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Short communication

Development of multiplex nucleic acid sequence-based amplification for detection of human respiratory tract viruses

Lok Ting Lau[a,b,c,d,e,f,g], Xiao Yan Feng[a,b,c,d,e], Tsz Yan Lam[e,g], Hung Kit Hui[e,g], Albert Cheung Hoi Yu[a,b,c,d,e,f,g,*]

[a] Neuroscience Research Institute, Peking University, Beijing, China
[b] Department of Neurobiology, School of Basic Medical Sciences, Peking University, Beijing, China
[c] Key Laboratory for Neuroscience (Ministry of Education), Peking University, Beijing, China
[d] Key Laboratory for Neuroscience (Ministry of Public Health), Peking University, Beijing, China
[e] Beijing Hai Kang DNA Chips Limited, Beijing Economic Development Area, Yizhuang, Beijing, China
[f] Infectious Disease Center, Peking University, Beijing, China
[g] Hai Kang Life Corporation Limited, Shau Kei Wan, Hong Kong SAR, China

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A B S T R A C T

A group of common lower respiratory tract infections, influenza A, influenza B, human parainfluenza virus 1–4 (HPIV1–4), respiratory syncytial virus (RSV), rubella virus (RV) and Coxsackie virus (CSV), were selected for the development of a multiplex nucleic acid sequence-based amplification (NASBA) assay. Quantifiable measurement utilizing an enzyme-linked oligonucleotide capture (EOC) optical detection method, which was described previously, alleviated the requirement of specialized instrumentation that is commonly used in other molecular techniques. Multiplex NASBA–EOC provided rapid and specific detection of a single virus from a multiplexed group, reducing laboratory testing time and enabling high throughput screening. The uniquely designed primers and probes proved to be highly sensitive and specific, exemplifying the robustness of the multiplex NASBA–EOC technique.

1. Introduction

Influenza virus A, influenza virus B, human parainfluenza types 1–4 (HPIV1–4), respiratory syncytial virus (RSV), rubella virus (RV) and Coxsackie virus (CSV) cause the majority of viral lower respiratory tract infections and are difficult to differentiate by the clinical presentation alone (Hall, 2001). Children, the elderly and immunocompromised patients are particularly susceptible (Whimbey et al., 1996; Ljungman et al., 2001). Conventional diagnostic methods such as antigen-based tests have lower sensitivity than molecular techniques such as the polymerase chain reaction (PCR) (Storch, 2003). However, PCR is not available in a number of laboratories, especially in rural areas and has high running costs, and expertise for test operation (Ellis et al., 1997). Nucleic acid sequence-based amplification (NASBA) is a homogeneous and direct RNA amplification process which was shown previously to have sensitivity comparable to PCR (Compton, 1991; Shan et al., 2003). The use of a thermal cycler is not required and a standardized isothermal reaction temperature is employed for highly reliable amplification of a RNA target (Collins et al., 2002a,b; Cui et al., 2007).

Multiplex diagnosis has enabled the screening for a range of diseases in a single sample test (Elnifro et al., 2000; Templeton et al., 2004). Molecular diagnostic techniques have been multiplexed previously for a number of diseases by PCR (Stockton et al., 1998; Elnifro et al., 2000; Bellau-Pujol et al., 2005; Hindiyeh et al., 2005), and by NASBA (van Deursen et al., 1999; Loens et al., 2008). However, the use of a specialized luminescence-based detector is required, which becomes a limiting factor for general use of molecular techniques in clinical settings. NASBA has been developed previously with an enzyme-linked oligonucleotide capture (EOC) optical detection method and shown to be comparable in sensitivity and specificity to electrochemiluminescence detection and PCR (Lau et al., 2006, 2008). In the EOC detection method, NASBA amplicons are immobilized by hybridization to a biotinylated oligonucleotide capture probe bound to a streptavidin-coated surface. Detection is then mediated by a digoxigenin (DIG)-labelled

Abbreviations: NASBA, nucleic acid sequence-based amplification; EOC, enzyme-linked oligonucleotide capture; ELISA, enzymes-linked immunosorbent assay.

