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One-Step Multiplex Reverse-Transcriptase PCR for Detecting Pandemic (H1N1) 2009 Influenza Virus

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ABSTRACT. Pandemic (H1N1) 2009 influenza has spread throughout the world since April 2009 and has caused many human deaths since its first report in humans. Pandemic (H1N1) 2009 influenza virus was first identified in a Canadian pig herd in April 2009 and has been reported in more than ten countries, including Korea. We developed a one-step multiplex reverse transcriptase polymerase chain reaction (RT-PCR) assay based on the matrix gene that discriminates pandemic (H1N1) 2009 influenza virus from endemic swine influenza viruses. The sensitivity of this assay was 100 copies of *in vitro*-transcribed target RNA and 0.01 tissue culture infective dose ($TCID_{50}$/m*l*) of virus and was as high as those of conventional influenza A virus common matrix reverse transcriptase PCR assays and real-time reverse transcriptase PCR assays (1 to 200 copies) developed for detecting pandemic (H1N1) 2009 influenza viruses from human and pig samples. This one-step multiplex RT-PCR assay would be a good tool in monitoring pandemic (H1N1) 2009 influenza virus among pig herds on a regular basis.

KEY WORDS: multiplex reverse transcriptase PCR, pandemic (H1N1) 2009 influenza, swine influenza.

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Influenza is a highly contagious, acute respiratory disease that affects people of all ages. It causes an average of 114,000 hospitalizations and 20,000 deaths each year in the United States [40]. The etiological agent, influenza A virus, belongs to the family of Orthomyxoviridae. The genome of influenza A virus consists of eight segments (PB2, PB1, PA, HA, NP, NA, M and NS) of negative strand RNA molecules [17]. Based on differences in the hemagglutinin (HA) and neuraminidase (NA) proteins, 16 HA and nine NA subtypes have been identified [8, 37, 47]. Genetic changes brought about by reassortment of genomic fragments, as well as by their error-prone replication, lead to two types of variations in both surface antigens of the influenza A viruses: antigenic drift and antigenic shift [17]. These variations cause the seasonal and pandemic flus in the human population.

Influenza A viruses infect a variety of animals, including humans, pigs, horses, sea mammals and birds [47]. They have different specificities against viral receptors in host cells [3, 22, 23, 35, 36]. They also replicate less efficiently in humans and primates; similarly, human viruses do not replicate efficiently in birds when introduced by a natural route [1, 11, 21, 23, 25]. Pigs, however, are susceptible to both avian and human influenza viruses because they have receptors to both viruses and hence, it has been suggested that pigs are a mixing vessel for influenza viruses [2, 13, 21, 22, 50]. So far, several subtypes of influenza A virus have been reported in pig herds and are known as swine influenza because they are common on pig farms. The most common subtypes found in pigs are H1N1, H1N2 and H3N2 [5, 45, 46, 51].

Pandemic (H1N1) 2009 influenza viruses were first reported in humans in North America in April 2009 and since then has quickly spread throughout the human population worldwide. With the increasing number of human infections and deaths, the World Health Organization (WHO) declared a level 6 pandemic alert on June 11, 2009. Since the first case of human death caused by pandemic (H1N1) 2009 influenza virus in April 2009, more than 18,000 people have died worldwide [48]. With the increasing number of human cases, pigs have had greater exposure to this virus. The extensive scientific effort to characterize pandemic (H1N1) 2009 influenza virus has determined that it is likely porcine in origin [6, 10, 28, 41]. It has been elucidated that among eight viral RNA segments, six segments (PB2, PB1, PA, HA, NP and NS) are from swine influenza of North American lineage, while the other two genes, NA and M, are from swine influenza of Eurasian lineage [10, 41, 43, 44]. Influenza A virus with this gene combination has not been identified in humans, pigs or any other species [6, 28, 41].

Since the first report of pandemic (H1N1) 2009 influenza virus infection in a pig in Alberta, Canada, in May 2009, many cases of pandemic (H1N1) 2009 influenza virus infecting swine in 19 countries (Argentina, Australia, Canada, Chile, China, Finland, Germany, Iceland, Indonesia, Ireland, Italy, Japan, South Korea, Mexico, Norway, Russia, Thailand, UK and U.S.A.) worldwide have been reported

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[30]. The Ministry of Agriculture, Forestry and Fisheries in Korea began monitoring pandemic (H1N1) 2009 influenza virus infections on May 20, 2009. The Korean national program faced a challenge in monitoring pandemic (H1N1) 2009 influenza virus infections among pigs because it was necessary to differentiate pandemic (H1N1) 2009 influenza viruses among endemic swine influenza viruses.

