COMPOSITIONS, KITS, METHODS FOR DETECTING AND IDENTIFYING PATHOGENS THAT CAUSE RESPIRATORY TRACT INFECTIONS AND USE THEREOF

TECHNICAL FIELD

[0001] The present application relates to the field of molecular biological detection, specifically, to the field of detection of pathogens that cause respiratory tract infections. More specifically, the present disclosure is capable of simultaneously detecting *Influenza A virus*, *Influenza B virus*, *Rhinovirus*, *Respiratory Adenovirus*, and *Respiratory syncytial virus* and *Mycoplasma pneumoniae*.

BACKGROUND

[0002] Infectious respiratory system diseases are the most common and frequently-occurring diseases in clinical practice. These diseases have similar clinical symptoms and epidemiological characteristics, and thus is difficult to be identified and determined for the pathogen species infected according to clinical symptoms and routine laboratory tests. Respiratory pathogens can be transmitted through the air. The pathogens that cause acute respiratory diseases have the characteristics of strong infectivity, rapid spread, short incubation period, and acute onset, and may cause a wide range of acute diseases of upper and lower respiratory tract, which seriously endanger human health. Common respiratory pathogens include the followings.

[0003] Influenza virus, also referred to as flu virus, is an RNA virus that causes influenza in humans and animals. Human influenza viruses, classified into three types of A, B, and C, are the pathogens of influenza (flu). Avian influenza (AI), also known as bird flu, is an infectious disease caused by a subtype of *Influenza A virus* (also known as avian influenza virus). Taxonomically, the influenza virus belongs to the Influenza virus genus in the family Orthomyxoviridae. It can cause acute upper respiratory tract infection and spread rapidly through the air, and often leads to periodic pandemics that occur globally, such as the “Spanish Influenza” that killed more than 20 million people worldwide in 1918-1919, the “Asian Influenza” that occurred in 1957, and the “Hong Kong Influenza” that occurred in 1968 and the “Russian Influenza” occurred in 1977, as well as the H7N9 avian influenza occurred in 2013.

[0004] *Respiratory syncytial virus* (RSV) is an RNA virus belonging to the Pneumonia genus in the family Paramyxoviridae. Clinical studies have shown that RSV is the most common pathogen causing viral pneumonia. RSV infection is more common in infants under three years of age, with the main symptoms being high fever, rhinitis, pharyngitis and laryngitis, and later manifesting as bronchiolitis and pneumonia, and may be complicated by otitis media, pleurisy and myocarditis, etc. in a small number of sick children. Infection in adults and older children mainly manifests upper respiratory tract

infections.

[0005] *Adenovirus* (Adv) belongs to the family Adenoviridae. According to their different immunological, biological and biochemical characteristics, *Adenovirus* is classified into 7 subspecies from A to G, with a total of 52 serotypes. Different serotypes have different organ affinities and cause corresponding clinical manifestations, and may infect the respiratory tract, gastrointestinal tract, urethra, bladder, eyes, liver, and the like.

[0006] Human *Rhinovirus* (HRV) is a non-enveloped, single-stranded RNA virus belonging to the genus Enterovirus in the Picornaviridae family. Human *Rhinovirus* has the same morphological structure and genome structure as enterovirus. It forms a spherical shape with a diameter of 15-30 nm, and has a nucleocapsid with icosahedral symmetry and no envelope. The genome is single-stranded, positive-strand RNA that is about 7500 nucleotides in length, and includes only one reading frame that is about 6500 nucleotides in length; the reading frame is initiated 611 nucleotides from 5' end, and stops 42 nucleotides from the poly A tail. Human *Rhinovirus* is a highly contagious and ubiquitous virus that is usually transmitted through direct contact with respiratory droplets/microdroplets, and also through contaminated surfaces, including person-to-person direct contact. HRV has three distinct subgroups A, B, and C consisting of 80, 30, and 56 types, respectively. It is the most serotyped virus among human infectious viruses. HRV's genetic diversity (>150 types) makes it very difficult to develop an effective vaccine. A certain degree of immune protection may be gained after infection, however, previous infection cannot provide complete immunity due to too many serotypes of HRV, that is to say, other serotypes may still lead to reinfection. HRV is the leading cause of the "common cold" manifesting as rhinorrhea, sore throat, cough, and malaise. HRV also causes many other respiratory diseases, such as asthma, chronic obstructive pulmonary disease, otitis media, angina, pneumonia and acute bronchiolitis. HRV co-infection with other respiratory viruses, more commonly *Respiratory syncytial virus*, *Adenovirus*, and the like, occurs frequently and aggravate the patient's condition, seriously affecting the life quality of the patients.

[0007] Mycoplasma pneumonia (MP) is a common pathogen of community-acquired pneumonia. Infection with MP can cause atypical pneumonias with pathological changes mainly manifesting as interstitial pneumonia, sometimes complicated by bronchial pneumonia. MP is mainly transmitted through droplets, has an incubation period of 2 to 3 weeks and presents the highest incidence in adolescents. MP causes mild clinical symptoms with only general respiratory symptoms such as headache, sore throat, fever and cough in most patients, and a few would have a persistent high fever, severe cough, and a rapidly progressing disease, leading to severe or critical conditions such as respiratory failure, multiple organ dysfunction in a short period of time.

[0008] Conventionally, some technologies are used for detecting respiratory tract infection pathogens, such as Chinese invention patent CN113186342A, which can detect

18 kinds of pathogens; CN111074001A, which can detect 9 kinds of pathogens. However, these techniques still face the problems of poor detectable rate and accuracy due to the complexity of the pathogens to be detected.

