathogens responsible for the respiratory tract infections (9-12). Researchers are constantly inventing and optimizing methods to detect adenovirus so as to avoid the overuse of antibiotics, improve the level of diagnosis and treatment, and provide a scientific basis for the prevention and control of HAdV causing respiratory illness.

Current standard diagnostic approaches rely mainly on cell culture and immunofluorescence assay (13). Cell culture is considered as gold standard because of its broad applicability and high specificity. However, it is time-consuming and

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usually takes 7-12 days to obtain a positive culture. Enzyme immunoassays and immunofluorescence give rapid results, but their relative lack of sensitivity and the availability of reactive antisera can be limiting factors (14-25). Advances in conventional reverse transcription-polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays have greatly facilitated the etiological study of respiratory infections due to their higher sensitivity and specificity. These assays can also reduce labor and cost by detecting more than one pathogen in a single reaction using multiple probes (26,27). Single and multiplex conventional and quantitative PCR assays using species- and type-specific primers/probes have been described for some HAdVs that cause respiratory tract infection. For example, Metzgar *et al* developed a series of single- and multiplex qPCR assays to detect and discriminate HAdVs implicated in adult epidemic acute respiratory diseases, including HAdV-B3, E4, B7, B11, B14, and B21 (28). Lu *et al* developed and validated sensitive and type-specific qPCR assays for detecting and identifying the epidemic-associated respiratory HAdVs, types B3, E4, B7, B11, B14, B16, and B21, to facilitate rapid outbreak response (29). Washington *et al* developed a multiplexed PCR assay for a similar panel of five HAdVs (HAdV types B3, E4, B7, B14, and B21) based on Luminex xMAP technology (30). HAdV types vary in terms of clinical manifestation and outbreak severity in respiratory infection. Accurate and rapid identification of HAdV infection in the respiratory tract not only avoids unnecessary antibiotic prescription but also prevents or inhibits HAdV-related outbreaks. Although many multiplex PCR assays have been developed and applied for detecting HAdV in respiratory tract infections, the assay covering all the serotypes (C1, C2, B3, E4, C5, C6, B7, B11, B14, B16, B21, B34, B35, B50, B55, and B66) of HAdV B, C and E has not been reported.

In this study, a multiplex qPCR assay was developed to detect HAdV B, C and E by employing four primer pairs and four virus-specific probes (including internal standard quality control). The study demonstrated that the aforementioned assay was a promising method to potentially reduce missed diagnosis or misdiagnosis in detecting HAdV, which could cause respiratory tract infections.

Materials and methods

Clinical samples collection. This study was approved by the ethics committee of Shenzhen Shajing Hospital affiliated to Guangzhou Medical University (Shenzhen, China) prior to commencement. After informed written consent from adult patients or legal representatives of children, throat swab samples, which are determined to be HAdV negative by D3 Ultra DFA Respiratory Virus Screening & ID kit (B&C Biological Technology Co., Shanghai, China), were collected using universal transport media (BD Biosciences, Franklin Lakes, NJ, USA). From February 2016 to June 2017, 3,160 cases of adenovirus-negative throat swab samples were collected from 3 districts (Baoan, Futian, Luohu) of Shenzhen.

Nucleic acid extraction. For nucleic acid extraction, all throat swab samples were centrifuged at 3,000 x g for 5 min at 4°C to collect virus attached to cells. Nucleic acid was extracted from 200 µl of each sample using the Nucleic Acid Extraction

kit according to the manufacturer's protocol (Huiyan Biotechnology Company, Shenzhen, China). Then, 200 µl of the sample was extracted, and nucleic acid was eluted into a volume of 150 µl. Nucleic acid was then processed immediately for qPCR.