In 2009, diagnosis of pandemic 2009 influenza virus among pig herds in Korea utilized conventional reverse transcriptase PCR (RT-PCR) [7, 24, 49] to detect influenza A virus common nucleoprotein (NP) or M gene in nasal swab samples after 1 or 2 passages in MDCK cells or embryonated eggs. Whenever there was a positive result against the M or NP gene, a hemagglutination inhibition test (HI) with H1-specific antiserum or a conventional RT-PCR assay for H1 subtyping was performed. If the results were positive for either test, then the sequence of the entire NA or M gene was analyzed to determine whether it was the pandemic (H1N1) 2009 influenza virus. Since Korean swine influenza isolates were closely related to the swine influenza viruses recently isolated from pigs in the United States and there have been no known cases of swine influenza viruses harboring Eurasian lineages of the NA or M gene in Korea [4, 15, 16, 39, 42], detecting a Eurasian lineage NA or M gene would mean the virus would be pandemic (H1N1) 2009 influenza virus. After our experience with the 2009 monitoring program, we attempted to simplify the diagnosis of pandemic (H1N1) 2009 influenza viruses among pigs for future monitoring by developing a one-step multiplex RT-PCR assay. Although high homology exists between the M gene of the endemic swine influenza viruses and the M gene of the pandemic (H1N1) 2009 influenza virus, we could still design primer sets that distinguished the two. This one-step multiplex RT-PCR system would be a good tool for future nationwide monitoring of pandemic (H1N1) 2009 influenza virus infection on swine farms in Korea.

MATERIALS AND METHODS

Viruses and cells: The Influenza A viruses are listed in Table 1. A total of 23 endemic swine influenza A viruses of different subtypes (H1N1, H1N2 or H3N2) and 18 pandemic (H1N1) 2009 influenza viruses isolated from humans or pigs were included. All of the endemic swine influenza viruses were isolated from nasal swab samples or lung samples collected from farms or slaughterhouses in Korea from 2005 to 2009. A/Korea/01/2009 is the first pandemic (H1N1) 2009 influenza virus isolated from a human in Korea and was kindly provided by Dr. Kang from National Institute of Health, Korea. These viruses were propagated in specific-pathogen-free (SPF) chicken embryonated eggs or in Madin Darby canine kidney (MDCK) cells. Allantoic fluids with viruses were harvested according to the methods described by the Office International des Epizooties (OIE). Briefly, SPF eggs incubated for 9 to 10 days were inoculated with the virus. After 48 to 72 hr of incubation at 37°C, the eggs were chilled to 4°C before harvesting the allantoic fluid. The viruses were divided into aliquots and kept frozen at –70°C until use. Each aliquot was used only once to avoid the loss of viral genomic material by repeated freezing and thawing. Virus titers were determined using the Reed-Münch method in MDCK cells with the presence of TPCK-trypsin, as described in the manual of the OIE [34] or hemagglutination (HA) test. The HA test was performed according to the WHO Manual on Animal Influenza Diagnosis and Surveillance. For the sensitivity assay of this one-step multiplex RT-PCR system, virus stocks were diluted in phosphate-buffered saline (PBS) from 10^0 to 10^{-9}. RNA was extracted from the diluted virus solution and applied for the assay.

Table 1. Influenza A viruses used for the assay

Influenza A viruses	Subtype	
A/Korea/01/2009	H1N1	Pandemic H1N1 2009 influenza viruses
A/swine/Korea/VD01/2009	H1N1	
A/swine/Korea/VD02/2009	H1N1	
A/swine/Korea/VD03/2009	H1N1	
A/swine/Korea/VD04/2009	H1N1	
A/swine/Korea/VD05/2009	H1N1	
A/swine/Korea/VD06/2009	H1N1	
A/swine/Korea/VD07/2009	H1N1	
A/swine/Korea/VD08/2009	H1N1	
A/swine/Korea/VD09/2009	H1N1	
A/swine/Korea/VD10/2009	H1N1	
A/swine/Korea/VD11/2009	H1N1	
A/swine/Korea/VD12/2009	H1N1	
A/swine/Korea/VD13/2009	H1N1	
A/swine/Korea/VD14/2009	H1N1	
A/swine/Korea/VD15/2009	H1N1	
A/swine/Korea/VD16/2009	H1N1	
A/swine/Korea/VD17/2009	H1N1	
A/swine/Korea/GN05K1/2005	H1N1	Influenza A viruses isolated from swine
A/swine/Korea/GC0503/2005	H1N1	
A/swine/Korea/CAS08/2005	H1N1	
A/swine/Korea/25–13/2009	H1N1	
A/swine/Korea/D180–3/2009	H1N1	
A/swine/Korea/251–1/2009	H1N1	
A/swine/Korea/G184/2009	H1N1	
A/swine/Korea/GC0502/2005	H1N2	
A/swine/Korea/R78–1/2005	H1N2	
A/swine/Korea/PZ4/2006	H1N2	
A/swine/Korea/MHT 70–2/2007	H1N2	
A/swine/Korea/CY08/2007	H1N2	
A/swine/Korea/194–1/2009	H1N2	
A/swine/Korea/160/2009	H1N2	
A/swine/Korea/274–3/2009	H1N2	
A/swine/Korea/103/2009	H1N2	
A/swine/Korea/104/2009	H1N2	
A/swine/Korea/76–1/2009	H1N2	
A/swine/Korea/100–4/2009	H1N2	
A/swine/Suwon/108/1998	H3N2	
A/swine/Korea/GC0407/2005	H3N2	
A/swine/Korea/CAS09/2006	H3N2	
A/swine/Korea/CY10/2007	H3N2	

