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Development and Evaluation of Multiplex Real-time RT-PCR Assays for Seasonal, Pandemic A/H1pdm09 and Avian A/H5 Influenza Viruses Detection

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Since the pandemic influenza A (H1N1) 2009 ((H1N1)pdm09) virus spread all over the world, the (H1N1)pdm09 virus has been circulating with seasonal influenza viruses. We developed rapid and sensitive one-step multiplex real-time RT-PCR assays (rRT-PCR) for simultaneous detection of influenza viruses currently circulating in humans, and the avian A/H5 virus. The detection limit of each assay was 4.8 to 1 copies per reaction and no cross-reactivity with other major respiratory pathogens was found. Analytical positive predictive value (PPV), negative predictive value (NPV) sensitivity and specificity were 100%, 94.1%, 93.7% and 100%, respectively. Clinical evaluation revealed that 1,976 (16.5%) of 11,963 throat swabs from patients with respiratory symptoms were confirmed as 1,651 (83.6%) A/H1pdm09, 308 (15.6%) A/H3 and 17 (0.8%) B virus during the 2010-2011 influenza season. Collectively, the multiplex rRT-PCR assays described here provide a practical tool for reliable implementation of influenza surveillance and diagnosis.

Keywords: influenza, real-time RT-PCR, surveillance, diagnosis

Introduction

Influenza viruses are the major causative agents of human acute respiratory disease worldwide, with potentially severe consequences for human health and the economy. The most common symptoms of the disease are chills, fever, sore throat, muscle pains, headache, coughing, fatigue, and general discomfort (Eccles, 2005). Although the incidence of influenza varies widely from year to year, approximately 36,000 deaths and more than 200,000 hospitalizations are directly associated with influenza every year in the United States (Thompson, *et al.*, 2003).

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A novel strain of the H1N1 virus that originated in swine was identified by the Centers for Disease Control and Prevention (CDC) in April 2009, becoming a pandemic strain and spreading across the world (CDC, 2009; Dawood *et al.*, 2009). In Korea, the first confirmed case was identified on May 2nd - a person who had travelled to Mexico following the outbreak there. Since then, the pandemic virus spread rapidly until early 2010 and by January 2010 there were 16,447 cases that had been confirmed by laboratories in public health institutes. At the beginning of the pandemic A (H1N1) 2009 ((H1N1)pdm09) virus outbreak, there was no specific molecular detection system until the World Health Organization (WHO) recommended protocol (CDC rRT-PCR swine Flu panel) became available (WHO, 30 April 2009). In-house conventional RT-PCR based on A/California/04/2009 virus genome sequences was the only applicable method in the Korea National Influenza Center (NIC). However, additional sequence analysis was needed for confirmation at that time. In May 2009, the Korea NIC introduced the CDC real-time RT-PCR (rRT-PCR) swine Flu panel with minor modifications and used it for clinical diagnosis until the end of the pandemic. Thereafter several multiplex rRT-PCR for large scale influenza surveillance and diagnosis have been developed by other groups (Chidlow *et al.*, 2010; Huber *et al.*, 2011).

In Korea, Seasonal H1N1 and H3N2 viruses dominantly circulated with distinct seasonality until the emergence of the (H1N1)pdm09 virus. Moreover, the highly pathogenic avian influenza (HPAI) H5N1 virus has prevailed continuously in domestic poultry farms in Korea since 2003 (Lee *et al.*, 2005, 2008). Therefore, an adequate detection system targeting currently circulating human strains, as well as HPAI H5N1, is also required.

This article describes the development and evaluation of rapid and sensitive one-step multiplex real-time assays that are capable of detecting influenza circulating in humans for large scale surveillance and diagnosis.

