

Comparative Analysis of the Multiple Test Methods for the Detection of Pandemic Influenza A/H1N1 2009 Virus

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Received: April 27, 2010 / Revised: June 29, 2010 / Accepted: July 15, 2010

Accurate and rapid diagnosis of Pandemic Influenza A/H1N1 2009 virus (H1N1 2009) infection is important for the prevention and control of influenza epidemics and the timely initiation of antiviral treatment. This study was conducted to evaluate the performance of several diagnostic tools for the detection of H1N1 2009. Flocked nasopharyngeal swabs were collected from 254 outpatients of suspected H1N1 2009 during October 2009. This study analyzed the performances of the RealTime Ready Inf A/H1N1 Detection Set (Roche), Influenza A (H1N1) Real-Time Detection Kit (Bionote), Seeplex Influenza A/B OneStep Typing Set [Seeplex Reverse Transcriptase PCR (RT-PCR)], BinaxNow Influenza A & B Test Kit [Binax Rapid Antigen Test (RAT)], and SD BIOLINE Influenza Ag kit (SD RAT). Roche and Bionote real-time RT-PCR showed identical results for the H1N1 2009 hemagglutinin gene. Compared with real-time RT-PCR, the sensitivities and specificities were 83.7% and 100% for Seeplex RT-PCR, 64.5% and 94.7% for Binax RAT, and 69.5% and 100% for SD RAT. The sensitivities of Seeplex RT-PCR, Binax RAT, and SD RAT in patients aged over 21 years were 73.7%, 47.4%, and 57.9%, respectively. The sensitivities of Seeplex RT-PCR, Binax RAT, and SD RAT on the day of initial symptoms were mostly lower (68.8%, 56.3%, and 31.3%, respectively). In conclusion, multiplex RT-PCR and RAT for the detection of H1N1 2009 were significantly less sensitive than real-time RT-PCR. Moreover, a negative RAT may require more sensitive confirmatory assays, because it cannot be ruled out from influenza infection.

Keywords: Influenza, H1N1, rapid antigen test, multiplex, RT-PCR

Influenza outbreaks occur annually during the late fall through early spring seasons, and past influenza pandemics have occurred at 10- to 40-year intervals during the last century [28]. Since the Pandemic Influenza A/H1N1 2009 virus (H1N1 2009) was identified in Mexico and the United States in March and early April 2009, the H1N1 2009 has been rapidly spreading from person-to-person worldwide [5]. Laboratory-confirmed cases of H1N1 2009 have been reported in more than 213 countries, and at least 17,700 H1N1-related deaths have been reported by the World Health Organization as of 4 April 2010 [27]. In Korea, since the first confirmed case in May 2009, the H1N1 2009 has spread rapidly to many areas with 243 confirmed deaths reported by the Korea Centers for Disease Control and Prevention as of 27 February 2010 [16].

Rapid diagnosis of influenza viruses is important for the prevention and control of influenza epidemics and for the timely initiation of antiviral treatment [2, 20]. Virus isolation has been the gold standard for diagnosis of influenza but requires the time needed to obtain a positive results [13, 15]. Immunofluorescence-based assay has the advantage of providing available results within hours after specimen submission but requires laboratory expertise and the high quality of the specimen collection [13]. Molecular diagnostic tests such as the reverse transcriptase-polymerase chain reaction (RT-PCR) are currently the methods of choice for the detection of influenza virus, owing to their high sensitivity [9, 24]. However, RT-PCR requires special equipment and expertise. Moreover, conventional RT-PCR is time-consuming because of the multiple processes involved in testing [15, 20].

Since the development of real-time RT-PCR detection of H1N1 2009 RNA by the Centers for Disease Control and Prevention (CDC) [6], real-time RT-PCR for the diagnosis of H1N1 2009 has been used in many laboratories. Viral antigen-based rapid antigen tests (RAT) have been used in

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many clinical settings because of the rapid results and ready availability of those tests [7], but its poor sensitivity has created many problems in the management of H1N1 2009 [8]. This study was conducted to evaluate the performances of several diagnostic tools for the detection of H1N1 2009.