* Corresponding author at: Neuroscience Research Institute, Peking University, 38 Xue Yuan Road, Beijing 100191, China. Tel.: +86 10 8280 5188; fax: +86 10 8280 5188.
E-mail addresses: achy@haikanglife.com, achy@hsc.pku.edu.cn, kinnie.ho@haikanglife.com (A.C.H. Yu).

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detection probe and an anti-DIG antibody–alkaline phosphatase conjugate, which provides the colorimetric end-product for detection using an enzymes-linked immunosorbent assay (ELISA) plate reader (Lau et al., 2008).

2. Experimental/materials and methods

All the virus primers and probes (Table 1) were designed using the Primer Premier 5.0 software program (Premier Biosoft, Palo Alto, CA). The expression vectors encoding influenza A, influenza B, HPIV1–4, RSV, RV, and CSV, were provided by Shanghai Sangon Biological Engineering Technology & Services. Amplification primers and capture probes for influenza A, influenza B, HPIV1–3, HPIV4, RSV, RV and CSV virus were all derived after comparison with conserved sequences of matrix protein 1, segment 8 complete sequence, hemagglutinin–neuraminidase, phosphoprotein, human respiratory syncytial virus N, and glycoprotein E1 (RV and CSV) genes, respectively. All the conserved sequences were selected by alignment of maximum strains sequences retrieved from the National Center for Biotechnology Information (NCBI, Bethesda, MD) GenBank database. Sequence alignments were performed using CLUSTALX software. To ensure the multiplexed primers would have no mis-combinations we used Beacon Designer 7.0 (Premier Biosoft, Palo Alto, CA) software to confirm the sequencing reaction.

The extraction of RNA was performed with the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using a sample volume of 200 μl. NASBA amplification was performed as reported previously (Lau et al., 2008). The solution of extracted RNA (5 μl) was added to a mixture of Tris pH 8.3, 24 mM $MgCl_2$, 140 mM KCl, 10 mM DTT, 2 mM dNTPs, 4 mM NTPs, and 30% (v/v) DMSO. The primer (0.4 μl) was added and the sample heated for 5 min at 65 °C. After cooling the sample solution to 41 °C, 5 μl of enzyme mix (6.4 U/μl T7 RNA polymerase, 1.3 U/μl AMV reverse transcriptase, 0.02 U/μl RNase H and 0.42 μg/μl BSA) was added and the sample was amplified for 90 min.

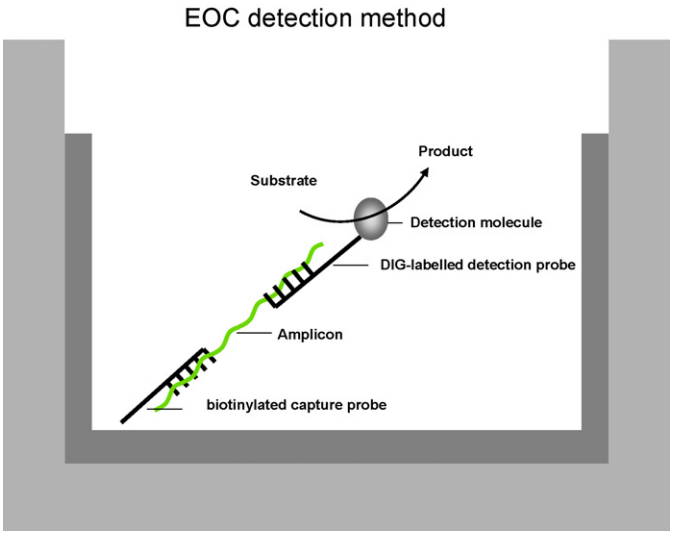

Fig. 1. Illustration of the principle of NASBA–EOC detection. The amplification reaction is immobilized by a biotinylated capture probe attached to the well of a streptavidin-coated microtitre plate (shaded grey). The digoxygenin (DIG)-labelled detection probe is added and binds to the immobilized amplicon. After addition of detection molecule and substrate, and incubation, reaction is stopped and the colorimetric signal generated is measured in a spectrophotometer, absorbance at 405 nm.