Materials and Methods

Design of primers and probes

The nucleotide sequences of the matrix (M), hemagglutinin (HA), and nucleoprotein (NP) genes of influenza viruses were obtained from the GenBank database. The M gene, conserved for influenza A, was selected for the detection of type A influenza virus. The M gene-specific primer and probe set was designed based on sequence comparison among

Table 1. Primer and probe sets of multiplex real-time RT-PCR assays

Multiplex set	Target gene	Primer/probe	Sequence (5′→3′)	Product size (bp)
1	Influenza type A Matrix	Forward	AAT CCT GTC ACC TCT GAC TAA GG	99
		Reverse	CAT TYT GGA CAA AKC GTC TAC G	
		Probe	**FAM**-TGC AGT CCT CGC TCA C-**MGBNFQ**	
	Influenza type B Nucleoprotein	Forward	GAA TGC TGT CAA TGA ATA TTG AGG G	77
		Reverse	CAT TGA GTC ATT CAT CAT CTT GAG TAG AT	
		Probe	**VIC**-TCC TTT GAC ATC TGC AT-**MGBNFQ**	
2	A/H1pdm09 Hemagglutinin	Forward	GCA CTC ATT CTG AGG GGA TCA G	67
		Reverse	TGC AAG CCC ATA CAC ACA AGC	
		Probe	**FAM**-CAC ATA AAT CCT GCC TGC-**MGBNFQ**	
	Avian A/H5 Hemagglutinin	Forward	GGT AAC GGT TGT TTC GAG TTC TAT CA	89
		Reverse	AAT ACT GCG GGT AGT CAT ACG TTC C	
		Probe	**NED**-ATG TGA TAA TGA ATG TAT GGA AAG T-**MGBNFQ**	
	GAPDH[a]	Forward	AGA TTT GGA CCT GCG AGC G	65
		Reverse	GAG CGG CTG TCT CCA CAA GT	
		Probe	**Cy5**-TTC TGA CCT GAA GGC TCT GCG CG- **BHQ**	
3	Seasonal A/H1 Hemagglutinin	Forward	CCC CAA GAC AAG TTC ATG GC	150
		Reverse	AGC ATG AGG ACA TGC TGC C	
		Probe	**FAM**- CAT GAC TCG AAC AAA GG -**MGBNFQ**	
	Seasonal A/H3 Hemagglutinin	Forward	TAG AAA ATG GTT GGG AGG GAA TG	102
		Reverse	CTG CTT GAG TGC TTT TAA GAT CTG C	
		Probe	**VIC**-TGG TAC GGT TTCA GGC AT-**MGBNFQ**	

[a] Glyceraldehyde 3-phosphate dehydrogenase : internal control.

different subtypes of over 200 strains of influenza A virus from human, swine and avian sources. Each subtype specific HA primer and probe set was designed based on the alignment of over 100 HA sequences of representative viruses from Asia, America and Europe. The influenza B primer and probe set was designed for the conserved NP gene between Victoria and Yamagata lineages. All primer and probe sets were designed by using PrimerExpress (Version5.0; Applied Biosystems, USA) software. Selected primer and probe sequences were compared with sequences submitted to the GenBank nucleotide database using a standard nucleotide–nucleotide comparison tool: BLASTN (Version 2.2.1; NCBI, MD, http://www.ncbi.nlm.nih.gov/). The probes were labeled with minor grove binding no fluorescence quencher (MGBNFQ) or black hole quencher (BHQ) at the 3′ end, and four different fluorescent reporter dyes (FAM, VIC, NED and CY5) at the 5′ end, so that the multiple genes of an influenza virus could been detected simultaneously in a single tube (Table 1).

Table 2. List of human respiratory pathogens used for specificity test

Viruses and bacteria[a]		Source[b]	Titer
RNA viruses	PIV1/2/3		> 5×10^5 $TCID_{50}$/ml
	RSV	ATCC VR-26	4×10^7 $TCID_{50}$/ml
	HCoV 229E	ATCC VR-740	3×10^4 $TCID_{50}$/ml
	HCoV OC43	ATCC VR-1558	4×10^5 $TCID_{50}$/ml
	Rhinovirus 13	ATCC VR-1123	1×10^6 $TCID_{50}$/ml
	Enterovirus 68	ATCC VR-561	5.2×10^5 $TCID_{50}$/ml
DNA viruses	HBoV		1×10^4 copies/ml
	Adenovirus 3		5×10^5 pfu/ml
	HSV-2	ATCC VR-540	2.5×10^4 $TCID_{50}$/ml
	HHV-6	ATCC VR-1480	3×10^4 $TCID_{50}$/ml
	VZV	ATCC VR-1503	4.5×10^4 $TCID_{50}$/ml
Bacteria	*Haemophilus influenzae*		2×10^5 cell/ml
	Legionella pneumophila		3×10^5 cell/ml
	Streptococcus pneumoniae		1.5×10^5 cell/ml
	Mycoplasma pneumoniae		3×10^6 cell/ml
	Chlamydia pneumoniae		1×10^3 cell/ml