Primers and TaqMan probe design. Potential primer and probe sequences were selected after being thoroughly analyzed using the Premier Biosoft software and the BLAST (National Centre of Biotechnology Information, National Institutes of Health, Bethesda, MD, USA) program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). BLAST analyses were performed to check the specificity of the primers and the probes against other closely related genome sequences. The Premier Biosoft software allowed the combination of existing proven primer and probe sequences with sequence targets to yield multiplexed assay designs and choose identical cycling parameters. The primer pairs and probes specific to each virus were grouped in a multiplex reaction on the basis of the following criteria: internal primer binding properties for hairpin and primer-dimer potential, length of the desired amplicon, G-C content, and melting temperatures (T_m) of the probes and primers. When the parameter settings were defined, Premier Biosoft software assessed each multiplex component as it was added to the assay pool, avoiding the risk of cross-reactivity issues. The primers and TaqMan probes were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Shanghai, China). TaqMan hydrolysis probes were labeled at the 5'-end with the reporter dyes and quenched with Blackhole Quencher 1 or 2 (BHQ 1 or 2) at the 3'-end. For more convenience, the method was somewhat modified and each TaqMan probe was labeled with a different fluorescent reporter dye (FAM, VIC, ROX, or CY5) (Table I).

Quality control/Internal standard plasmid construction. A 1,000-bp random DNA sequence was generated using the DNAMAN software (Lynnon Biosoft, Vaudreuil, QC, Canada), and four bases (A, T, C, and G) accounted for 25%, respectively. The generated sequence was then synthesized by Takara and introduced into plasmid pBSK.

Standard curve. Three inactivated HAdV isolates were provided as gifts by Professor Rong Zhou (State Key Laboratory of Respiratory Diseases, Guangzhou Medical University, Guangdong, China). These clinical isolates were sent to Hexin Health Technology Company (Guangdong, China) and identified using DHI, immunofluorescence, flow cytometry, and qPCR. The fragments amplified with HAdV primers were sequenced and compared with HAdV sequences in NCBI (National Centre of Biotechnology Information, National Institutes of Health). After comparison, the three clinical isolates were confirmed as HAdV B3, C2, and E4. DNA of three isolates was extracted from 200 µl samples as described earlier.

The concentration of three isolates was detected using the QX100 drop digital PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The droplet digital PCR (ddPCR) reaction mixture consisted of 10 µl of a 2 x ddPCR Master Mix (Bio-Rad Laboratories, Inc.), 2 µl of HAdV primers/probes, and 5 µl of sample nucleic acid solution in a final volume of

Table I. Primers and probes used in this study.

Virus serotypes	Oligo	Primer/probe sequence (5'-3')	5' label	3' label	Genomic region	Amplicon size (bp)
HAdV-B	Forward	CACATGGGAGCCAGGAGT	NO	NO	1285-1303	118
	Reverse	RAACATGGCCAGATCGCAC	NO	NO	1383-1402	
	Probe	TCTGTCCGAGGTCCTGACGGTT	6-FAM	BHQ1	1322-1344	
HAdV-C	Forward	YAACCCCTTYTCKGGACCTC	NO	NO	316-337	118
	Reverse	CAGTTGCTCTGCCTCTCCA	NO	NO	375-394	
	Probe	CATTCAGTCGTAGCCGTCCGC	VIC	BHQ1	349-370	
HAdV-E	Forward	CCTGCATGAAAGTCTTTGTTGTC	NO	NO	637-660	114
	Reverse	GTGAAGGTCAGAGACTGGTTG	NO	NO	730-751	
	Probe	CTGAGATCAGCGACTACTCCGGAC	ROX	BHQ2	685-709	
HAdV-Ref	Forward	GCTAGTCTCAAGAGTCTGGAAG	NO	NO	NO	91
	Reverse	ACGCCTTCGTCTTGGTGTC	NO	NO	NO	
	Probe	CCGTCAGCATCCTCGCATCAAGCA	CY5	BHQ2	NO	

20 μ l. The entire reaction mixture was loaded into a disposable plastic cartridge (Bio-Rad Laboratories, Inc.) together with 70 μ l of droplet generation oil (Bio-Rad Laboratories, Inc.) and placed in the droplet generator (Bio-Rad Laboratories, Inc.). After processing, the droplets generated from each sample were transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany). PCR amplification was carried out on a T100 thermal cycler (Bio-Rad Laboratories, Inc.) using a thermal profile beginning at 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec, 1 cycle of 95°C for 10 min, and ending at 12°C. After amplification, the plate was loaded on the droplet reader (Bio-Rad Laboratories, Inc.) and the droplets from each well of the plate were read automatically at a rate of 32 wells per hour. ddPCR data were analyzed with Quanta Soft analysis software (Bio-Rad Laboratories, Inc.), and the quantification of the target molecule was presented as the number of copies per μ l of PCR mixture.