Nasal swab samples: A total of 150 nasal swab samples

Table 2. Oligonucleotides Used

Name	Target	Oligonucleotide sequence	Size	Note
M30F2/08–1	Matrix	5'-ATGAGYCTTYTAACCGAGGTCGAAACG-3'	242 bp	Ref. (49)
M264R3/08–242		5'-TGGACAAANCGTCTACGCTGCAG-3'	AA 1-80[a)]	FJ969513
M-NF-339F	Matrix	5'-GGAGGTGTCACTAAGCTATTCA-3'	452 bp	FJ969513
M-NF-790F		5'-CCCAATGATATTTGCTGCAATG-3'	AA 113–264	

a) AA amino acid *.

collected from slaughterhouses were tested to evaluate this one-step multiplex RT-PCR assay. Pig nasal discharge was swabbed in a polyester tipped applicator and stored in 3 m*l* viral transport medium (BD, Franklin Lakes, NJ, U.S.A.) in a 15 m*l* conical tube. After brief vortexing, the sample was centrifuged at 3000 rpm for 5 min, transferred to a new tube and stored at –70°C until use.

Primer design: The M gene sequences of three pandemic (H1N1) 2009 influenza viruses (NCBI GenBank accession numbers: GQ131025 (human), FJ969513 (human), GU324342 (swine)) and 26 influenza A viruses (NCBI GenBank accession numbers: EU798798 (H1N1, swine), EU798800 (H1N2, swine), EU798810 (H3N2, swine), CY009629 (H1N1, swine), M55481 (H1N1, swine), CY028790 (H1N1, swine), DQ280196 (H1N1, swine), CY035071 (H1N1, swine), M63517 (H1N1, swine), CY025003 (H1N1, swine), AF389121 (H1N1, human), CY034045 (H2N2, human), CY005814 (H3N8, duck), CY014649 (H4N6, duck), DQ107453 (H5N3, duck), L25831 (H6N5, shearwater), DQ107475 (H7N1, duck), CY005828 (H8N4, duck), DQ067438 (H9N2, turkey), CY014672 (H10N7, chicken), CY014680 (H11N6, duck), CY021302 (H12N5, mallard), CY014695 (H13N6, gull), CY014605 (H14N5, duck), CY005725 (H15N9, wedge-tailed sheawater), CY004562 (H16N3, duck)) were retrieved from the GenBank database and analyzed using the Vector NTI Advance 9.0 software. One set of primers was designed to amplify pandemic (H1N1) 2009 influenza virus specific M gene sequences. Primers targeting the common influenza A virus M gene site were selected from the established WHO protocol for the laboratory diagnosis of pandemic (H1N1) 2009 influenza virus infections in humans [49]. The primers were synthesized commercially (Bioneer, Chonan, Korea). The primer sequences are listed in Table 2.

One-step multiplex RT-PCR: Viral RNA genomes were purified using an RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. Briefly, 300 μl of viral solution harvested from inoculated allantoic fluid or cell culture supernatant were used, and RNA was eluted in 40 μl of distilled water. The RT-PCR was performed using a one-step RT-PCR kit (Qiagen). The reaction mixture contained 5 μl of 5X RT-PCR buffer (2.5 mM $MgCl_2$), 0.4 mM dNTP, 0.5 μM of each of the four primers shown in Table 2, 1 μl of enzyme mix and 5 μl of RNA in a final volume of 25 μl. Viral genes were amplified using the following conditions: reverse transcription at 50°C for 30 min; an initial denaturation at 95°C for 15 min, followed by 40 cycles of 94°C for 40 sec, 55°C for 40 sec, 72°C for 50 sec; and final extension at 72°C for 10 min. Negative reagent controls were included in each assay.

