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Simultaneous detection of SARS coronavirus and influenza A viruses using real-time polymerase chain reaction

Key Messages

1. Simultaneous detection of SARS-CoV and influenza A virus is described.
2. The rapid diagnostic method is less expensive than other methods.
3. Automation of molecular diagnosis is possible.

Introduction

In early 2003, severe acute respiratory syndrome (SARS) was recognised as a newly emerging pneumonic disease and a novel coronavirus, named SARS coronavirus (SARS-CoV), was implicated as the causative agent of the epidemic. In Hong Kong, the outbreak affected more than 1700 people with 290 deaths up to July 2003. Specific laboratory tests to detect viral RNA and antibody responses were used to establish a cause in patients suspected to have SARS. As one of the World Health Organization SARS network laboratories, the Department of Microbiology, Queen Mary Hospital, The University of Hong Kong provided rapid laboratory diagnoses (within 24 hours) for clinically suspected SARS cases using a polymerase chain reaction (PCR) technology. The first-generation PCR protocol was found to be highly specific but insensitive for rapid diagnosis of SARS-CoV infection.

During the latter part of 2003, a viral influenza A epidemic in North America and Europe caused considerable morbidity and mortality. The overlapping of SARS and the influenza season in Hong Kong created additional difficulty for establishing an early clinical diagnosis. The situation became more complicated when fatal cases of avian influenza H5N1 were reported in Southeast Asia. Rapid laboratory confirmation of both SARS-CoV and influenza virus is important not only to facilitate timely therapeutic interventions and clinical management, but also to identify the beginning of any new influenza or SARS epidemic.

In this project, a highly sensitive multiplex real-time PCR was developed for simultaneous rapid diagnosis of both SARS-CoV and influenza A viruses (including human seasonal influenza [eg H3N2] and avian influenza H5N1 causing epidemics in Southeast Asia). It included a retrospective analysis of 500 stored respiratory samples collected from confirmed cases of SARS or viral influenza infection in 2003, and another 500 samples recruited for prospective study in 2006.

Methods

A total of 1150 nasopharyngeal aspirates were collected from patients suffering respiratory tract infections or flu syndrome between 2003 and 2007. Conventional laboratory diagnosis for influenza A and B viruses, parainfluenza virus types 1, 2, and 3, respiratory syncytial virus, and adenovirus were performed using an immunofluorescence, commercial screening kit and viral culture on MDCK, HepII and LLCMK2 cells. Patient serum samples were also collected for serologic testing for SARS-CoV. RNA extraction was performed automatically by QIAamp Virus BioRobot 9604 (Qiagen, Hilden, Germany) for total viral nucleic acid load (Table 1). Initial processing of specimens was performed under biohazard level-2 containment. According to the manufacturer's instructions, a sample volume of 220 μL was mixed with 240 μL of AL buffer and 40 μL protease solution. The mixture was incubated at 60ºC for 10 minutes before transferring to the rack of the Robotic 9604 System containing 96 samples tubes. Nucleic acid precipitation was initiated by addition of 275 μL absolute ethanol

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Table 1. Workflow and manipulation time for multiplex real-time polymerase chain reaction (PCR) assays

Workflow	Hands-on time for 32 samples
Decontamination and lysis	45 min
Automatic RNA extraction, purification and concentration (waiting time 40 min)	0 (The second batch of samples can be processed)
Setting master mix for multiplex real-time PCR	30 min
Real-time PCR (waiting time 45 min)	0 (The third batch of samples can be processed)
Turnaround time	2 hr 40 min

and 250 μL AW1 buffer followed by transferring to the QIAamp 96 well vacuum manifold. RNA extracted was washed once with 360 μL AW1 buffer and twice with 1 μL AW2 buffer. A final volume of 50 to 60 μL total nucleic acid was eluted by addition of 86 μL AVE elution buffer. The sequential steps of nucleic acid precipitation, washing and elution were operated automatically. Each run of nucleic acid extraction for 96 samples required 3 hours. A multiplex real-time PCR was designed against SARS-CoV (P gene) and influenza A (M gene) with confirmation by specific fluorescent labelled hybridisation probes.