Then, three HAdV isolates B3, C2, and E4 were equally mixed, and standard curves for adenovirus were drawn by performing qPCR on tenfold serial dilution in DNase- and RNase-free H₂O of the corresponding standard virus templates ranging from 10⁸ to 10² copies (equivalent genomes)/ml. The standard curves for multiplex qPCR were obtained by linear regression analysis of the threshold cycle (C_T) value (y axis) vs. the log of the initial copy number present in each sample dilution (x axis), allowing the determination of the correlation coefficient (R²). The amplification efficiencies (E) of reactions were calculated from the curves using the equation: $E = (10^{(-1/\text{slope})} - 1) \times 100\%$ (31,32). The resulting curves were used to determine the number of viral genome copy corresponding to each virus found in the experimental samples.

Multiplex qPCR. For the multiplex assay, the reaction mixture was prepared, containing 25 μ l of iQ Multiplex Powermix (Bio-Rad Laboratories, Inc.), 0.5 μ l of each primer (final concentrations, 200 nM), and 0.375 μ l of each probe (final concentrations, 75 nM). The final volume of the mixture was adjusted to 30 μ l with DNase- and RNase-free H₂O (Nacalai

Tesque, Inc., Kyoto, Japan). Finally, 20 μ l of sample DNA was added to the reaction mixture on ice. qPCR was performed using a Roche Light Cycler 480 (LC480) (Roche Diagnostics, Basel, Switzerland) with the following amplification protocol: Pre-denaturation for 2 min at 95°C; 40 cycles of denaturation for 10 sec at 95°C, and annealing and primer extension for 30 sec at 60°C. The fluorescence emitted by FAM, VIC, ROX, or CY5 dyes was measured simultaneously and independently at the end of the annealing step. For a background control, no template control (NTC, water) was used.

The results were expressed as the quantification cycle (C_q, cycle at which the fluorescence surpasses the background level). Lower C_q values corresponded to a greater amount of initial template and a negative result was considered to have a C_q value of 40 or more cycles. So the cutoff point was fixed empirically at a C_q value of 40. The status of the sample (positive or negative) was determined first using this chosen cutoff to assess the variability of the observed C_q values. However, the use of such arbitrary cutoffs was not ideal because they might be either too low (eliminating valid results) or too high (increasing false-positive results) (33). Hence, those PCR products, which were initially identified as adenovirus-positive, were sent to the Invitrogen Biotechnology Company (Invitrogen, Shanghai, China) for conventional Sanger sequencing. The nucleotide sequences of HAdV B, C, and E were screened using the BLAST program on the NCBI website to determine the sequence origin.

For a good analysis of the presented results, the performances of the multiplex assays were also assessed using the confusion matrix that contained information about the two assays. The following parameters were defined: True positive (TP): The number of 'positive' samples categorized as 'positive'; false positive (FP): The number of 'negative' samples categorized as 'positive'; false negative (FN): The number of 'positive' samples categorized as 'negative'; true negative (TN): The number of 'negative' samples categorized as 'negative'. Based on these four metrics, the sensitivity [$S_n = TP / (TP + FN)$] and the specificity [$S_p = TN / (TN + FP)$] of the multiplex assay could be evaluated (33).