Real-time RT-PCR as the evaluation assay: Each RNA sample and standard was amplified by a quantitative RT-PCR assay performed on an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Foster City, CA, U.S.A.) with a SuperScript™ III Platinum® One-Step Quantitative Kit (Invitrogen, Carisbad, CA, U.S.A.). All primers were taken from the protocol developed for detecting pandemic (H1N1) 2009 influenza viruses from swine samples [20]. All procedures followed the manufacturer's protocol for real-time RT-PCR. Briefly, the 25 μl reaction volume for each sample contained 1 μl of extracted RNA, 12.5 μl of 2 × PCR master mix buffer, 0.5 μl of *Taq* mix, 10 μM (0.5 μl) of probe (M(99)-Probe: 5'-CGC GCA GAG ACT GGA AAG TGT C-3'), 40 μM of each primer (M(76)-For and M(234)-Rev: 5'-TCA GGC CCC CTC AAA GCC GA-3': 5'-GGG CAC GGT GAG CGT GAA CA-3' respectively,) and 9.5 μl of nuclease-free water. Reactions were first incubated at 50°C for 15 min. After a 2-min denaturation at 95°C, the reactions were thermal-cycled for 40 cycles (95°C for 15 sec and 60°C for 30 sec). Negative control reactions without RNA were included in each test.

RNA standard for sensitivity: Standard RNA was generated for the sensitivity test of this one-step multiplex RT-PCR system. The full M gene of pandemic (H1N1) 2009 influenza virus, A/Korea/01/2009, was amplified using primers Bm-M-1 (forward, 5'-TAT TCG TCT CAG GGA GCA AAA GCA GGT AG-3') and Bm-M-1027R (reverse, 5'-ATA TCG TCT CGT ATT AGT AGA AAC AAG GTA GTT TTT-3') and SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen). PCR was performed as described previously by other researchers [12]. The PCR product was cloned into a pGEMT-easy vector system (Promega, Madison, WI, U.S.A.), and the DNA was linearized using a restriction enzyme. *In vitro* transcription was performed using a MEGAscript T7 Kit (Ambion, Austin, TX, U.S.A.) following the manufacturer's recommendations. Briefly, a reaction mixture with a final volume of 20 μl containing 500 *n*g of the linear DNA template, 2 μl of each nucleotide triphosphate, 2 μl of enzyme mix and 2 μl of 10 × reaction buffer was incubated at 37°C for 4 hr. After the incubation, 1 μl of DNase was added, and the mixture was incubated at 37°C for 15 min. The RNA transcripts were purified with MEGAclear Kit (Ambion) and quanti-