[0009] Therefore, there is a need in the art for a detection kit for rapidly and accurately detecting the most common respiratory tract infection pathogens with a high detectable rate, accuracy and sensitivity.

SUMMARY

[0010] Accordingly, the detection of various viruses is required to be specifically configured in order to improve the detectable rate and accuracy. Taking the nucleic acid detection for *Rhinovirus* as an example, since both human *Rhinovirus* and enterovirus belong to the genus Enterovirus, many reagents or methods for the detection cannot distinguish between human *Rhinovirus* and enterovirus, or can distinguish them but with poor specificity. The reason why these reagents cannot accurately detect *Rhinovirus* is that only the conservative property of 5' terminal untranslated region of the genus Enterovirus is considered when designing primers and probes, so *Rhinovirus* cannot be well differentiated from enterovirus. Against gene sequences of different mutation types of *Rhinovirus*, the inventors have designed two probes that can cover various mutation types of *Rhinovirus* without non-specific binding to enterovirus. By using such two probes with completely different sequences but the same fluorescent labels, the *Rhinovirus* can be detected accurately and effectively.

[0011] Taking the detection of *Respiratory Adenovirus* as an example, two forward primers and two identical fluorescence-labeled probes are designed for various genotypes of *Adenovirus* in order to ensure the accuracy of the detection results, since there are 52 serotypes of *Adenovirus* among which types 3, 7, 11, 14, 16, 21, 34, 35, 50 and 55, etc. capable of causing respiratory infections in humans and recombination of 2 or more *Adenovirus* strains can produce a new type of *Adenovirus*.

[0012] In a first aspect, the present application provides a composition for detecting and identifying a pathogen that causes a respiratory tract infection, the composition including:

a first nucleic acid composition, including:

an *Influenza A virus* forward primer as set forth in SEQ ID NO:1, an *Influenza A virus* reverse primer as set forth in SEQ ID NO:2, and an influenza A probe as set forth in SEQ ID NO:3;

an *Influenza B virus* forward primer as set forth in SEQ ID NO:4, an *Influenza B virus* reverse primer as set forth in SEQ ID NO:5, and an influenza B probe as set forth in SEQ ID NO:6;

a *Rhinovirus* forward primer as set forth in SEQ ID NO:7, a *Rhinovirus* reverse primer as set forth in SEQ ID NO:8, a *Rhinovirus* probe as set forth in SEQ ID NO:9, and a *Rhinovirus* probe as set forth in SEQ ID NO:10; and

a second nucleic acid composition, including:

a *Respiratory Adenovirus* forward primer as set forth in SEQ ID NO: 11, a *Respiratory Adenovirus* forward primer as set forth in SEQ ID NO: 12, a *Respiratory Adenovirus* reverse primer as set forth in SEQ ID NO: 13, a *Respiratory Adenovirus* probe as set forth in SEQ ID NO: 14, and a *Respiratory Adenovirus* probe as set forth in SEQ ID NO: 15;

a *Respiratory syncytial virus* forward primer as set forth in SEQ ID NO:16, a *Respiratory syncytial virus* reverse primer as set forth in SEQ ID NO:17, and a *Respiratory syncytial virus* probe as set forth in SEQ ID NO:18;

a *Mycoplasma pneumoniae* forward primer as set forth in SEQ ID NO:19, a *Mycoplasma pneumoniae* reverse primer as set forth in SEQ ID NO:20, and a *Mycoplasma pneumoniae* probe as set forth in SEQ ID NO:21;

wherein, fluorescent groups in the first group (the first nucleic acid composition) are different from each other and do not interfere with each other, and fluorescent groups in the second group (the second nucleic acid composition) are different from each other and do not interfere with each other.

[0013] The expression "different from each other and do not interfere with each other" herein means that the fluorescent groups used by respective probe in the composition are different, and will not affect the detection of each other, that is, can be detected using different. For example, FAM, HEX, ROX and CY5 may be used. These groups that do not have approximate absorbance values allow the selection of different channels, and thus will not interfere with each other.

[0014] In a further embodiment, the composition includes an internal standard forward primer, an internal standard reverse primer and an internal standard probe, that are configured for monitoring.

[0015] In one specific embodiment, the composition further includes an internal standard forward primer as set forth in SEQ ID NO: 22, an internal standard reverse primer as set forth in SEQ ID NO: 23, and an internal standard probe as set forth in SEQ ID NO: 24.

[0016] In the present application, the fluorescent reporter group may be selected from a group consisting of FAM, HEX, ROX, VIC, CY5, 5-TAMRA, TET, CY3 and JOE, but is not limited thereto.

[0017] In one specific embodiment, the fluorescent reporter group of the *Influenza A virus* probe as set forth in SEQ ID NO:3 is FAM; the fluorescent reporter group of the *Influenza B virus* probe as set forth in SEQ ID NO:6 is HEX; the fluorescent reporter group of the human *Rhinovirus* probe as set forth in SEQ ID NOs: 9 and 10 is CY5.

[0018] In one specific embodiment, the fluorescent reporter group of the *Respiratory Adenovirus* probe as set forth in SEQ ID Nos: 14 and 15 is FAM; the fluorescent reporter group of the *Respiratory syncytial virus* probe as set forth in SEQ ID NO: 18 is HEX; the fluorescent reporter group of the *Mycoplasma pneumoniae* probe as set forth in SEQ ID

NO: 21 is CY5.