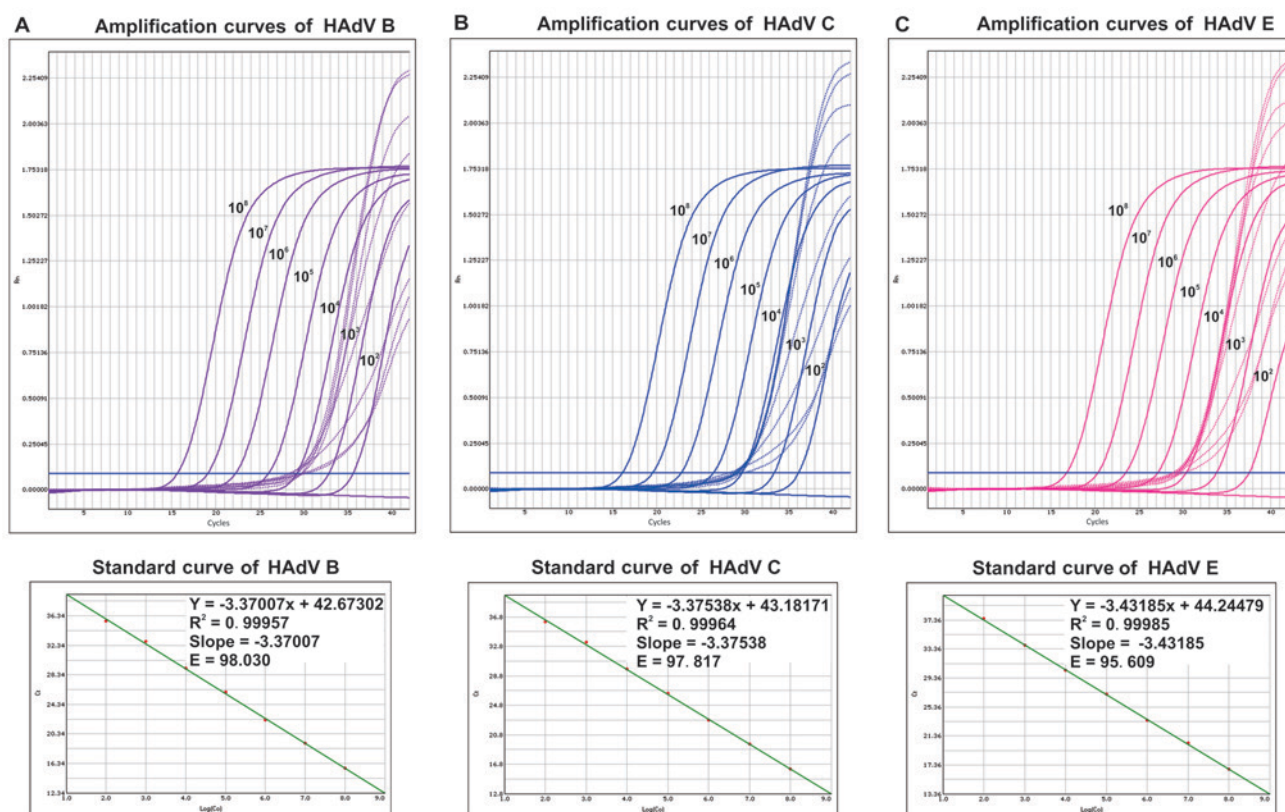


Figure 1. Amplification plot and standard curve of adenovirus serotypes (A) B3, (B) C2 and (C) E4 in multiplex qPCR assay. 10-fold serial dilutions of HAdV serotypes C2, B3, and E4 DNA were used for standard curve construction. DNA concentrations were (from left to right) 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , and blank. For each dilution, the normalized fluorescence signal (Rn) was plotted against the PCR cycle number. A representative amplification plot and a standard curve plot are shown. qPCR, quantitative polymerase chain reaction; HAdV, human adenovirus.