MATERIALS AND METHODS

Patient Population and Specimen Collection

Clinical specimens were obtained from 254 outpatients who were evaluated for H1N1 infection during October 2009 at Soonchunhyang University Cheonan Hospital, Korea. The age of the patients ranged from 5 months to 78.3 years, with a median age of 13.9 years. The patients were categorized into 4 age groups: 0 to 7 years (60 patients), 7 to 14 years (68), 14 to 21 years (64), and over 21 years (62). The male/female ratio was 0.97:1 (125:129). The most common presenting symptoms of patients were fever (90.2% of patients), cough (78.3%), and sore throat (59.8%). Three swabs per patient were collected using flocked swabs (Copan Diagnostics, Murrieta, CA, U.S.A.) from each patient's nasopharynx. One swab was placed into a test tube containing viral transport medium for RT-PCR, and the remaining two swabs were placed separately into two test tubes each containing different types of suitable buffer solutions for RAT.

Extraction of Viral RNA

The viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from 140 µl of clinical samples in viral transport medium, in accordance with the manufacturer's instructions. The RNA was eluted from columns with 50 µl of RNase-free water.

Real-Time RT-PCR

Real-time RT-PCR was conducted using the RealTime Ready Inf A/H1N1 Detection Set (Roche Real-Time RT-PCR; Roche Applied Science, Mannheim, Germany) and Influenza A (H1N1) Real-Time Detection Kit (Bionote Real-Time RT-PCR; Bionote, Hwasung, Korea) for comparison.

Each reaction mixture for the Roche Real-Time RT-PCR was prepared in accordance with the manufacturer's instructions. Briefly, for detection of the type A influenza matrix gene and RNase P gene, RNA (5 µl), water (4.6 µl), Enzyme Blend (0.4 µl), Primer/Probe Mix Inf A/M2 (3 µl), Primer/Probe Mix/Human Nucleic Acid Extraction Control (3 µl), and Reaction Buffer (4 µl) were used in a total reaction volume of 20 µl. The RNase P gene serves as an internal control for human nucleic acid. For detection of the hemagglutinin gene specific for H1N1 2009, the final reaction volume of 20 µl included RNA (5 µl), water (7.6 µl), Enzyme Blend (0.4 µl), Primer/Probe Mix InfA/H1 (3 µl), and Reaction Buffer (4 µl). Reverse transcription, amplification, and detection were performed on a LightCycler 480 (Roche Applied Science, Mannheim, Germany) with the following conditions: initial holds at 58°C for 8 min and 95°C for 30 s, followed by 45 cycles at 95°C for 1 s, 60°C for 20 s, and 72°C for 1 s.

The Bionote RT-PCR was carried out in a 25 µl volume containing RNA (5 µl), Detection Solution Inf A & RNaseP or Detection Solution Novel Inf A/H1 (7 µl), 2× Enzyme Buffer (12.5 µl), and RT Mix (0.5 µl), according to the protocol recommended by the CDC [6]. Reverse transcription, amplification, and detection were performed on the 7500 Real-Time PCR System (Applied Biosystems,

Foster City, U.S.A.). PCR conditions were initial holds at 58°C for 30 min and 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 55°C for 32 s.

Conventional Multiplex RT-PCR

Conventional multiplex RT-PCR was conducted using the Seeplex Influenza A/B OneStep Typing Set (Seeplex RT-PCR; Seegene, Seoul, Korea). The panel of Seeplex RT-PCR assays was capable of detecting influenza A, influenza B, and 3 subtypes of influenza A (H1, H3, and H1N1 2009). Briefly, reverse transcription and PCR amplification were performed on the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, U.S.A.) in a 50 µl volume containing RNA (10 µl), 5× Flu A/B OneStep Typing PM (10 µl), 5× OneStep RT-PCR Buffer (10 µl), 8-Mop solution (16 µl), dNTP Mix (2 µl), and OneStep RT-PCR Enzyme Mix (2 µl), in accordance with the manufacturer's instructions. PCR conditions were initial holds at 50°C for 30 min and 95°C for 15 min, followed by 45 cycles at 94°C for 30 s, 60°C for 90 s, and 72°C for 60 s. Amplicons were detected by gel electrophoresis.

Influenza RAT

Influenza RAT was conducted using the BinaxNow Influenza A & B Test Kit (Binax RAT; Inverness Medical Innovations, Hague, the Netherlands) and SD BIOLINE Influenza Ag Kit (SD RAT; Standard Diagnostics, Yongin, Korea), in accordance with the manufacturer's instructions.