The EOC detection protocol was employed as described previously (Lau et al., 2008). This enzyme-based colorimetric detection method is illustrated in Fig. 1. For each sample, 2 μl of probe solution, comprising a 1:1 mixture of 26 μM biotinylated capture probe (biotin–AT GCA AGG TCG CAT ATG AGT, Invitrogen) and 26 μM DIG-

Table 1
Nucleotide sequences and genomic location used in the multiplex NASBA assay.

Pathogen	Name	Sequence (5′–3′)	Virus isolates	Sequence coordinates
Influenza A	InfluA-T7 F	GATGCAAGGTCGCATATGAG–CTTCTAACCGAGGTCGAAACGTA	A/brown-headed gull/Thailand/VSMU-28-SPK/2005(H5N1)	32–54
	InfluA-T7	AATTCTAATACGACTCACTATAGGGAGAAGG–A(A/G)GGCATT(C/T)TGGACAAA(G/T)CGTCTA		253–274
	InfluA-CP	DIG–CCGTCAGGCCCCCTCAAAGCCGA		71–93
Influenza B	InfluB-T7 F	GATGCAAGGTCGCATATGAG–ATCGGATCCTCAACTCACTCTTCGA	B/Taiwan/70028/2007	716–740
	InfluB-T7	AATTCTAATACGACTCACTATAGGGAGAAGG–TCTTCTGGTGATAATCGGTGCTCTT		815–839
	InfluB-CP	DIG–ACAT(C/T)CAAAGCCAATTCGAGCAG		756–778
HPIV1	HPIV1-T7 F	GATGCAAGGTCGCATATGAG–TATCAGTTATGCTCCTTGCCCACT	Human parainfluenza virus 1	858–881
	HPIV1-T7	AATTCTAATACGACTCACTATAGGGAGAAGG–TTTTCAAT(C/T)TTTATCCCACTTCCTA		1027–1051
	HPIV1-CP	DIG–GACCATCCTTTTTCTGCAATGTATC		996–1027
HPIV2	HPIV2-T7 F	GATGCAAGGTCGCATATGAG–TGAATCATACAATGGGACGCCTA	Human parainfluenza virus 2	515–537
	HPIV2-T7	AATTCTAATACGACTCACTATAGGGAGAAGG–AGATGCCGTGAAATC(A/G)AGACAATC		666–689
	HPIV2-CP	DIG–CCCAGCTTTATCCCCTCAGCAACAT		561–585
HPIV3	HPIV3-T7 F	GATGCAAGGTCGCATATGAGC–TCGGGTATGGAGGTCTTGAACAT	Human parainfluenza virus type 3	1076–1099
	HPIV3-T7	AATTCTAATACGACTCACTATAGGGAGAAGGC–T(C/T)CT(A/G)TCTGAAAACCA(G/T)GGACTATG		1174–1200
	HPIV3-CP	DIG–TGAGAATGCAATCTGCAACACAACT		1108–1132
HPIV4	HPIV4-T7 F	GATGCAAGGTCGCATATGAG–GGAGTCCCATCAAAAGTAAGTCTCAG	Parainfluenza virus type 4B	951–976
	HPIV4-T7	AATTCTAATACGACTCACTATAGGGAGAAGG–GGGTCTTGCTAA(C/T)G(A/G)(A/G)TCAAGTGTA		1064–1088
	HPIV4-CP	DIG–GG(A/C)GA(C/T)GTCTCAAAATTTGTTGATC		1025–1050
RSV	RSV-T7 F	GATGCAAGGTCGCATATGAG–C(C/T)T(A/C)(A/C)TACC(A/C)AAGGA(C/T)ATAGC(C/T)AACA	Human respiratory syncytial virus	1681–1710
	RSV-T7	AATTCTAATACGACTCACTATAGGGAGAAGG–AT(C/T)CCTTCAACTCTACT(A/G)CC(A/C)CCT		1687–1710
	RSV-CP	DIG–ACC(A/C)AAGGA(C/T)ATAGC(C/T)AACAG(C/T)T		1786–1809
RV	RV-T7 F	GATGCAAGGTCGCATATGAG–ACTGAACACCC(A/G)TTCTGCAACA	Rubella virus	8788–8810
	RV-T7	AATTCTAATACGACTCACTATAGGGAGAAGG–TGATTGCC(A/G)GTGTAATTCATA(A/G)TGT		8884–8891
	RV-CP	DIG–(C/T)GGGGACCTGGTTGAGTACAT(C/T)AT		8867–8884
CSV	CSV-T7 F	GATGCAAGGTCGCATATGAG–CTGAATGCGGCTAATCC(C/T)AAC(C/T)	Coxsackie virus B1	457–479
	CSV-T7	AATTCTAATACGACTCACTATAGGGAGAAGG–ATTGTCACCATAAGCAGCCA		581–600
	CSV-CP	DIG–CGACTACTTTGGGTGTCCGTGTT		541–565