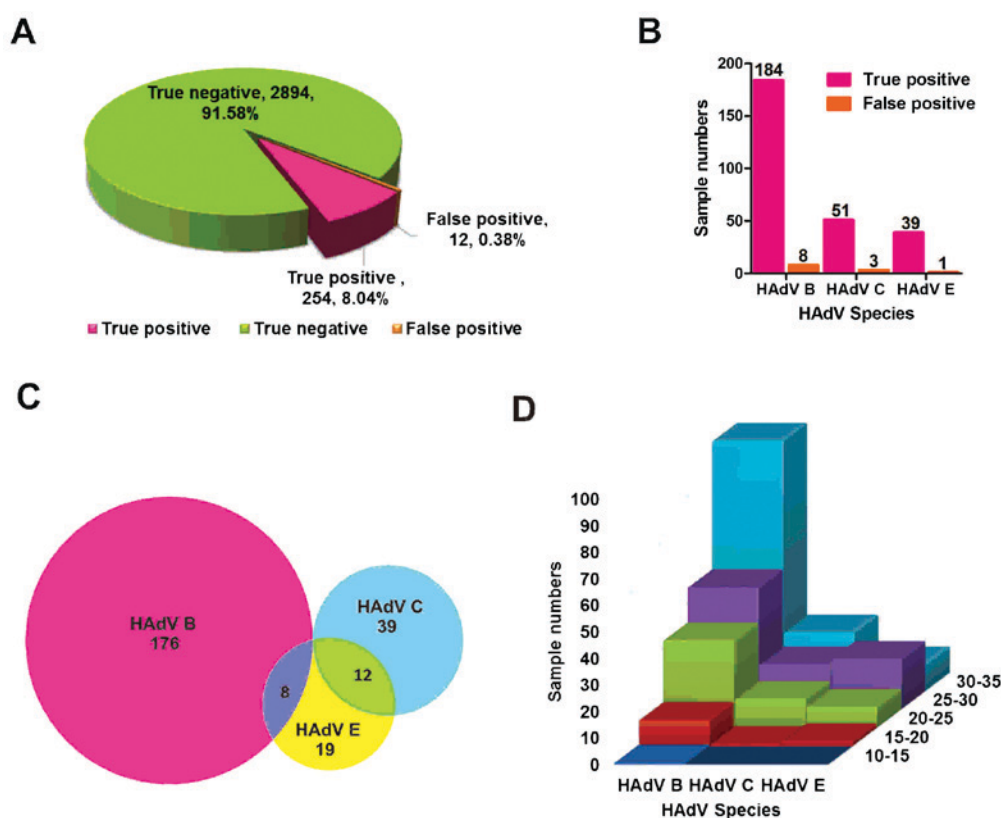


Figure 2. Detection and distribution of adenovirus in swab samples. (A) Detection of adenovirus in adenovirus-negative throat swab samples tested using immunofluorescence assay. (B) Distribution of HAdV B, C, and E in true-positive and false-positive samples. (C) Distribution of HAdV B, C, and E in true-positive samples. (D) Distribution of Ct values of HAdV B, C, and E in true-positive samples. HAdV, human adenovirus.

Table II. Comparison of the primers of using multiplex qPCR assay to detect adenovirus serotypes in respiratory tract infection.

Adenovirus species	Serotypes	Target gene	References
B	B1: 3, 7, 16, 21, 50 and 66; B2: 11, 14, 34, 35 and 55	E3	This study
C	1, 2, 5 and 6	E3	
E	4	E3	
A	12	Hexon	Pierce <i>et al</i> (34), 2012
B	3, 7, 16, 21 and 34	Hexon	Popowitch <i>et al</i> (35), 2013
C	2, 5	Hexon	Rand <i>et al</i> (39), 2011
D	17, 48	Hexon	Poritz <i>et al</i> (40), 2011
F	40, 41	Hexon	Heim <i>et al</i> (46), 2003
E	4	Hexon	Loeffelholz <i>et al</i> (36), 2011
B	3	Hexon	Loeffelholz <i>et al</i> (36), 2011
C	1,5	Hexon	
B	3,7,11, 16, 21, 34 and 35	Fiber	Xu <i>et al</i> (37), 2000
C	2,5	Fiber	
E	4	Fiber	
B	3,7, 11, 14, 16, 21 and 35	Hexon E2A fiber	Metzgar <i>et al</i> (38), 2005
B	3, 7	Hexon	Fujimoto <i>et al</i> (33), 2004
B	3,7, 11, 16, 21, 34 and 35	Hexon	Echavarria <i>et al</i> (10), 2003
C	1, 2, 5, 6	Hexon	
E	4	Hexon	

qPCR, quantitative polymerase chain reaction.