Viral Culture and Sequencing

All specimens that produced discrepant results between real-time RT-PCR and multiplex RT-PCR were tested further for viral culture and sequencing. Viral culture was conducted in Madin-Darby canine kidney (MDCK) cells. Each culture tube containing MDCK cells in minimum essential medium supplemented with glucose solution, vitamin solution, trypsin-EDTA, and antibiotic-antimycotic solution was inoculated with 0.2 ml of clinical specimens and was incubated at 35°C with 5% CO₂ until the appearance of influenza-like cytopathic effects or until 14 days after inoculation with no detectable cytopathic effects.

In all samples that were positive for H1N1 2009 in real-time RT-PCR testing but negative in multiplex RT-PCR tests, fragments from the hemagglutinin genes were sequenced. The real-time RT-PCR products (139 bp) were cloned into the pDrive cloning vector (Qiagen). After cloning, the nucleotide sequences were determined using the BigDye Terminator Cycle sequencing kit (Applied Biosystems Foster City, U.S.A.) with the M13 forward (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-AACAGCTATGACCATG-3') primers in the 3730xl DNA Analyzer (Applied Biosystems, Foster City, U.S.A.). The sequences obtained were confirmed as H1N1 2009 by comparison with those in the GenBank (National Center for Biotechnology Information) database using the Basic Local Alignment Search Tool (BLAST).

RESULTS

Real-Time RT-PCR

Among 254 specimens, 141 tested positive for the H1N1 2009 hemagglutinin gene in the Roche and Bionote real-

time RT-PCR tests. All specimens that were positive for the hemagglutinin gene were positive for the type A influenza matrix gene in the Roche real-time RT-PCR. Among them, 140 specimens were positive for the matrix gene in the Bionote real-time RT-PCR. One specimen that was positive for the hemagglutinin gene and was negative for the matrix gene in the Bionote real-time RT-PCR was confirmed as H1N1 2009 by sequencing. A total of 113 specimens were negative for the hemagglutinin gene and the matrix gene in the Roche and Bionote real-time RT-PCR. The average crossing-point (Cp) values of hemagglutinin gene-positive samples in the Roche real-time RT-PCR was 24.6 and the average cycle threshold (Ct) of hemagglutinin gene-positive samples in the Bionote real-time RT-PCR was 24.1.

Among 254 patients, 141 patients (55.5%) were confirmed as H1N1 2009 positive in the real-time RT-PCR. The age of H1N1 2009-confirmed patients ranged from 7 months to 53 years, with a median age of 14.1 years. The male/female ratio was 1.01:1 (71:70). The most common presenting symptoms of H1N1 2009 patients were fever (96.5% of patients), cough (90.8%), and sore throat (65.2%).

Conventional Multiplex RT-PCR

Among all the samples, 118 were positive for H1N1 2009 in the Seeplex RT-PCR. All positive samples in the Seeplex RT-PCR were also positive in the real-time RT-PCR. Among the 136 Seeplex RT-PCR-negative samples, 23 samples tested positive for H1N1 2009 in the real-time RT-PCR. All samples that were negative in the Seeplex RT-PCR but positive in the real-time RT-PCR were confirmed as H1N1 2009 positive using sequencing. Based on maximum identity, all the sequences indicating the hemagglutinin genes of H1N1 2009 matched to those in the GenBank, with homology ranging from 98% to 100% (100% identity, 11 samples; 99% identity, 8 samples; 98% identity, 4 samples). Among 23 samples in the that were