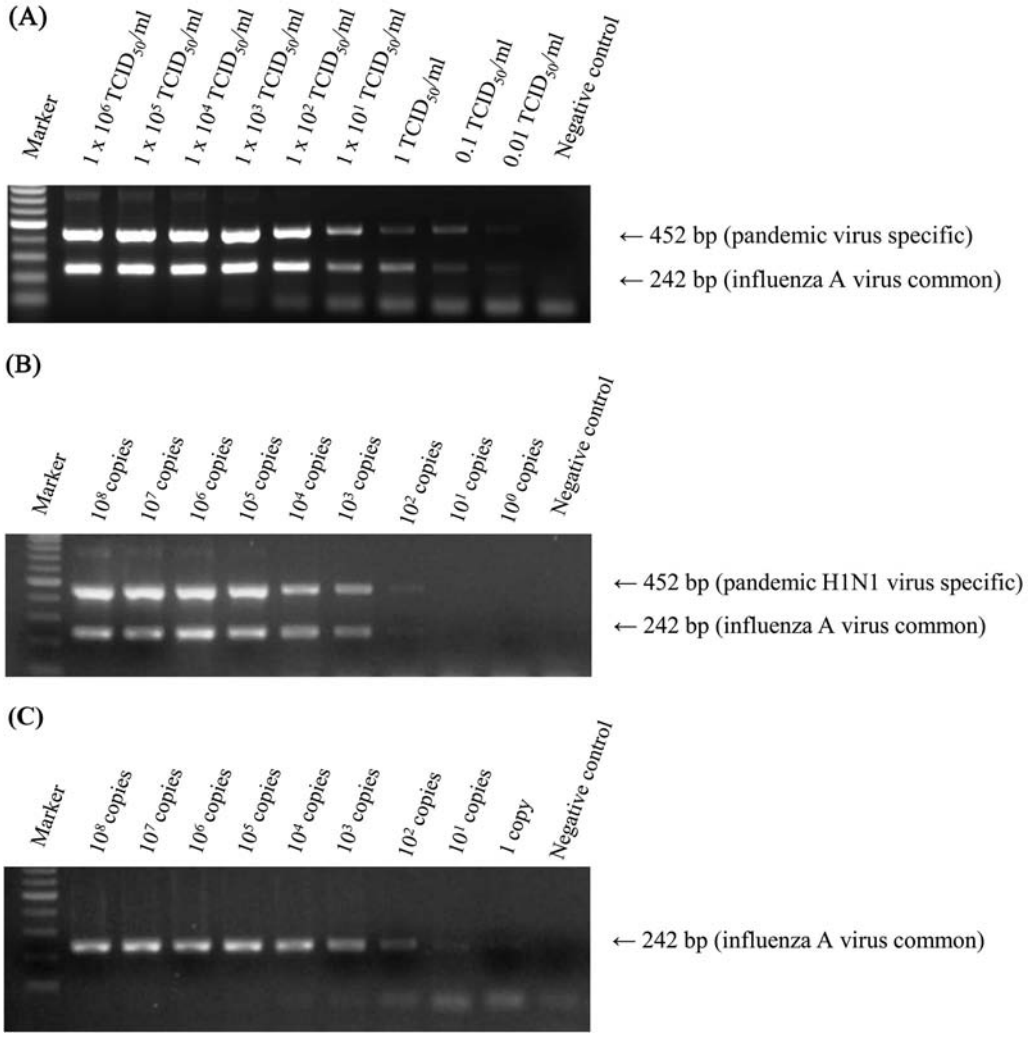

Fig. 1. Sensitivity of the one-step multiplex RT-PCR in detecting pandemic (H1N1) 2009 influenza virus. (A) One-step multiplex RT-PCR for pandemic (H1N1) 2009 influenza virus. Serial dilutions of pandemic (H1N1) 2009 influenza virus, A/Korea/01/2009 (H1N1), representing 1×10^6 to 0.01 $TCID_{50}$/m*l*, were applied to one-step multiplex RT-PCR. PCR products were visualized in 1% agarose gels. (B) One-step multiplex RT-PCR for A/Korea/01/2009 M gene RNA standards. Tenfold serial dilutions of *in vitro* transcribed M gene RNA, representing 10^8 to 10^0 copies RNA/μl of template, were applied to the one-step multiplex RT-PCR assay. PCR products were visualized in 1% agarose gels. (C) Influenza A virus common M RT-PCR for A/Korea/01/2009 M gene RNA standards. One-step RT-PCR assay with WHO influenza A common M gene primers was performed for the RNA standards representing 10^8 to 10^0 copies to compare the sensitivity of the two assays. PCR products were visualized in 1% agarose gels.

fied using ND-1000 NanoDrop spectrophotometer (NanoDrop Technology, Wilmington, DE, U.S.A.). Tenfold serial dilutions of the transcripts in distilled water, representing 10^8 to 10^0 copies RNA/μl of template, were prepared aliquoted and stored at –80°C.

RESULTS

Sensitivity of the one-step multiplex RT-PCR in detecting pandemic (H1N1) 2009 influenza viruses: Pandemic (H1N1) 2009 influenza virus, A/Korea/01/2009 (H1N1), was adjusted to contain 1×10^6 $TCID_{50}$/m*l* and serially diluted in PBS from 10^0 to 10^{-8}, giving virus titers of from 1×10^6 to 0.01 $TCID_{50}$/m*l*; RNA was then extracted. The one-step multiplex RT-PCR assay was performed with the RNA, and products were visualized in 1% agarose gels. A positive amplification could be visualized at up to 10^{-8} dilution, which meant that the lowest concentration detectable was 0.01 $TCID_{50}$/m*l* (Fig. 1A). Both the 242-bp band from the common M gene and the 452-bp band from the pandemic (H1N1) 2009 influenza virus-specific M region were visualized. The one-step multiplex RT-PCR assay was also