Results

Design of primers and probes. In the study of adenovirus infection in the respiratory tract, most of the literature reported primers and probes based on the hexon and fiber genes (Table I). HAdV serotypes that could be detected by designing primers for the hexon gene included HAdV-C1, C2, B3, E4, C5, C6, B7, B11, B14, B16, B21, B34, and B35. HAdV serotypes that could be detected by designing primers for the fiber gene included HAdV-C2, B3, E4, C5, B7, B11, B16, B21, B34, and B35. As more new adenovirus serotypes (HAdV-B50, B55, and B60) cause respiratory infections, primers for multiplex qPCR should be able to detect adenovirus serotypes as comprehensively as possible. Primers and probes were designed to detect 16 types so as to achieve the coverage of the main HAdV types that cause the respiratory tract infections: 3, 7, 11, 14, 16, 21, 34, 35, 50, 55, and 66 of HAdV species B; 1, 2, 5, and 6 of HAdV species C; 4 of HAdV species E. HAdVs associated with respiratory tract infections were subjected to a conservative analysis. Not only hexon and fiber genes were found to be highly conserved, but E3 genes were also highly conserved. Unique sets of PCR primers and TaqMan probes were designed to target highly conserved sequences in each viral hexon, fiber, and E3 genes. After thorough analysis using the Premier Biosoft software and the BLAST program, sets of primers and TaqMan probes designed for the E3 gene were finally selected for subsequent experiments.

Standard curve and dynamic range of the multiplex assay. Serial dilutions of mixed adenovirus standards were tested using multiplex qPCR, and individual standard curves were

constructed from the C_q values (Fig. 1). The multiplex assay could amplify each plasmid standard between 10^2 and 10^8 copies per reaction mixture with a strong linear relationship between the C_q values and the \log_{10} of the input number of copies (HAdV B3, $r^2=0.99957$; HAdV C2, $r^2=0.99964$; HAdV E4, $r^2=0.99985$). The slopes of the standard curves were -3.37007 for HAdV B3, -3.37538 for HAdV C2, and -3.43185 for HAdV E4.

The defined acceptance criteria for accurate and reproducible quantification were as follows: qPCR $R^2 \geq 0.98$; qPCR E among 90 and 110% (corresponding to the slope of the regression curve that should be between -3.1 and -3.6). For the multiplex assay, the qPCR E ranged from 80 to 120% (corresponding to the slope of the regression curve that should be between -3.9 and -2.9) (31). The regression analysis of the multiplex assay demonstrated that the R^2 and the PCR amplification E were all within the suitable range. The R^2 of the standard curves of each multiplex reaction were all greater than 0.99 along with the E values ranging from 95 to 98%, which was considered as acceptable for a multiplex qPCR assay.

Detection of clinical samples. A total of 3,160 throat swab samples that tested adenovirus negative by the immunofluorescence assay were retested using multiplex qPCR assay. The sensitivity and specificity of the multiplex qPCR assay were compared with those of the immunofluorescence assay. Further, 2,894 samples were detected as adenovirus negative, which was consistent with the findings of immunofluorescence assay. However, 266 samples were detected as adenovirus positive. The samples were sent for conventional Sanger sequencing to verify whether 266 adenovirus-positive

Table III. Different detection methods to detect the types of pathogens.