[0019] In one specific embodiment, the fluorescent reporter group of the internal standard probe as set forth in SEQ ID NO: 24 is ROX.

[0020] The internal standard is the primers and probe that detect human housekeeping gene GAPDH gene sequence. GAPDH gene sequence (SEQ ID NO: 25) is:

GGGGAGCCAAAAGGGTCATCATCTCTGCCCCCTCTGCTGATGCCCCCA TGTTCGTCATGGGTGTGAACCATGAGAAGTATGACAACAGCC

[0021] In a further embodiment, the detection primers are used in the composition in an amount of 0.2-0.4 pM; the detection probes are used in the composition in an amount of 0.1-0.3 pM; the internal standard primers are used in the composition in an amount of 0.2 pM; and the internal standard probe is used in the composition in an amount of 0.1 pM.

[0022] In the present application, the terms "detection primers" and "detection probes" refer to the primers and probes for amplification and detection of a pathogen.

[0023] In the present application, the terms "internal standard primer" and "internal standard probe" refer to a primer and a probe for amplification and detection of an internal standard.

[0024] In one specific embodiment, two nucleic acid compositions of the composition of the present application are respectively present in separate packages.

[0025] In a further specific embodiment, each component in each nucleic acid composition of the compositions of the present application is present in a mixed form.

[0026] In a second aspect, the present application provides use of the above-mentioned composition of the present application for the preparation of a kit for detecting and identifying a pathogen that causes a respiratory tract infection.

[0027] The pathogen that causes a respiratory tract infection includes *Influenza A virus*, *Influenza B virus*, *Respiratory Adenovirus*, human *Rhinovirus*, *Respiratory syncytial virus* and *Mycoplasma pneumoniae*.

[0028] In a third aspect, the present application provides a kit for detecting and identifying a pathogen that causes a respiratory tract infection, the kit including the above-mentioned composition of the present application.

[0029] In a further embodiment, the kit further includes a nucleic acid release reagent and a PCR amplification system.

[0030] In a further embodiment, the kit also includes at least one of a nucleic acid release reagent, dNTP, reverse transcriptase, DNA polymerase, a PCR buffer and Mg^{2+}.

[0031] Common PCR buffers include Tris-HCl, $MgCl_2$, KCl, Triton X-100 and other buffer systems. Generally, a total volume in a single PCR reaction tube is 20~200μl.

[0032] In a further embodiment, the detection primers are used in the composition in an amount of 25-500 nM; the detection probes are used in the composition in an amount of 20-500 nM; the internal standard primers are used in the composition in an amount of 12.5~250 nM; the internal standard probe is used in the composition in an amount of

10~250 nM; and dNTP are used is in an amount of 0.2-0.3 mM.

[0033] In further embodiment, the reverse transcriptase is at a concentration of 5 U/μL to 15 U/μL, and the reverse transcriptase may be, for example, murine leukemia reverse transcriptase (MMLV) or Tth enzyme. The DNA polymerase is at a concentration of 5 U/μL to 15 U /μL, and the DNA polymerase may be, for example, Taq enzyme.

[0034] In a further embodiment, the kit contains a positive control and a negative control. The negative and positive controls need to be processed simultaneously with the sample to be tested. The positive control, which simulates actual clinical specimens, is a mixture of artificially synthesized lentiviral particles containing specific nucleic acid sequences of *Influenza A virus*, *Influenza B virus*, human *Rhinovirus* and *Respiratory syncytial virus*, and artificially synthesized plasmids containing specific nucleic acid sequences of *Adenovirus* and *Mycoplasma pneumoniae*. The negative control, which completely simulates throat swab samples of normal people, is composed of artificially synthesized lentiviral particles containing the target fragment (SEQ ID NO: 25) of GAPDH housekeeping gene.

[0035] In a fourth aspect, a method for detecting and identifying a pathogen that causes a respiratory tract infection is provided. The method includes the following steps:

[0036] 1) releasing a nucleic acid in a sample to be detected;

[0037] 2) performing a fluorescence quantitative PCR on the nucleic acid obtained in step 1) using the above-mentioned composition or the above-mentioned kit of the present application; and

[0038] 3) obtaining a result and analyzing the result.

[0039] In the present application, the sample to be tested may be a throat swab, sputum, bronchoalveolar lavage fluid, blood, etc., but is not limited thereto.

[0040] In a further embodiment, the fluorescent quantitative PCR is performed under the following conditions:

[0041] 1 cycle of pre-denaturation and enzyme activation at a temperature of 95℃ for 1 to 10 minutes;1 cycle of reverse transcription at a temperature of 60℃ for 25 to 35 minutes; 1 cycle of cDNA pre-denaturation at a temperature of 95℃ for 1 to 10 minute; 40 to 50 cycles of denaturation at a temperature of 95℃ for 10 to 20 seconds and annealing at a temperature of 60℃ for 20 to 40 seconds.

[0042] In one specific embodiment, a method for detecting and identifying a pathogen that causes a respiratory tract infection for non-diagnostic purposes is provided. The method includes the following steps:

[0043] 1) releasing a nucleic acid in a sample to be detected;

[0044] 2) performing a fluorescence quantitative PCR on the nucleic acid obtained in step 1) using the above-mentioned composition or the above-mentioned kit of the present application; and

[0045] 3) obtaining a result and analyzing the result.