HSV-2, Herpes simplex virus 2; HHV-6, Human herpesvirus 6; VZV, Varicella-zoster virus; PIV1/2/3, Parainfluenza virus 1/2/3; RSV, Respiratory syncytial virus; HCoV, Human coronavirus; HBoV, Human bocavirus

[a] These bacteria were provided by Division of Bacterial Respiratory Infections, Center for Infectious Diseases, Korea National Institute of Health.

[b] If not specified, strains are clinical isolates.

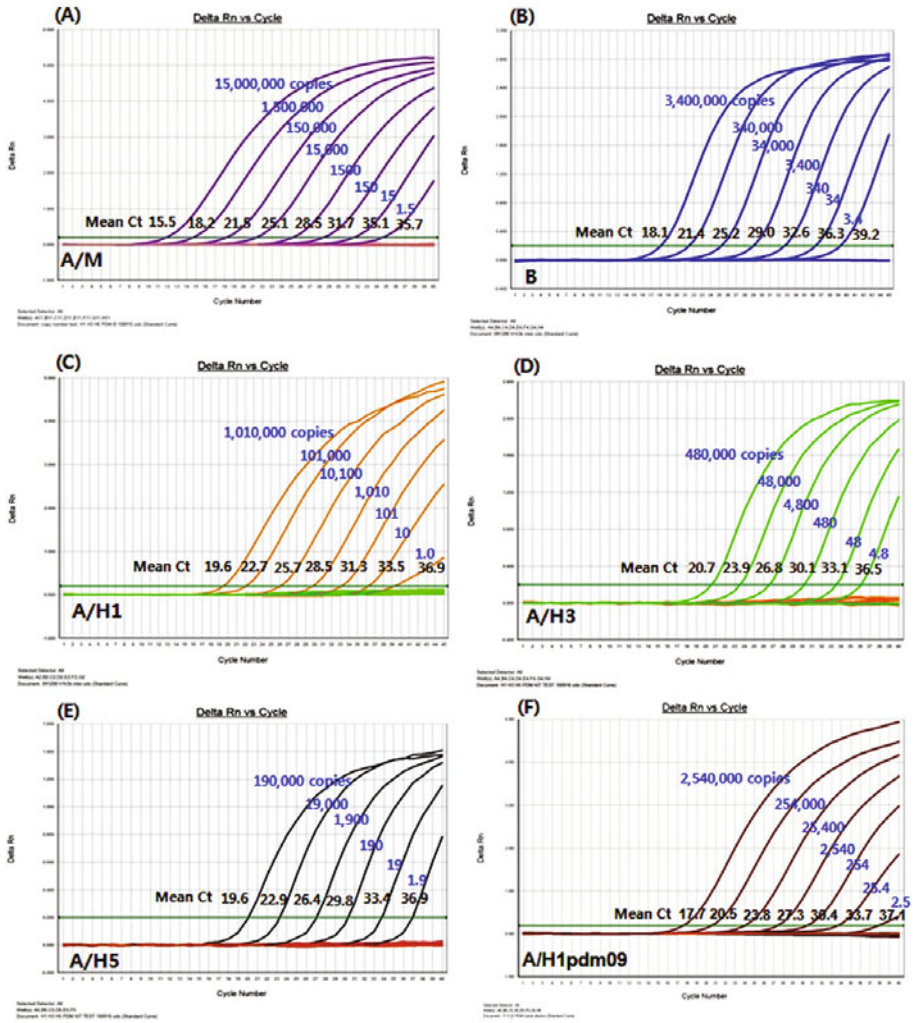

Fig. 1. Representative amplification plots of multiplex real-time RT-PCR assays. The dynamic range and detection limit were evaluated by testing 10-fold serial diluted viral RNAs of each virus ranging from 1 to 1×10^6 copies for seasonal A/H1 (A), 3.4 to 3.4×10^6 copies for influenza B (B), 9.4 to 9.4×10^5 copies for seasonal A/H1 (C), 4.8 to 4.8×10^5 copies for A/H3 (D), 1.9 to 1.9×10^5 copies for avian A/H5 (E) and 2.5 to 2.5×10^6 copies for A/H1pdm09 (F). The limit of the detection was as low as 9.4, 4.8, 1.9, 2.5, and 3.4 copies/reaction, corresponding to A/H1, A/H3, A/H5, A/H1pdm09, and B viral RNAs, respectively.