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Table 2
Analytical specificity results for the multiplex NASBA–EOC assay for the detection of human respiratory viruses. Human RNA was used as interference in the sample solution to exemplify the robustness. An OD_{405} value above 0.45 was considered positive with one value per experiment.

Template	Interference	OD_{405}		Result
		Monoplex	Multiplex	
InfluA	–	0.837/1.896	1.091/1.174	Positive
InfluA	Human RNA	ND[a]	0.809/0.753	Positive
InfluB	–	1.467/1.463	1.292/1.423	Positive
HPIV1	–	1.307/1.437	1.446/1.352	Positive
HPIV1	Human RNA	ND[a]	0.767/0.706	Positive
HPIV2	–	1.291/1.299	0.950/1.224	Positive
HPIV2	Human RNA	ND[a]	0.586/0.542	Positive
HPIV3	–	1.303/1.319	1.341/1.358	Positive
HPIV4	–	1.465/1.466	1.082/1.243	Positive
RSV	–	1.062/0.638	0.809/0.942	Positive
RV	–	0.695/0.644	0.779	Positive
CSV	–	1.334/1.443	1.269/1.307	Positive
CSV	Human RNA	ND[a]	0.886/0.889	Positive
NASBA water	–	0.229/0.220	0.235/0.153	Negative
NASBA water	Human RNA	ND[a]	0.129/0.117	Negative

[a] Not done.

labelled detection probe (Table 1) was added to 5 ml of the NASBA amplicon and 93 ml hybridization buffer [20× SSPE, pH 7.4; 50 mM Tris–HCl, pH 8.8; 1% (w/v) BSA], mixed thoroughly and the mixture aliquoted into a 96-well streptavidin-coated microtitre plate (Nalge Nunc International Corp., Naperville, IL) and incubated at 41 °C for 1 h. The supernatant was discarded and the wells were washed three times with 0.2 ml 1× TBS. Detection solution (0.1 ml, 1:500 monoclonal α-DIG–alkaline phosphatase conjugate, Sigma Chemical Co., St. Louis, MO) was added and incubated at room temperature for 30 min. The supernatant was discarded and the wells were washed three times with 0.2 ml 1× TBS. Substrate solution (0.1 ml, p-nitrophenyl phosphate liquid substrate system, Sigma Chemical Co.) was added to each well and the plate was incubated in the dark for 5 min at room temperature. The reaction was terminated by the addition of 0.1 ml 3 M NaOH. The absorbance at 405 nm (with a 630 nm reference filter) was measured in a microtitre plate spectrophotometer (Titertek Multiskan® PLUS MKII). The cut-off value had been calculated previously as 0.45 and any readings above this value were considered positive (Lau et al., 2008).