[0046] The compositions of the present application can be used to simultaneously detect six pathogens that cause respiratory tract infection, leading to simple, quick, and objective detection of the respiratory tract pathogens, thereby realizing identification of pathogens, early diagnosis, rational guidance of clinical medication, reduced waste of medical resources, and reduced psychological burden on patients and society. At the same time, it has a higher detectable rate than that of the existing respiratory pathogen detection composition, and is more accurate in detection.

[0047] The composition of the present application, combined with the fluorescent probe method, enables the simultaneous use of two tubes in one test, and thus has low cost and high throughput. The information of four target points can be given in one tube in a single test with simple and convenient operation, and the results can be read by judgment with CT values. The whole process of the detection is carried out in a single tube under sealing, which avoids false positives and environmental contamination caused by cross-over between samples.

BRIEF DESCRIPTION OF THE DRAWINGS

[0048] Fig. 1 is a graph showing the result of the detection using the composition of the present application.

[0049] Figs. 2-7 are graphs showing the sensitivity of the compositions of the present application.

[0050] Figs. 8-9 are graphs showing the specificity of the compositions of the present application.

[0051] Fig. 10A is a graph showing the effect of the compositions of the present application, and Fig. 10B is a graph showing the effect when the *Respiratory syncytial virus* in the present application is replaced by SARS-CoV-2.

[0052] Fig. 11A is a graph showing the effect of the compositions of the present application, and Fig. 11B is a graph showing the effect when the *Rhinovirus* of the present application is replaced by metapneumovirus.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0053] Hereinafter, the present application will be described in detail with reference to specific embodiments and examples, and the advantages and various effects of the present application will be more clearly presented therefrom. It is understood by those skilled in the art that these specific embodiments and examples are intended to illustrate, but not to limit the present application.

[0054] Example 1. Primers and probes used in the present application

[0055] The primers and probes used in the present application are shown in Table 1 below (SEQ ID NOs: 1 to 24 corresponds to the base sequences only, and the labeling modes here are shown as specific examples only):

[0056] Table 1

Name	Detection target	Sequences and labeling modes (5'-3')
SEQ ID NO:1	*Influenza A virus*	TCTCATGGAATGGCTAAAGACAA
SEQ ID NO:2		AGGGCATTTTGGACAAAGC
SEQ ID NO:3		FAM-CACCGTGCCCAGTGAGCGAGGA-BHQ1
SEQ ID NO:4	*Influenza B virus*	TCACTCTTCGAGCGTTTTAATGAAG
SEQ ID NO:5		TAATCGGTGCTCTTGACCAAAT
SEQ ID NO:6		HEX-AGCCAATTCGAGCAGCTGAAACTGCG-BHQ1
SEQ ID NO:7	Human *Rhinovirus*	GCCCCTGAATGCGGCTAA
SEQ ID NO:8		GAAACACGGACACCCAAAGTAGT
SEQ ID NO:9		CY5-TAATGAGCAATTGCGGGATGGGACC-BHQ2
SEQ ID NO:10		CY5-CTAGTGCATACAATCCAGTGTGTRGCTAG-BHQ2
SEQ ID NO:11	*Respiratory Adenovirus*	GGATCCACCTCAAAAGTCAT
SEQ ID NO:12		TGGGCGGAGTTGGCGTAGA
SEQ ID NO:13		GTTAAGAGTATTACCCAGAAAAAGTT
SEQ ID NO:14		FAM-ACATGAAGTTGCTGGAGAAGGGGAT-BHQ1
SEQ ID NO:15		FAM-CCCATGGACATAAAGTTACTGGAGAAGG-BHQ1
SEQ ID NO:16	*Respiratory syncytial virus*	AGAACAAGATGGGGCAAATATG
SEQ ID NO:17		TATGTTGATGCTTGCAAGTTCTTTTAT
SEQ ID NO:18		HEX-CGTGAACAAGCTTCACGAAGGCTCCACA-BHQ1
SEQ ID NO:19	*Mycoplasma pneumoniae*	CCGTTTCTCCACCGGGTT
SEQ ID NO:20		GTTTTAGGCGCGGTTATATCATC
SEQ ID NO:21		CY5-CCCTGGATTGGGAATGGGTACAGGTATG-BHQ2
SEQ ID NO:22	Human Housekeeping Gene	GGGGAGCCAAAAGGGTCAT
SEQ ID NO:23		GGCTGTTGTCATACTTCTCATGGTT
SEQ ID NO:24		ROX-ATCTCTGCCCCCTCTGCTGATGCCC-

	Internal Standard	BHQ2

[0057] Example 2. Methods for detecting pathogens that cause respiratory tract infections and identifying the pathogens

[0058] In the present application, the samples to be detected were throat swabs, sputum and bronchoalveolar lavage fluid, and blood. Viral nucleic acid was extracted using the magnetic bead method. The following operation was performed in a sample processing chamber.

[0059] 2.1. An appropriate number of 1.5 mL sterilized centrifuge tubes were labeled for negative control, positive control and samples to be tested, and 300 μL of RNA extraction solution 1 was added to each tube;

[0060] 2.2. 200 μL of the sample to be tested, negative control or positive control was added per tube; and the tubes were capped, mixed by shaking for 10 seconds, and subjected to a short spin;

[0061] 2.3. 100 μL of RNA extraction solution 2-mix (which should be mixed well before pipetted) was added to each tube, and the tubes were mixed by shaking for 10 seconds, then left standing at room temperature for 10 minutes;

[0062] 2.4. After a short spin, the centrifuge tubes were placed on a separator for 3 minutes, and the solution was gently pipetted out (be careful not to touch the brown substance adsorbed on the tube wall);

[0063] 2.5 600 μL of RNA extraction solution 3 and 200 μL of RNA extraction solution 4 were added to each tube, and the centrifuge tubes were mixed by shaking for 5 seconds, subjected to a short spin, and placed on the separator again;

[0064] 2.6. After about 3 minutes, the supernatant was divided into two layers, a pipette tip was inserted into the bottom of the centrifuge tube to gently pipette the liquid out from the bottom completely and discard the liquid, and then the tube was left standing for 1 minute, and then the residual liquid at the bottom of the tube was pipetted out completely and discarded.