Viral RNA extraction

Viral RNA was extracted from 140 µl of viral transport medium (VTM) or tissue culture fluid of each sample using a QIAmp viral RNA mini kit (Qiagen, UK), according to the manufacturer's instructions.

Multiplex rRT-PCR

A one-step multiplex rRT-PCR was carried out using the Agpath ID One-Step RT-PCR system (Ambion, USA). Multiplex sets of primers and Taq-Man probes are described in Table 1. The reaction mixture contained 5 µl of RNA template, 590 nM primers, 140 nM probe, 10 µl of 2× reaction buffer, 0.8 µl of enzyme Mixture, and RNase-free water with a final volume of 20 µl. One-step multiplex real-time RT-PCR was performed on an ABI 7500Fast (Applied Biosystems). The thermocycler conditions included a reverse transcription step at 50°C for 30 min, and an activation hot start DNA Taq polymerase at 95°C for 10 min, followed by amplification that was performed during 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 55°C for 30 sec. Multiple fluorescent signals were obtained once per cycle upon completion of the extension step. Data acquisition and analysis of the real-time PCR assay were performed using SDS software Version 1.4 (Applied Biosystems).

Specificity and sensitivity

Analytical specificity was evaluated by cross-reactivity with other subtypes of influenza viruses, respiratory viruses and bacteria (Table 2). The analytical sensitivity was tested using 10-fold serial diluted viral RNAs in triplicate, and the copy numbers of viral RNA were determined according to M (influenza A) and NP (influenza B) gene standards. In order to generate standard RNA transcripts for the sensitivity test, in vitro transcription was carried out using a MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. The concentration of the *in vitro* transcribed RNAs was calculated by measuring absorbance at 260 nm and the rests of RNAs were kept at -70°C until use.

WHO external quality assessment program (EQAP) panels

The assays were introduced to the WHO 10th EQAP panel which was comprised of 10 RNA samples and 2 inactivated viruses and that had been received from the WHO EQAP team in Hong Kong SAR.

Table 3. Evaluation of the multiplex real-time RT-PCR using the 10th WHO EQAP[a] panel

EQA panel	Expected result[b]	A/M	A/H5	A/H1(2009)	A/H3	A/H1	B	Copies / μl	Clade
2011-11	B	-	-	-	-	-	26.9	5.670×10^{2}	
2011-12	A/H5	27.8	27.6	-	-	-	-	1.149×10^{3}	2.3.2
2011-13	B	-	-	-	-	-	25.7	1.990×10^{3}	
2011-14	A/H5	31.3	31.2	-	-	-	-	2.462×10^{2}	2.3.4
2011-15	A/H3	31.6	-	-	31.6	-	-	7.180×10^{2}	
2011-16	A/H5	25.2	24.8	-	-	-	-	4.107×10^{3}	2.3.4
2011-17	A/H1pdm09	30.7	-	29.2	-	-	-	4.948×10^{2}	
2011-18	A/H5	27.8	27.0	-	-	-	-	9.142×10^{2}	2.3.2
2011-19	Neg	-	-	-	-	-	-	-	
2011-20	A/H1pdm09	30.6	-	29.7	-	-	-	3.407×10^{2}	
V03-2011	A/H3	36.3			35.2			1.310×10^{2}	
V04-2011	A/H3	36.2			34.8			8.310×10^{1}	

[a] WHO External Quality Assessment Program
[b] Each test was performed in triplicate and results were expressed by mean Ct value.