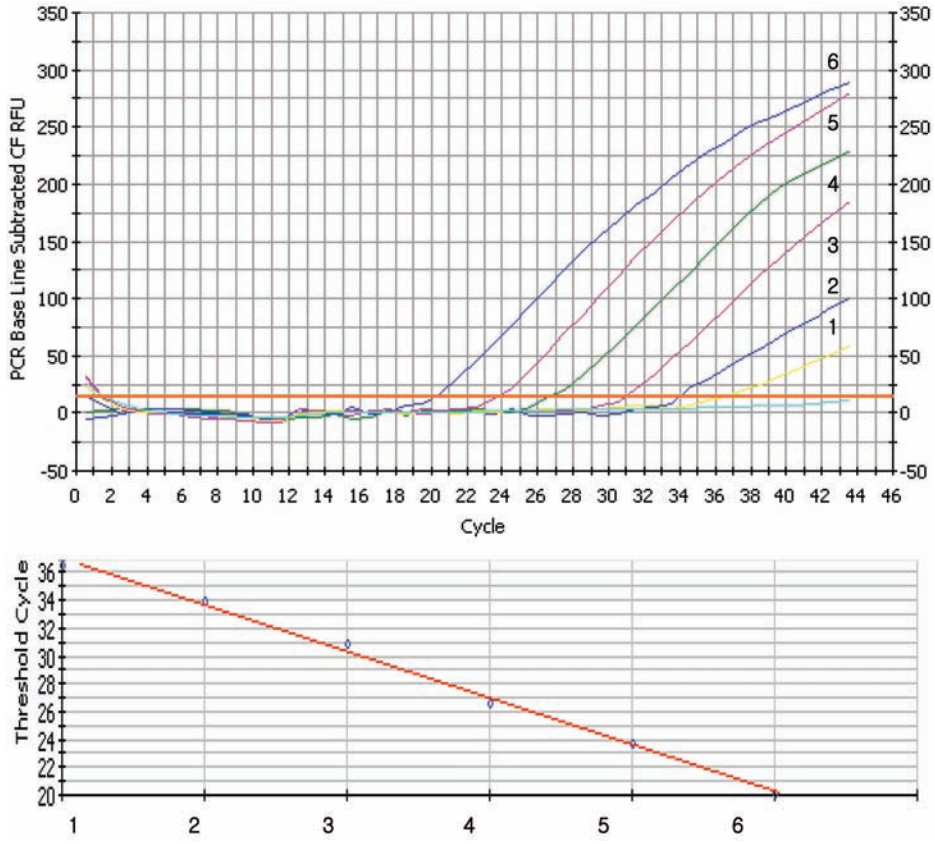

Fig. 2. Amplification of A/Korea/01/2009 (H1N1) M gene RNA standards by real-time RT-PCR. The six curves on the top show the amplification of six serial 10-fold dilutions of RNA standards from 10^1 to 10^6 copies. The numbers 1 to 6 represent 10^1 to 10^6 RNA copies, respectively. The features of the curves were as follows: slope of -3.341 and R^2 of 0.998.

performed with the RNA standard to determine the exact sensitivity in copy numbers (Fig. 1B). The minimum copy number of matrix gene RNA detectable with our system was approximately 10^2 copies of *in vitro* transcribed RNA. When the one-step RT-PCR assay with WHO influenza A common M gene primers was performed for the RNA standards, the detection limit was 10^1 RNA copies (Fig. 1C). Real-time RT-PCR for diagnosing pandemic (H1N1) 2009 influenza virus was performed for the RNA standard to compare the sensitivity of detection (Fig. 2). The minimum copy number of RNA detectable by real-time RT-PCR was around 10 copies of *in vitro* transcribed RNA, which was ten-fold lower than with the one-step multiplex RT-PCR system.

Specificity of the one-step multiplex RT-PCR with endemic swine influenza viruses and pig nasal swab samples: The specificity of this one-step multiplex RT-PCR system was tested using more than 23 endemic swine influenza viruses. RNA was extracted from 300 μl of virus solution, in which the titers ranged from 10^4 to 10^6 $TCID_{50}$/m*l*. The RNA was applied for our one-step multiplex RT-PCR. Only the 242-bp bands were visualized. The results demonstrated that this system could differentiate pandemic (H1N1) 2009 influenza virus from other swine influenza viruses (Fig. 3). A total of 150 pig nasal swab samples collected from slaughterhouses were tested using both the influenza A virus common M RT-PCR assay [49] and one-step multiplex RT-PCR assay. All 150 samples were negative in both assays (Table 3).

Detection of pandemic (H1N1) 2009 influenza virus isolated from pigs: Seventeen pandemic (H1N1) 2009 influenza viruses isolated from pig herds in 2009 were used to evaluate the performance of this assay. Both the 242-bp band for the common M gene region and the 452-bp band for the pandemic (H1N1) 2009 influenza virus specific M gene region were visualized for all 17 pandemic (H1N1) 2009 influenza viruses (Fig. 4).

DISCUSSION

When the first pig infection of pandemic (H1N1) 2009 influenza virus was reported in Alberta, Canada, in April 2009 with sequential outbreaks in other countries, the Korean government listed pandemic (H1N1) 2009 influenza