[0065] 2.7. Elution was performed by adding 50μL of TE buffer (pH8.0) to each tube, then all the brown mixture obtained after elution was transferred into a 0.2mL of PCR reaction tube as a sample to be tested, and then the tubes were capped, and transferred to an amplification detection area.

[0066] A real-time fluorescent PCR reaction system was configured as follows:

[0067] PCR reaction system A:

Components	Volume/Concentration in each reaction
PCR buffer	19.20 μL
dNTP (100mM)	0.50 μL

1mol/L $MgCl_2$	0.20 μL
Primer SEQ ID NO:1 (50 pmol/μL)	0.40 μL
Primer SEQ ID NO:2 (50 pmol/μL)	0.40 μL
Primer SEQ ID NO:4 (50 pmol/μL)	0.40 μL
Primer SEQ ID NO:5 (50 pmol/μL)	0.40 μL
Primer SEQ ID NO:7 (50 pmol/μL)	0.30 μL
Primer SEQ ID NO:8 (50 pmol/μL)	0.30 μL
Primer SEQ ID NO:22 (50 pmol/μL)	0.2 μL
Primer SEQ ID NO:23 (50 pmol/μL)	0.2 μL
Probe SEQ ID NO:3 (50 pmol/μL)	0.20 μL
Probe SEQ ID NO:6 (50 pmol/μL)	0.30 μL
Probe SEQ ID NO:9 (50 pmol/μL)	0.20 μL
Probe SEQ ID NO:10 (50 pmol/μL)	0.10 μL
Probe SEQ ID NO:24 (50 pmol/μL)	0.10 μL
RNasin	0.125 μL
0.1% DEPC water	Make up to 43.5 μL

[0068] PCR reaction system B:

Component	Volume/Concentration in each reaction
PCR buffer	19.20 μL
dNTP(100mM)	0.5 μL
1mol/L MgCl2	0.20 μL
Primer SEQ ID NO:11 (50 pmol/μL)	0.40 μL
Primer SEQ ID NO:12 (50 pmol/μL)	0.20 μL
Primer SEQ ID NO:13 (50 pmol/μL)	0.40 μL
Primer SEQ ID NO:16 (50 pmol/μL)	0.30 μL
Primer SEQ ID NO:17 (50 pmol/μL)	0.30 μL
Primer SEQ ID NO:19 (50 pmol/μL)	0.30 μL
Primer SEQ ID NO:20 (50 pmol/μL)	0.30 μL
Primer SEQ ID NO:22 (50 pmol/μL)	0.20 μL
Primer SEQ ID NO:23 (50 pmol/μL)	0.20 μL
Probe SEQ ID NO:14 (50 pmol/μL)	0.20 μL
Probe SEQ ID NO:15 (50 pmol/μL)	0.10 μL
Probe SEQ ID NO:18 (50 pmol/μL)	0.20 μL
Probe SEQ ID NO:21 (50 pmol/μL)	0.20 μL
Probe SEQ ID NO:24 (50 pmol/μL)	0.10 μL
RNasin	0.125 μL

Sterilized purified water	Make up to 43.5 μL

[0069] An enzyme mix consisting of Neoscript RT reverse transcriptase and H - Taq enzyme was obtained by mixing H-Taq enzyme (15 U/μL) and Neoscript RT enzyme (18 U/μL) according to a certain ratio (1 μL of H-Taq enzyme and 0.5 μL of Neoscript RT enzyme mix per person).

[0070] A PCR amplification program was set up as follows:

Step		Temperature	Time	Cycle Number
1	reverse transcription	50℃	30 minutes	1
2	pre-denaturation and enzyme activation	95℃	1 minute	1
3	denaturation	95℃	15 seconds	45
	annealing, extension and fluorescence acquisition	60℃	30 seconds	
4	instrument cooling	25℃	10 seconds	1

[0071] Result analysis:

[0072] 1) the detection signal for the target was FAM, HEX (or VIC) and CY5, and the detection signal for internal reference was ROX;

[0073] 2) Baseline setting: Baseline was generally set to 3-15 cycles, which could be adjusted according to the actual situation. The adjustment principle was to select a region where fluorescence signal is relatively stable before the exponential amplification part, with a start point (Start) avoiding the signal fluctuation in the initial stage of fluorescence acquisition, and an end point (End) being less by 1-2 cycles than the Ct of a sample showing the earliest exponential amplification. Threshold setting: the setting principle was to make a threshold line just exceeded the highest point of a normal negative control.