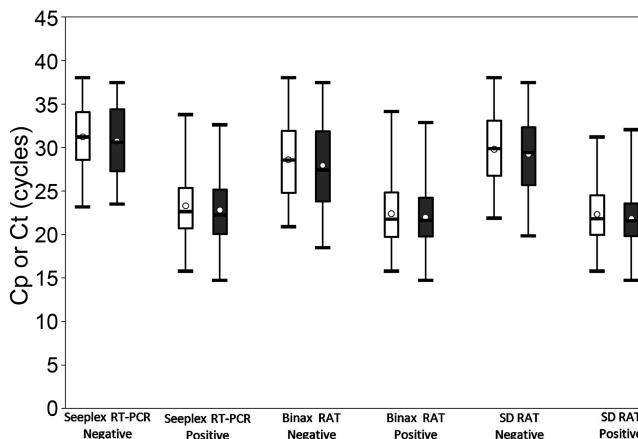


Fig. 1. Box plot showing Cp values (open box) of Roche real-time RT-PCR and Ct values (hatched box) of Bionote real-time RT-PCR for H1N1 2009 hemagglutinin gene in H1N1 2009 patients. The ends of the box are the 25th and 75th quartiles, respectively. The open circles are average Cp or Ct values, and the lines across the middle of the box identify the median values.

negative in the Seeplex RT-PCR but positive in the real-time RT-PCR, 18 samples were positive for viral culture.

The sensitivity, specificity, positive predictive value (percent of patients with positive results who were infected; PPV) and negative predictive value (percent of patients with negative test results who were not infected; NPV) of Seeplex RT-PCR compared with real-time RT-PCR were 83.7%, 100%, 100%, and 83.1%, respectively (Table 1). The average Cp and Ct values of real-time RT-PCR in the Seeplex RT-PCR-positive samples were 23.8 and 22.8, respectively. However, the average Cp and Ct values of real-time RT-PCR in the Seeplex RT-PCR-negative samples that tested positive by real-time RT-PCR were 31.2 and 30.7, respectively (Fig. 1). The sensitivity of Seeplex RT-PCR was 88.2% in the 0- to 7-year-old group,

Table 1. Results of Seeplex RT-PCR, Binax RAT, and SD RAT Compared with Real-Time RT-PCR for the Detection of H1N1 2009.

Test methods	Real-time RT-PCR		Sensitivity (95% CI ^a)	Specificity (95% CI)	PPV ^b	NPV ^c
	Positive (n=141)	Negative (n=113)				
Seeplex RT-PCR						
Positive	118	0	83.7% (76.7~88.9%)	100% (96.7~100%)	100%	83.1%
Negative	23	113				
Binax RAT						
Positive	91	6	64.5% (56.4~72.0%)	94.7% (88.9~97.5%)	93.8%	68.2%
Negative	50	107				
SD RAT						
Positive	98	0	69.5% (61.5~76.5%)	100% (96.7~100%)	100%	72.4%
Negative	43	113				

^aCI, confidence interval.
^bPPV, positive predictive value.
^cNPV, negative predictive value.

Table 2. Sensitivities of Seeplex RT-PCR, Binax RAT, and SD RAT for the Detection of H1N1 2009 According to Age Groups.

Age group	Sensitivity (95% CI ^a)		
	Seeplex RT-PCR	Binax RAT	SD RAT
0–7 (n=60)	88.2% (65.7–96.7)	64.7% (41.3–82.7)	64.7% (41.3–82.7)
7–14 (n=68)	83.0% (70.8–90.8)	62.3% (48.8–74.1)	67.9% (54.5–78.9)
14–21 (n=64)	86.5% (74.7–93.3)	73.1% (59.7–83.2)	76.9% (63.9–86.3)
>21 (n=62)	73.7% (51.2–88.2)	47.4% (27.3–68.3)	57.9% (36.3–76.9)

^aCI, confidence interval.

83% in the 7- to 14-year-old group, 86.5% in the 14- to 21-year-old group, and 73.7% in the over 21-year-old group (Table 2).

Influenza RAT

Of 254 specimens, 97 were positive and 157 were negative for influenza A in the Binax RAT. Among 97 specimens that tested positive in the Binax RAT, 91 tested positive and 6 tested negative in the real-time RT-PCR. Of 157 Binax RAT-negative samples, 50 were positive and 107 were negative in the real-time RT-PCR. The sensitivity, specificity, PPV, and NPV of Binax RAT compared with those of the real-time RT-PCR were 64.5%, 94.7%, 93.8%, and 68.2%, respectively (Table 1). The average Cp and Ct values of the samples that tested positive in the Binax RAT and real-time RT-PCR were 22.4 and 22.0, respectively. The average Cp and Ct values of the Binax RAT-negative samples that tested positive in the real-time RT-PCR were 28.6 and 27.9, respectively (Fig. 1). The sensitivity of the Binax RAT in the 14- to 21-year-old group was mostly higher (73.1%) and the sensitivity in the over 21-year-old group was mostly lower (47.4%) (Table 2).