Real-time PCR was performed using LightCycler PCR (Roche Diagnostics GmbH, Manheim, Germany) with the following conditions: 61ºC for 20 minutes, 95ºC for 30 seconds; amplification at 95ºC for 5 seconds, 55ºC for 15 seconds, and 72ºC for 13 seconds for 50 cycles; cooling at 40ºC for 30 seconds. Positive and negative controls were included in each run, the National Committee for Clinical Laboratory Standards for the molecular diagnosis of infectious diseases were adopted to prevent cross contamination.

Results

Among the 1150 samples tested, 323 positive samples were obtained from patients during the 2003 SARS epidemic (Table 2). No SARS-CoV was detected in samples obtained between 2004 and 2007. During the SARS epidemic in 2003, 638 paired (10-21 days apart) serum samples were available for parallel testing with 680 nasopharyngeal aspirate samples. Among 470 nasopharyngeal aspirate samples collected from late 2003 to 2007, only 65 paired samples for SARS-CoV serology assay were available due to early discharge of patients. For 405 single serum samples collected during the early onset of disease, a serology result of ≥1:20 dilution was interpreted as positive. Influenza A viruses (H1 and H3 subtypes) were detected among 275 samples and no H5 subtype was identified. All 278 samples positive for SARS-CoV by multiplex PCR for the P gene were confirmed by real-time PCR for the N gene on the same DNA extract. The resolved performance of real-time PCR was validated against conventional laboratory diagnosis with 86% and 93% sensitivity for SARS-CoV and influenza A viruses, respectively. The real-time PCR assay exhibited 100% specificity for the detection of both viral pathogens.

Discussion

Sero-diagnosis for SARS-CoV infection is reliable and specific, but sero-conversion can only be detected by about day 10 of the illness. When patients are treated with immunomodulator drugs such as steroids, the detection may be delayed until the third or fourth week of illness. The first-generation in-house RT-PCR assay has been shown to be highly specific but insensitive. In the previous study, manual RNA extraction was performed using 140 μL sample volume. In this study, the automatic system used a larger initial sample volume (220 μL) for viral RNA extraction, thus providing higher diagnostic sensitivity with a 30% saving of manpower. The multiplex real-time PCR assay also increased the specificity of this assay for the differential diagnosis of SARS-CoV and influenza A virus and was suitable for routine application.

Rapid diagnosis of SARS-CoV by PCR assays is useful during an epidemic. In the post-epidemic period, no SARS-

Table 2. Performance of multiplex real-time polymerase chain reaction (PCR) for rapid detection of SARS-CoV and influenza A viruses on nasopharyngeal aspirate specimens

No. of specimens tested	Sero-conversion of SARS-CoV	Conventional diagnosis of influenza A			No. of specimens positive for real-time PCR			
		H1	H3	H5	SARS-CoV	H1	H3	H5
323	+*	–	–	–	278†	0	0	0
186	–	–	+	–	0	0	177‡	0
89	–	+	–	–	0	79	0	0
897	–	–	–	–	0	0	0	0
264§	–	–	–	–	0	0	0	0

* 703 paired serum (10-21 days apart) and 405 single serum at early onset of disease were available. A 4-fold rise in paired serum titre was interpreted as positive. For single serum, a positive serology result for ≥1:20 dilution was interpreted as positive

† The 278 samples positive for the P gene were confirmed by N gene real-time PCR assay

‡ Two samples were positive for both influenza A and adenovirus

§ Samples positive for other viral pathogens included 12 parainfluenza type 1, two parainfluenza type 2, 39 parainfluenza type 3, 121 respiratory syncytial virus (RSV) and 87 adenovirus, five with co-infections (three RSV & parainfluenza type 1, two RSV & parainfluenza type 3)

CoV was detected by the multiplex real-time PCR assay. To monitor reemerging of SARS-CoV infection, rapid diagnosis is important not only for timely therapeutic interventions but also to identify the beginning of a new outbreak. Our study highlights the high throughput and performance of automatic RNA extraction and multiplex real-time PCR assay, which appear suitable for large-scale routine diagnosis in the event of future SARS and/or influenza epidemics.

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