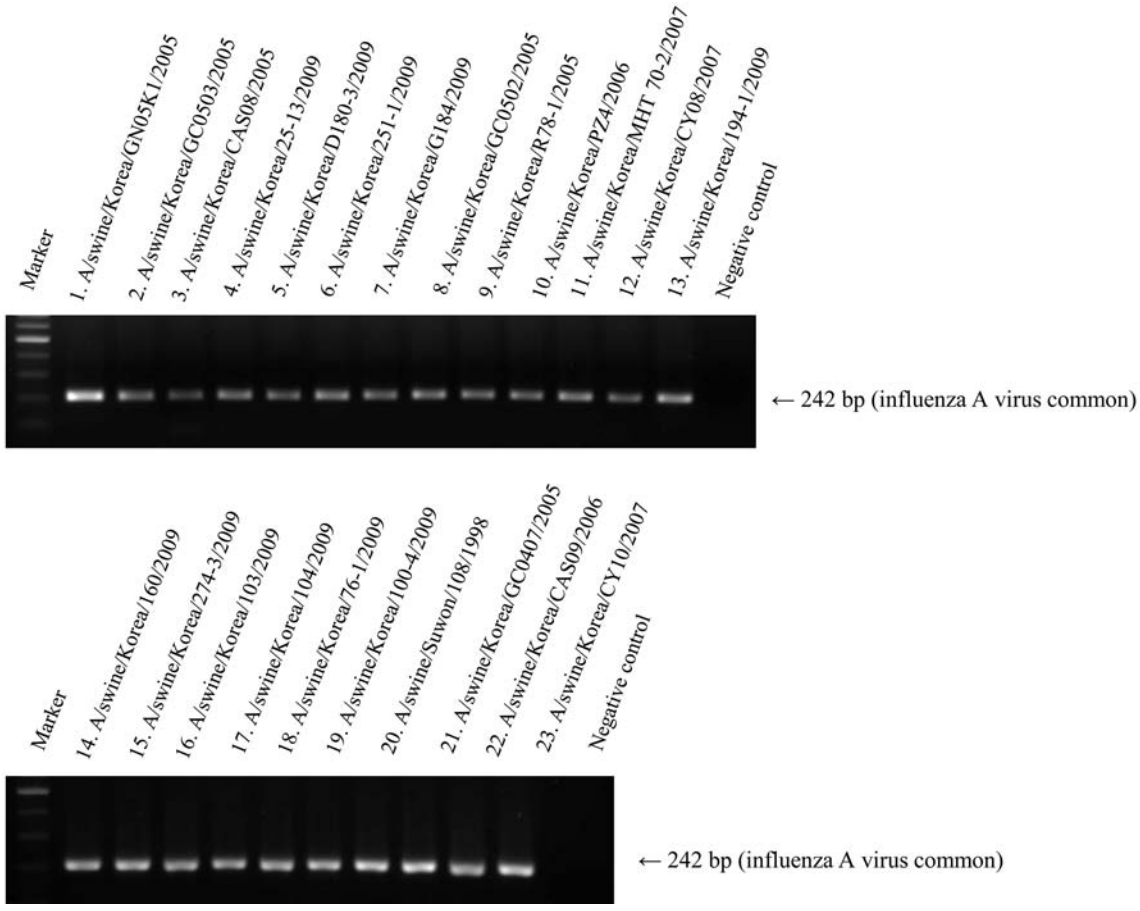

Fig. 3. One-step multiplex RT-PCR with endemic swine influenza A viruses. Twenty-three endemic swine influenza viruses, for which the titers ranged from 10^4 to 10^6 $TCID_{50}$/m*l*, were applied to the one-step multiplex RT-PCR assay. PCR products were visualized in 1% agarose gels.

Table 3. Influenza A virus common M RT-PCR and one-step multiplex RT-PCR results for pig nasalswab samples from slaughterhouses

		One-step multiplex RT-PCR		Total
		Positive	Negative	
Influenza A virus common M RT-PCR	Positive	0	0	0
	Negative	0	150	150
Total		0	150	150

virus infections as a second-degree notifiable animal disease according to the Enforcement Decree in the Act on the Prevention of Contagious Animal Diseases on August 21, 2009. Public awareness of pandemic (H1N1) 2009 influenza virus infections has driven the Korean government to prove that the Korean pork industry is safe from the pandemic flu. The Korean government began monitoring pandemic (H1N1) 2009 influenza virus infections in pigs in May 2009 and found that 17 farms were contaminated with this virus [29]. The government plans to monitor pandemic (H1N1) 2009 influenza virus infections in pigs every year, including 2010, for the time being.

Therefore, we developed a one-step multiplex RT-PCR for routine pandemic (H1N1) 2009 influenza monitoring of pigs for our national plan in the future. With the application of this one-step multiplex RT-PCR system, it would be possible to shorten the time for detecting pandemic (H1N1) 2009 influenza viruses. Using a conventional M or NP RT-PCR assay, it would take more time to obtain sequencing data. This detection system could differentiate pandemic (H1N1) 2009 influenza viruses from endemic swine influenza A viruses using one RT-PCR reaction and allow rapid action to be taken to prevent viral dissemination from contaminated farms or pigs. Of course, we eventually need to