Assay	The full name	Pathogens types	References
Multiplex PCR	Multiplex qPCR assay	HAdV-B3, 7, 11, 14, 16, 21, 34, 35, 50, 55, 66; HAdV-C1, 2, 5, 6; HAdV-E4	This study
IF assay	Immunofluorescence assay	AdV, influenza virus A, influenza virus B, PIV1, -2, -3; RSV	This study
Film Array RP	The FilmArray respiratory panel multiplexed nucleic acid amplification test	AdV; CoV HKU1, NL63; influenza virus A (H1/2009, H1, H3); influenza virus B; MPV; PIV1, -2, -3, -4; RSV; RhV/EV	Pierce <i>et al</i> (34), 2012 Loeffelholz <i>et al</i> (36), 2011 Rand <i>et al</i> (39), 2011
PCR	qPCR	AdV; RSV -A,-B; influenza virus A (H1/2009, H1, H3), influenza virus B; MPV; PIV1, -2, -3; RSV; RhV/EV	Poritz <i>et al</i> (40), 2011 Pierce <i>et al</i> (34), 2012
DFA	Direct fluorescent antibody	AdV; hMPV; Flu A; Flu B; PIV1; PIV2; PIV3; RSV	Poritz <i>et al</i> (40), 2011
xTAG RVP (xTAG)	The Luminex xTAG RVP	AdV; influenza virus A (H1, H3); influenza virus B; MPV; PIV1, -2, -3; RSV (A/B); RhV/EV	Rand <i>et al</i> (39), 2011
Prodesse assays	Prodesse qPCR assays	Adenovirus; human metapneumovirus; influenza A virus; influenza B virus; parainfluenza viruses 1 to 3; respiratory syncytial virus	Loeffelholz <i>et al</i> (36), 2011

samples were TP. Through the BLAST online software sequence alignment, 254 samples were verified as TP and 12 samples were verified as FP (Fig. 2A). Among the 254 TP samples, HAdV B was detected in 184 samples, HAdV C was detected in 51 samples, and HAdV E was detected in 39 samples. Among the 12 FP samples, HAdV B was detected in 8 samples, HAdV C was detected in 3 samples, and HAdV E was detected in 1 sample (Fig. 2B). Co-infections with two HAdVs B and E were detected in 8 samples and co-infections with HAdVs C and E in 12 samples. No detection of co-infections of more than two HAdVs was reported (Fig. 2C). Briefly, the distribution frequencies of C_q values for a set of 254 analyzed adenovirus-positive samples is displayed in Fig. 2D. Further, 66 positive samples had a C_q value less than 25, 14 a C_q value less than 20, and one with a C_q value less than 15 in HAdV B species. The aforementioned data indicated that the sensitivity and the specificity of multiplex qPCR were much better than those of immunofluorescence assay.

Comparison of the proposed assay with the reported assays. Multiplex qPCR methods in other studies were used to detect not only adenovirus but also other respiratory pathogens (Tables II and III). The multiplex qPCR in this study was used to detect only HAdV B, C and E. 16 HAdV serotypes associated with respiratory infections could be detected using primers and probes in this study. By comparing the number of detected serotypes, sensitivity and specificity, the more comprehensive the serotype detection, the higher the sensitivity of the assay. Therefore, the sensitivity and specificity of this study were still 100% (254/254) and 99.6% (2894/2906), respectively, in the large sample size of the respiratory tract.

Discussion

In this study, a multiplex qPCR assay was developed that could simultaneously quantify 16 kinds of HAdV serotypes causing respiratory tract infections. The multiplex qPCR in this study could not only simultaneously and accurately detect the three species of HAdV (HAdV B, C and E), but also had a high sensitivity (100%).

The multiplex qPCR assay to detect throat swab samples found that 254 patients were adenovirus positive but these adenovirus-positive patients did not get timely diagnosis and treatment due to the poor sensitivity of the immunofluorescence method. Therefore, it is not appropriate to use DFA to detect swab samples and obtain the epidemiological conclusion of adenovirus negative. This indicated that the sensitivity of multiplex qPCR was higher than that of the immunofluorescence assay. Compared with the traditional cell culture, the whole experiment took only about 1.5 h from the extraction of nucleic acid to qPCR results. This could not only timely make an accurate diagnosis based on the qPCR result but also improve work efficiency.