Clinical specimen evaluation

The clinical throat swab specimens from outpatients with acute respiratory symptoms were collected from September 2008 to October 2010 through the Korea influenza surveillance system. A total of 319 specimens were tested retrospectively for evaluation of assay performance. Among them, 159 were influenza virus culture positive (seasonal 19 A/H1, 47 A/H3, 63 A/H1pdm09 and 30 B viruses) and 160 were culture negative. These samples were confirmed by real-time RT-PCR described here after viral culture. In addition, this assay was introduced to nationwide surveillance from September 2010 to August 2011, and 11,963 throat swab specimens were collected from sentinel clinics in 10 provinces and 7 metropolitan cities, and were evaluated at provincial health institutes. Informed consent was obtained from the patients or the parents of minor patients.

Results

Analytical sensitivity and specificity

The analytical sensitivities of each assay were determined by using triplicate 10-fold serial diluted viral RNAs of each virus ranging from 9.4 to 9.4×10^{5} copies for A/H1, 4.8 to 4.8×10^{5} copies for A/H3, 3.4 to 3.4×10^{6} copies for influenza B, 1.9 to 1.9×10^{5} copies for A/H5 and 2.5 to 2.5×10^{6} copies for A/H1pdm09 virus. The limits of detection were as low as 1, 4.8, 1.9, 2.5 and 3.4 copies per reaction, corresponding to A/H1, A/H3, A/H5, A/H1pdm09 and B viral RNAs, respectively (Fig. 1). The specificity of each assay was evaluated by testing other respiratory viruses, including parainfluenza viruses 1/2/3, RSV, respiratory syncytial virus, coronavirus 229E/OC43, rhinovirus, enterovirus, human bocavirus and adenovirus. In addition, respiratory pathogenic bacteria such as *Haemophilus influenzae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* were assessed by applying approximately 200 ng of DNAs (Table 2). No cross-reaction was observed against those pathogens and among different influenza subtypes.

WHO EQAP panel evaluation

The assay performance was evaluated using the WHO 10th EQAP panel. Seven of the 10 RNA panels were identified as influenza A viruses: 4 A/H5, 2 A/H1 (2009) and 1 A/H3 and the remaining 3 panels were identified as being 2 influenza B viruses and 1 negative. Two inactivated virus panels were determined to be A/H3 (Table 3). All results were consistent with intended results.

Clinical specimen evaluation

The clinical performance of the assays was tested by using 319 throat swab samples, including 129 influenza A (19 H1, 47 H3 and 63 H1pdm09) and 30 influenza B samples pre-

Table 4. The performance of multiplex Real-time RT-PCR with 319 clinical specimens

Virus	Viral culture[a]		Multiplex Real-time RT-PCR		Assay performance[b]			
	Positive	Negative	Positive	Negative	PPV[c]	NPV[d]	Sensitivity	Specificity
Influenza A	129	190	119	200	100%	95.0%	92.2%	100%
Influenza B	30	289	30	289	100%	100%	100%	100%
A/H1	19	300	19	300	100%	100%	100%	100%
A/H3	47	272	40	279	100%	97.4%	85.1%	100%
A/H1pdm09	63	256	60	259	100%	98.8%	95.2%	100%
Total (n = 319)	159	160	149	170	100%	94.1%	93.7%	100%

[a] Virus culture was performed on MDCK cells.
[b] The A/H5 assay test was excluded.
[c] Positive predictive value
[d] Negative predictive value

viously cell culture positive, and 160 negative samples. Of these, the multiplex rRT-PCR assays subtyped 149 samples which were consistent with viral culture as 119 influenza A (19 H1, 40 H3 and 60 H1pdm09) and 30 influenza B virus. However, the assays could not subtype 10 (7 A/H3 and 3 A/H1pdm09) viral culture positive samples. With viral culture as the reference, the overall positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity, except for the A/H5 assay, was 100%, 94.1%, 93.7%, and 100%, respectively (Table 4).

During the 2010-2011 season, a total of 11,963 throat swab samples collected through the Korea influenza surveillance system were tested, and 1,959 (16.3%) influenza A viruses (1,651 H1pdm09 and 308 H3) and 17 (0.1%) influenza B viruses were identified. However, no seasonal A/H1 virus which had circulated before the pandemic 2009 H1N1 virus was detected in these samples. The age distribution of the 1,976 influenza positive patients was 148 (7.4%) 0–2 years; 366 (18.5%) 3–6 years; 700 (35.4%) 7–19 years; 572 (28.9%) 20–49 years; 152 (7.6%) 50–64 years; 38 (1.9%) >64 years. The male to female ratio was 48.0% and positive rate of the vaccinated group was 21.1% (418 / 1,976) among influenza positive patients. The major clinical symptoms were fever and respiratory complications.