[0074] A sample was confirmed positive if obvious sigmoid amplification curves occurred in FAM, HEX and CY5 channels and the Ct value was less than or equal to 40; and a sample was confirmed negative if no amplification curve occurs in FAM, HEX and CY5 channels (i.e., No Ct) or the Ct value was greater than 40, and the ROX internal standard channel was positive (Ct value ≤ 40). The details were as follows:

Amplification results / reaction solution	FAM channel		HEX channel		CY5 channel		ROX channel	
	Ct value ≤ 40	No Ct or Ct value > 40	Ct value ≤ 40	No Ct or Ct value > 40	Ct value ≤ 40	No Ct or Ct value > 40	Ct value ≤ 40	No Ct or Ct value > 40

PCR mix A	Flu A **positive**	Flu A negative	Flu B **positive**	Flu B negative	HRV **positive**	HRV negative	Internal Standard **positive**	Internal Standard negative
PCR mix B	ADV **positive**	ADV negative	RSV **positive**	RSV negative	MP **positive**	MP negative		

[0075] Example 3. Detectable rate of the compositions of the present application

[0076] The detection was carried out using the compositions in Table 1 according to the method described in Example 2 of the present application, and a series of controls (other dual-probe schemes: probe 1 (SEQ ID NO:26): CY5-CTAACCTTAACCCCGCAGC-BHQ2; probe 2 (SEQ ID NO:27): CY5-AATCCTAACCATGGAGCAAG- BHQ2) were also designed for the detection as well. The detection results are shown in Tables 2 and 3.

[0077] Table 2. Statistical table of Ct value results of different *Rhinovirus* detection schemes

Sample number	Scheme of the present application	Other dual-probe schemes	Single-probe scheme	Sequencing results of 5' untranslated region
1	13.88	26.13	17.76	*Rhinovirus* A
2	22.81	34.03	32.22	*Rhinovirus* A39
3	31.40	40.47	NoCt	*Rhinovirus* A
4	NoCt	31.82	23.9	*Coxsackievirus* EV71
5	29.03	33.38	NoCt	*Rhinovirus* C
6	22.33	31.62	24.79	*Rhinovirus* A22
7	39.89	NoCt	NoCt	*Rhinovirus* A22
8	19.11	28.55	21.21	*Rhinovirus* A22
9	21.66	32.13	NoCt	*Rhinovirus* A
10	19.18	24.28	21.8	*Rhinovirus* A
11	39.67	NoCt	NoCt	*Rhinovirus* A
12	39.68	37.64	NoCt	*Rhinovirus* A
13	31.46	38.04	NoCt	*Rhinovirus* A10
14	26.90	NoCt	NoCt	*Rhinovirus* A
15	24.28	32.12	28.19	*Rhinovirus* A46
16	19.66	30.85	23.33	*Rhinovirus* A103
17	23.78	27.22	25.31	*Rhinovirus* A
18	25.71	36.52	NoCt	*Rhinovirus* A
19	24.18	30.43	26.32	*Rhinovirus* A22
20	NoCt	NoCt	30.79	*Coxsackievirus* A6

[0078] Notes: a larger Ct value means a worse detection effect, and No Ct means negative.

[0079] It could be seen from the detection results in the above table that, enteroviruses

(*Coxsackievirus* EV71, *Coxsackievirus* A6) would not be detected by using the scheme of the present application; while for *Rhinovirus* positive samples, Ct values obtained by detecting with the scheme of the present application were smaller, indicating that it has better detection effect than that of the other dual-probe scheme and single-probe scheme.

[0080] Table 3. Summary of Ct value results of different *Adenovirus* detection schemes

Sample Number	The Present Application Scheme	The present application scheme without SEQ ID NO: 12	The present application scheme without SEQ ID NO:15	The present application scheme without SEQ ID NO: 12 and SEQ ID NO: 15
1	31.08	29.17	41.27	NoCt
2	33.16	NoCt	NoCt	NoCt
3	34.92	33.89	NoCt	NoCt
4	34.56	34.84	NoCt	NoCt
5	25.89	37.27	NoCt-	NoCt
6	34.36	NoCt	NoCt	NoCt
7	21.55	21.72	21.48	32.72
8	20.37	20.45	20.78	40.89
9	34.29	33.79	NoCt	NoCt
10	36.09	36.24	33.16	NoCt
11	20.51	20.63	20.97	NoCt
12	23.74	23.70	23.94	34.04
13	22.45	22.63	22.95	NoCt
14	35.43	35.24	35.25	NoCt
15	26.84	26.70	27.83	37.85
16	34.78	34.24	34.65	NoCt
17	34.20	33.59	33.25	38.15
18	23.01	22.33	23.25	26.09
19	31.62	31.47	32.08	32.38
20	30.65	29.76	31.17	31.84
21	28.43	29.61	28.11	29.42
22	30.29	30.82	30.26	33.56

[0081] Note: a larger Ct value means a worse detection effect, and No Ct means negative.

[0082] It could be seen from the detection results in the above table that, all 22 positive samples could be detected by using the compositions of the present application; where there was no primer SEQ ID NO: 12, 2 positive samples were not detected; where there was no probe SEQ ID NO: 12, 6 positive samples were not detected; where there was neither SEQ ID NO: 12 nor SEQ ID NO: 15, 13 positive samples were not detected.

[0083] Example 4. Sensitivity of the compositions of the present application

[0084] Six pathogens were tested using the compositions of the present application, and the detection results are shown in FIG. 1, indicating that the compositions of the present application were capable of detecting six pathogens.