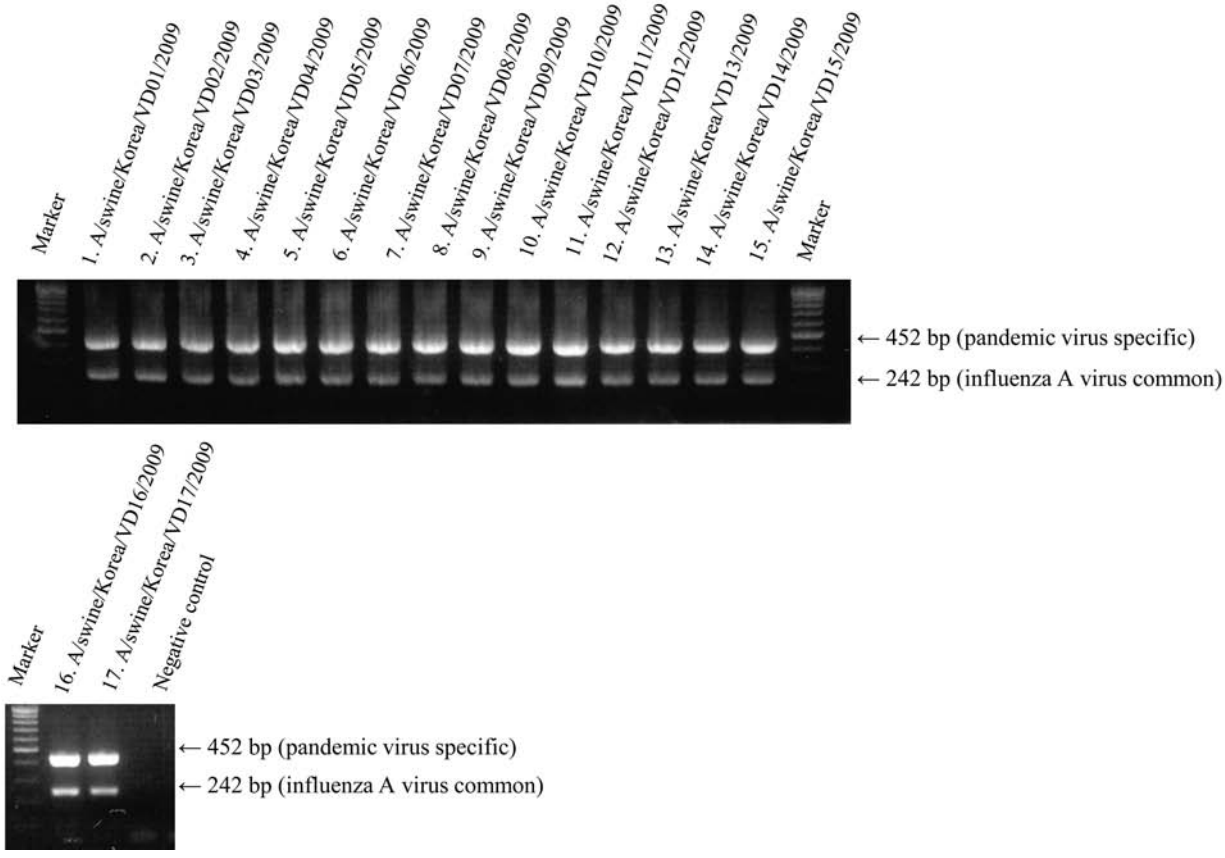

Fig. 4. Detection of pandemic (H1N1) 2009 influenza viruses isolated from pigs. Seventeen pandemic (H1N1) 2009 influenza viruses isolated from pig herds in 2009 were applied to the one-step multiplex RT-PCR assay. PCR products were visualized in 1% agarose gels.

analyze the entire 8 gene sequences of the newly detected pandemic (H1N1) 2009 influenza virus to determine if there are mutations or reassortment in the genes, but this is not required for diagnostic purposes.

The WHO and CDC distributed real-time RT-PCR protocols for detection and characterization of swine influenza from human samples in April 2009. These methods are applicable only for humans because they are not able to differentiate pandemic (H1N1) 2009 influenza viruses from swine influenza viruses. In the case of pandemic 2009 (H1N1) influenza viruses in the human population, detection of the swine influenza viral gene would be enough to warrant preventive measures in controlling the disease and to start medical treatment for patients because there have not been many human cases of swine influenza virus infection in the last half century [26, 27, 31, 33].

Other groups have reported real-time RT-PCR methods that are able to differentiate pandemic (H1N1) 2009 influenza viruses from endemic swine influenza viruses among pig samples [19, 20], but our system is the first one-step multiplex RT-PCR system that detects pandemic (H1N1) 2009 influenza virus infections from swine samples. The detection limits of most of the real-time RT-PCR methods for pandemic (H1N1) 2009 influenza virus have been between 1 and 200 copies [14, 18, 20, 32], and our system's detection limit is approximately 100 copies. There is a possibility that the sensitivity of the this assay might be lower than we verified if we were to test field nasal swab samples, which include many biological substances such as blood, nasal mucosa and nasal mucus. We plan to evaluate this assay with field samples if there are additional pandemic (H1N1) 2009 influenza outbreaks on pig farms in Korea.

Genetic and biologic observations suggest that pigs may serve as "mixing vessels" for the generation of reassortant influenza A viruses because pigs have cell surface receptors for both human and avian influenza A viruses [9, 13, 38]. It is likely that any reassortant virus between pandemic (H1N1) 2009 influenza viruses and endemic swine influenza viruses would have unpredictable pathogenicity. The prompt detection of pandemic (H1N1) 2009 influenza viruses in pig herds and reasonable action for controlling the disease would benefit both pigs and humans. Our system would primarily be used to monitor our pig herds efficiently, especially where real-time PCR equipment is not available.

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