Multiplex PCR is achieved using separate probe and primer sets for each target sequence, but it requires careful optimization of assay parameters and primer/probe design. During qPCR, each amplicon simultaneously amplifies and is independently detected by different fluorescence spectra. In previous studies, most of the primers were designed based on the hexon and fiber genes used to detect adenovirus in the respiratory tract (10,32,34-38). However, these primers did not fully detect the adenovirus serotypes that caused respiratory infections. Therefore, in this study, the highly conserved E3 gene was selected to design primers and probes. The multiplex qPCR was linear from 10^2 to 10^8 copies/ml. The amplification

Table IV. Comparison of the sensitivity and specificity of using multiplex qPCR assay to detect adenovirus in respiratory tract infection.

Sample numbers detected	Sample numbers				Sensitivity		References
	(TP) ^a +/+	(FP) ^b +/-	(FN) ^c -/+	(TN) ^d -/-	TP/(TP+FN) (%)	TN/(TN+FP) (%)	
Multiplex PCR/IF assay	254	12	0	2,894	254/254 (100)	2,894/2,906 (99.6)	This study
Film Array RP/PCR	11	0	13	191	11/24 (45.8)	191/191 (100)	Pierce <i>et al</i> (34), 2012
Film Array RP/xTAG RVP	9	0	1	190	9/10 (90)	190/190 (100)	Rand <i>et al</i> (39), 2011
Film Array RP/DFA	22	32	4	270	22/26 (84.6)	270/302 (89.4)	Poritz <i>et al</i> (40), 2011
Film Array RP/ Prodesse assays	6	0	5	181	6/11 (54.5)	181/181 (100)	Loeffelholz <i>et al</i> (36), 2011

^aTP, true positive; ^bFP, false positive; ^cFN, false negative; ^dTN, true negative; qPCR, quantitative polymerase chain reaction.

efficiency of HAdV B, C, and E reached more than 99%. The multiplex qPCR was specific for three adenovirus species independently, without mutual cross-reactivity. The results indicated that the E3 gene was selected to design primers and probes, which not only detected more adenovirus serotypes but also had good sensitivity and specificity.

Over the past few years, multiplex qPCR assays have significantly contributed to the rapid identification of the virus associated with acute respiratory infections, allowing the quick adoption of the measures and preventive strategies to avoid the spread of the disease with high transmissibility (34,35,37,39,40). The sensitivity of multiplex qPCR is an important consideration in the development of diagnostic assays; even extremely low adenoviral levels can cause respiratory infections. Many newly identified adenovirus serotypes, which are not included in the detection range of multiplex qPCR, can also cause respiratory infections. Table IV shows that the sensitivity and specificity of detecting adenovirus in this study were compared with those in previous multiplex qPCR studies. It was noteworthy that 100% sensitivity could be achieved for a relatively large number of samples, compared with 45.8, 54.5, 84.6, and 90% in previous studies. Multiplex qPCR in this study may be helpful in reducing missed diagnosis effectively by detecting a wider range of adenovirus serotypes.

In this study, a multiplex qPCR was developed that could rapidly detect and accurately quantify HAdV B, C, and E in the respiratory tract with high sensitivity and specificity. All the serotypes of HAdV B, C, and E in the respiratory tract could be detected using this assay. The assay simplified the routine service and decreased the overall costs (41-44). These benefits had a positive effect on clinical service, allowing clinicians to tailor patient management or initiate antiviral therapy more promptly (45).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YHD, JKD and HLL conceived and designed the experiments. YXL, CFM, HJZ, QL, HLL and YT performed the

experiments. YXL, JKD, HLL and RC analyzed the data. YHD and HLL contributed reagents/materials/analysis tools. YXL and JKD wrote the paper.

Ethics approval and consent to participate

The medical ethics committee of the Shenzhen Shajing Hospital Affiliated to Guangzhou Medical University approved the present study. All patients provided written consent.

Patient consent for publication

Written informed consent was obtained.

Competing interests

The authors declare that they have no competing interests.

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