[0085] In addition, experiments were carried out with samples at different concentrations to detect the compositions of the present application for sensitivity using the compositions in Table 1 according to the method described in Example 2 of the present application. The positive samples for each target sensitivity were diluted in detection gradient, and the samples diluted to the detection limit were used as the test samples for detection, and each channel was detected 20 times. The analysis of sensitivity test results showed that, detection limits of this kit for *Influenza A virus*, *Influenza B virus*, human *Rhinovirus*, *Respiratory syncytial virus*, *Adenovirus* and *Mycoplasma pneumoniae* were respectively: 2.0 TCID50/mL, 2.0 TCID50/mL, 500.0 copies/mL, 500.0 copies/mL, 500.0 copies/mL, 500.0 copies/mL, as shown in Figs. 2-7.

[0086] Example 5. Specificity of the compositions of the present application

[0087] The compositions of the application invention were used to detect other common respiratory pathogens. The experiment showed that the compositions of the present application had no cross-reactivity with common respiratory pathogens (measles virus, mumps virus, rubella virus, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, human parainfluenza virus I, metapneumovirus, enterovirus 71, coronavirus 229E, cytomegalovirus, *Coxsackievirus* A, *Bordetella pertussis*, chlamydia pneumoniae, *haemophilus influenzae*, *streptococcus salivarius*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Mycobacterium tuberculosis*, human parainfluenza viruses 2 and 3, Epstein-Barr virus, Cryptococcus, *Aspergillus fumigatus*, *Aspergillus flavus*, *Candida albicans*, *Legionella pneumophila*, Boca Virus, herpes simplex virus 1, varicella zoster virus, *corynebacterium diphtheria*, *Lactobacillus bulgaricus*, *Moraxella catarrhalis*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, Pneumocystis, *Klebsiella pneumoniae*, etc.). The experimental results are shown in Figs. 8-9 (Fig. 8 shows the result of PCR reaction system A, and Fig. 9 shows the result of PCR reaction system B).

[0088] Example 6. Joint detection of multiple pathogens, mutual interference between primers and probes

[0089] Due to the principle of complementary base pairing, dimers would form between primers and/or probes, but this probability was rare and could be ruled out at the beginning of the design. However, when multiple pathogens were jointly detected, there were many primers and probes, and dimers were prone to form between primers and primers, between probes and probes, or between primers and probes. To ensure the conservative property of

the design (the conservative property was crucial to the accuracy of detection), and to consider the mutual interference between various primers and probes, the primers and probes needed to be carefully designed.

[0090] In the present application, the inventors replaced the *Respiratory syncytial virus* in the present application with SARS-CoV-2, or replaced the *Rhinovirus* in the present application with metapneumovirus. However, upon the experiments, it was found that the addition of SARS-CoV-2 and metapneumovirus would seriously affect the detection sensitivity of the method of the present application (experimental results are shown in Figs. 10-11).

CLAIMS

What is claimed is:

1. A composition for detecting and identifying a pathogen that causes a respiratory tract infection, comprising:

a first nucleic acid composition:

an *Influenza A virus* forward primer as set forth in SEQ ID NO:1, an *Influenza A virus* reverse primer as set forth in SEQ ID NO:2, and an influenza A probe as set forth in SEQ ID NO:3;

an *Influenza B virus* forward primer as set forth in SEQ ID NO:4, an *Influenza B virus* reverse primer as set forth in SEQ ID NO:5, and an influenza B probe as set forth in SEQ ID NO:6;

a *Rhinovirus* forward primer as set forth in SEQ ID NO:7, a *Rhinovirus* reverse primer as set forth in SEQ ID NO:8, a *Rhinovirus* probe as set forth in SEQ ID NO:9, and a *Rhinovirus* probe as set forth in SEQ ID NO:10; and

a second nucleic acid composition:

a *Respiratory Adenovirus* forward primer as set forth in SEQ ID NO: 11, a *Respiratory Adenovirus* forward primer as set forth in SEQ ID NO: 12, a *Respiratory Adenovirus* reverse primer as set forth in SEQ ID NO: 13, a *Respiratory Adenovirus* probe as set forth in SEQ ID NO: 14, and a *Respiratory Adenovirus* probe as set forth in SEQ ID NO: 15;

a *Respiratory syncytial virus* forward primer as set forth in SEQ ID NO:16, a *Respiratory syncytial virus* reverse primer as set forth in SEQ ID NO:17, and a *Respiratory syncytial virus* probe as set forth in SEQ ID NO:18;

a *Mycoplasma pneumoniae* forward primer as set forth in SEQ ID NO:19, a *Mycoplasma pneumoniae* reverse primer as set forth in SEQ ID NO:20, and a *Mycoplasma pneumoniae* probe as set forth in SEQ ID NO:21;

wherein, fluorescent groups in the first nucleic acid composition are different from each other and do not interfere with each other, and fluorescent groups in the second nucleic acid composition are different from each other and do not interfere with each other.

2. The composition of claim 1, wherein the composition further comprises an internal standard forward primer, an internal standard reverse primer and an internal standard probe, that are configured for monitoring.

3. The composition of claim 1, wherein the composition further comprises an internal standard forward primer as set forth in SEQ ID NO: 22, an internal standard reverse primer

as set forth in SEQ ID NO: 23, and an internal standard probe as set forth in SEQ ID NO: 24.

4. The composition of claim 1, wherein the fluorescent group may be selected from a group consisting of FAM, HEX, ROX, VIC, CY5, 5-TAMRA, TET, CY3 and JOE.

5. The composition of any one of claims 1 to 4, wherein a fluorescent reporter group of the *Influenza A virus* probe as set forth in SEQ ID NO:3 is FAM; a fluorescent reporter group of the *Influenza B virus* probe as set forth in SEQ ID NO:6 is HEX; fluorescent reporter groups of the human *Rhinovirus* probe as set forth in SEQ ID NOs: 9 and 10 are CY5.