Discussion

In Korea, the nationwide human influenza surveillance system adopted viral culture followed by conventional RT-PCR for human influenza virus detection and subtyping, until the 2009 pandemic outbreak. However, these methods are laborious, time-consuming and hard to standardize among the laboratories in local public health institutes which participate in surveillance. As the pandemic (H1N1)pdm09 virus emerged in April 2009, Korea NIC introduced rRT-PCR which was the WHO-recommended US CDC rRT-PCR Swine Flu Panel (WHO, 30 April 2009) to detect (H1N1)pdm09 virus. Although it showed invaluable performance during the early phase of the outbreak, it had some drawbacks (relatively low sensitivity to swH1 compared to the InfA assay, cross-reactivity with A/H5 of swInfA and single assay format) that made it undesirable to introduce for routine surveillance and diagnosis, in terms of cost-effectiveness and user convenience (Shu *et al.*, 2011). Thereby, the development of an adequate and fine-tuned molecular detection system for influenza surveillance and diagnosis was required.

In the present study, the one-step multiplex rRT-PCR assays were developed and optimized for simultaneous detection of influenza type A and B and four subtypes of currently circulating human influenza, as well as avian influenza H5N1. Each assay was performed over a wide dynamic range and showed no cross-reactivity with other circulating respiratory viruses or bacteria. The assay also incorporates an internal control Glycrealdehyde 3-phosphate dehydrogenase (GAPDH) probe to screen possible PCR inhibitor or viral RNA extraction error. We also utilized the WHO EQAP panel for the evaluation of the A/H5 assay because there has so far been no H5N1 human infection case in Korea. The WHO panel consists of RNAs of seasonal influenza and avian influenza A/H5 with relatively low copy number, and inactivated seasonal influenza viruses (Table 3). For the Korea NIC, it is crucial to detect HPAI A/H5N1 human infection, because it has prevailed continuously in poultry farms since 2003 (Lee *et al.*, 2005, 2008).

In comparison with other recently reported multiplex real-time RT-PCR assays, ours could detect currently circulating influenza viruses in humans, as well as avian A/H5 virus, implying that these are practical tools for influenza detection (Huber *et al.*, 2011; Shisong *et al.*, 2011). The detection limit of multiplex rRT-PCR described in this report is lower than 10 copies per reaction (Fig. 1), which suggests that these are comparable to other published assays (Chidlow *et al.*, 2010; Chen *et al.*, 2011). Moreover, we evaluated the assays by introducing them to nationwide influenza surveillance. Thereby, the assays could be validated in different work environments (17 public health institutes which participated in surveillance) with very large numbers of clinical specimens, and have shown excellent performance with reduced working time and costs compared to previous single type or two-step rRT-PCR assays (He, 2009; Nakauchi *et al.*, 2011; WHO, 30 April 2009). However, the assays missed 10 influenza A (7 H3 and 3 H1pdm09) culture positive samples, because these showed signals which were near the detection limit (Ct values of >35 in A/M and subtypes) even after viral culture (Table 4).

One of our major concerns is laboratory contamination by PCR amplicons which cause false positive results, since conventional RT-PCR had previously been applied for influenza detection until the introduction of rRT-PCR to surveillance. Thus, viral RNA extraction, reaction mixture preparation and sample loading were carried out in a separated room to prevent possible contamination. We also tried to adjust assay sensitivity between the typing (multiplex set for influenza A or B type) and the subtyping assays to avoid inconclusive results in the influenza A sample that require further sequencing or tissue culture analysis.

In conclusion, well-balanced single-step multiplex rRT-PCR assays described here could be a useful tool for large scale surveillance and influenza diagnosis, as well as the detection of a possible HPAI A/H5 virus human infection.

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Competing interests

None declared

Ethic approval

This study was approved by Korea Center for Disease Control and Prevention institutional review board (IRB). The IRB approval number is 2011-06EXP-01-C.

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