3. Results

RNA templates synthesized artificially and mimicking various viruses were synthesized to test the efficacy of the primer and probe sequences in the multiplex NASBA assays. The monoplex NASBA assay was used as a reference. Studies to evaluate the effect of interference were conducted with unrelated healthy human RNA present in the template solution for diseases influenza A, HPIV1 and 2 and CSV. Blank controls of the NASBA water and human RNA were also tested. The assays were successful (Table 2). The number of non-specific interactions between RNA templates and primers was low and the specificity of monoplex assay was retained when transferred to the multiplex configuration. Positive results were recorded for the multiplex assays in the presence of human RNA interference, exemplifying the usefulness of the NASBA technique for transfer to clinical use.

Table 3
Analytical sensitivity of the monoplex NASBA–EOC assay for the detection of human respiratory viruses. An OD_{405} value above 0.45 was considered positive with one value per experiment.

Template	Monoplex	
	Detection limit (dilution)	OD_{405}
InfluA	10^{-10}	1.266/0.839
InfluB	10^{-11}	0.702/0.794
HPIV1	10^{-8}	0.500
HPIV2	10^{-10}	0.608/0.549
HPIV3	10^{-9}	1.491/1.128
HPIV4	10^{-9}	1.438/1.286
RSV	10^{-8}	2.562/3.129
CSV	10^{-8}	0.899/1.269

The sensitivity of the EOC optical detection method was assessed using the RNA amplification products for each virus in the monoplex assay. The concentration of RNA was measured at OD_{260} and a stock solution was prepared, from which 10-fold serial dilutions were investigated until a positive reading could no longer be observed. The detection limits are shown in Table 3. In general, the NASBA monoplex assay recorded extremely low detection limits, ranging from 10^{-11} dilution for influenza B virus, 10^{-10} dilution for influenza A virus and HPIV1, 10^{-9} dilution for HPIV3 and 4 and 10^{-8} dilution for HPIV1, RSV and CSV. For comparison, the influenza A virus template was assessed in both the monoplex and multiplex NASBA–EOC assay (Table 4). A detection limit of 10^{-7} dilution was observed by the multiplex assay while monoplex assay can reach 10^{-10} dilution, showing that multiplex assay has lower sensitivity than a monoplex assay. This was in agreement with a previous demonstration that multiplex assay has lower sensitivity than the monoplex assay (Loens et al., 2008).

Lower respiratory tract infections are often difficult to distinguish from the clinical presentation alone (Hall, 2001). The rapid and accurate detection of the virus is important for determining which therapeutic measures to initiate (Ljungman et al., 2001). In this study NASBA–EOC was evaluated as a method to amplify and detect RNA homogeneously by a multiplex assay for a range of common lower respiratory tract infections. The ability of nucleic acid amplification testing for multiplex diagnosis has been shown previously to be successful for specific identification from a range of respiratory viruses (Templeton et al., 2004; Mahony, 2008; Bellau-Pujol et al., 2005). However, multiplex diagnosis using PCR was found to be dependent on the first few rounds of thermal cycling (Elnifro et al., 2000). Hence, NASBA offers advantages for multiplex diagnosis because it uses a continuous isothermal reaction. Also, the performance of the EOC optical detection method increases the availability of molecular testing to be conducted in laboratories equipped with ELISA plate readers. The EOC has been shown to have

Table 4
Analytical sensitivity of the multiplex assay for influenza A compared to monoplex. An OD_{405} value above 0.45 was considered positive with one value per experiment.

Template	Detection limit (dilution)	Monoplex		Multiplex	
		OD_{405}	Result	OD_{405}	Result
InfluA	10^{-6}	ND[a]	–	0.787/0.695	Positive
InfluA	10^{-7}	ND[a]	–	0.742/0.718	Positive
InfluA	10^{-8}	3.087/3.107	Positive	0.437/0.338	Negative
InfluA	10^{-9}	1.304/1.432	Positive	ND[a]	–
InfluA	10^{-10}	1.266/0.839	Positive	ND[a]	–
InfluA	10^{-11}	0.190/0.222	Negative	ND[a]	–

[a] Not done.

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sensitivity comparable to PCR (Lau et al., 2008). This development of NASBA–EOC increases the availability for high throughput routine screening by multiplex diagnosis. Additional work is recommended to validate fully the assay.

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