6. The composition of any one of claims 1 to 4, wherein a fluorescent reporter group of the *Respiratory Adenovirus* probe as set forth in SEQ ID Nos: 14 and 15 is FAM; a fluorescent reporter group of the *Respiratory syncytial virus* probe as set forth in SEQ ID NO: 18 is HEX; a fluorescent reporter group of the *Mycoplasma pneumoniae* probe as set forth in SEQ ID NO: 21 is CY5.

7. The composition of any one of claims 1 to 4, wherein two nucleic acid compositions of the composition are respectively present in separate packages.

8. The composition of any one of claims 1 to 4, wherein the detection primers in the composition in an amount of 0.2–0.4 pM; and/or the detection probes are used in the composition in an amount of 0.1–0.3 pM.

9. A method for the preparation of a kit for detecting a pathogen that causes a respiratory tract infection by using the composition of any one of claims 1 to 8.

10. The method of claim 9, wherein the pathogen that causes a respiratory tract infection comprises *Influenza A virus*, *Influenza B virus*, *Respiratory Adenovirus*, human *Rhinovirus*, *Respiratory syncytial virus* and *Mycoplasma pneumoniae*.

11. A kit for detecting a pathogen that causes a respiratory tract infection and identifying the pathogen, comprising the composition of any one of claims 1 to 8.

12. The kit of claim 11, wherein the kit further comprises a nucleic acid release reagent and a PCR amplification system.

13. The kit of claim 11 or 12, wherein the detection primers are used in the composition in an amount of 25-500 nM; the detection probes are used in the composition in an amount of 20-500 nM; the internal standard primers are used in the composition in an amount of 12.5~250 nM; the internal standard probe is used in the composition in an amount of 10~250 nM.

14. A composition for use in the preparation of a reagent or a kit for detecting and identifying a pathogen that causes a respiratory tract infection, comprising the following steps:

1) releasing a nucleic acid in a sample to be detected;

2) performing a fluorescence quantitative PCR on the nucleic acid obtained in step 1) using the composition of any one of claims 1 to 8 or the kit of any one of claims 11 to 13; and

3) obtaining a result and analyzing the result.

15. A composition for detecting and identifying a pathogen that causes a respiratory tract infection, comprising:

a first nucleic acid composition:

an *Influenza A virus* forward primer as set forth in SEQ ID NO:1, an *Influenza A virus* reverse primer as set forth in SEQ ID NO:2, and an influenza A probe as set forth in SEQ ID NO:3;

an *Influenza B virus* forward primer as set forth in SEQ ID NO:4, an *Influenza B virus* reverse primer as set forth in SEQ ID NO:5, and an influenza B probe as set forth in SEQ ID NO:6; and

a *Rhinovirus* forward primer as set forth in SEQ ID NO:7, a *Rhinovirus* reverse primer as set forth in SEQ ID NO:8, a *Rhinovirus* probe as set forth in SEQ ID NO:9, and a *Rhinovirus* probe as set forth in SEQ ID NO:10;

wherein, fluorescent groups in the first nucleic acid composition are different from each other and do not interfere with each other.

16. The composition of claim 15, wherein a fluorescent reporter group of the *Influenza A*

virus probe as set forth in SEQ ID NO:3 is FAM; and/or a fluorescent reporter group of the *Influenza B virus* probe as set forth in SEQ ID NO:6 is HEX; and/or a fluorescent reporter group of the human *Rhinovirus* probe as set forth in SEQ ID NOs: 9 and 10 is CY5.

17. A kit for detecting a pathogen that causes a respiratory tract infection and identifying the pathogen, comprising the composition of claim 15.

18. A composition for detecting and identifying a pathogen that causes a respiratory tract infection, comprising:

a second nucleic acid composition:

a *Respiratory Adenovirus* forward primer as set forth in SEQ ID NO: 11, a *Respiratory Adenovirus* forward primer as set forth in SEQ ID NO: 12, a *Respiratory Adenovirus* reverse primer as set forth in SEQ ID NO: 13, a *Respiratory Adenovirus* probe as set forth in SEQ ID NO: 14, and a *Respiratory Adenovirus* probe as set forth in SEQ ID NO: 15;

a *Respiratory syncytial virus* forward primer as set forth in SEQ ID NO:16, a *Respiratory syncytial virus* reverse primer as set forth in SEQ ID NO:17, and a *Respiratory syncytial virus* probe as set forth in SEQ ID NO:18; and

a *Mycoplasma pneumoniae* forward primer as set forth in SEQ ID NO:19, a *Mycoplasma pneumoniae* reverse primer as set forth in SEQ ID NO:20, and a *Mycoplasma pneumoniae* probe as set forth in SEQ ID NO:21;

wherein, fluorescent groups in the second nucleic acid composition are different from each other and do not interfere with each other.

19. The composition of claim 18, wherein a fluorescent reporter group of the *Respiratory Adenovirus* probe as set forth in SEQ ID Nos: 14 and 15 is FAM; and/or a fluorescent reporter group of the *Respiratory syncytial virus* probe as set forth in SEQ ID NO: 18 is HEX; and/or a fluorescent reporter group of the *Mycoplasma pneumoniae* probe as set forth in SEQ ID NO: 21 is CY5.

20. A kit for detecting a pathogen that causes a respiratory tract infection and identifying the pathogen, comprising the composition of claim 18.