Among 254 clinical samples, the SD RAT identified 98 of 141 real-time RT-PCR-positive influenza infections. All specimens that tested negative by real-time RT-PCR were negative for influenza A in the SD RAT. The sensitivity, specificity, PPV, and NPV of the SD RAT compared with those of the real-time RT-PCR were 69.5%, 100%, 100%, and 72.4%, respectively (Table 1). The average Cp and Ct values of the samples that tested positive in the SD RAT and real-time RT-PCR were 22.3 and 21.8, respectively. The average Cp and Ct values of the SD RAT-negative samples that tested positive in the real-time RT-PCR were 29.8 and 29.3, respectively (Fig. 1). The sensitivity of the SD RAT in the 14- to 21-year-old group was mostly higher (76.9%) and the sensitivity in the over 21-year-old group was mostly lower (57.9%) (Table 2).

Sensitivities of Multiplex RT-PCR and RAT According to Symptom Duration

Among 248 patients who presented with flu-like symptoms such as fever, cough, and sore throat, 57.9% of patients

were diagnosed with H1N1 2009. The 248 patients with flu-like symptoms were categorized into 4 groups based on duration of initial symptoms: 0 days (26 patients), 1 day (107), 2 days (44), and 3 days or more (71). The positive rate for H1N1 2009 for symptom duration was 61.5% for 0 days, 63.6% for 1 day, 56.8% for 2 days, and 45.1% for 3 days or more. The sensitivities of the Seeplex RT-PCR, Binax RAT, and SD RAT for symptom duration of 0 days were mostly lower (68.8%, 56.3%, and 31.3%, respectively). The sensitivities of the Seeplex RT-PCR, Binax RAT, and SD RAT for symptom duration of 2 days were mostly higher (100%, 88%, and 88%, respectively) (Fig. 2).

DISCUSSION

Most patients with influenza present with typical flu-like symptoms. Some authors have reported the sensitivity and specificity of fever and cough for the diagnosis of influenza during flu season as 64–78% and 55–67%, respectively [1, 17, 30]. It has also been reported that the most common presenting symptoms of 642 H1N1 2009-confirmed cases from April 15 through May 5, 2009 were fever (94%), cough (92%), and sore throat (66%) [19]. During the

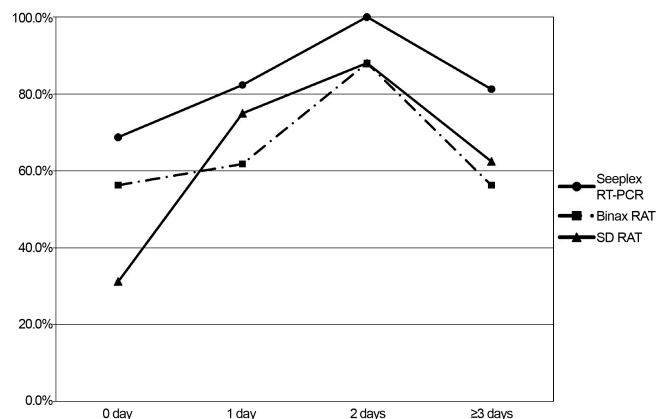


Fig. 2. Sensitivities of the Seeplex RT-PCR, Binax RAT, and SD RAT for the detection of H1N1 2009 according to symptom duration.

H1N1 2009 pandemic, our results showed that most patients with H1N1 2009 were presenting with fever (96.5%), cough (90.8%) and sore throat (65.2%). Furthermore, when making a clinical diagnosis on the basis of fever and cough, the sensitivity of diagnosis for H1N1 2009 was relatively high (87.9%) but the specificity was relatively low (51.3%) (data not shown). However, the clinical diagnosis of influenza on the basis of symptoms alone may be inaccurate, because symptoms caused by influenza cannot be distinguished from those caused by other respiratory pathogens, and patients with influenza do not always present with flu-like symptoms [3, 10, 18].

RT-PCR for the detection of virus-specific RNA is used to diagnose influenza in many laboratories because of its high sensitivity [9, 15, 24]. Because of the little available data on the performance of RT-PCR in detecting H1N1 2009, our study included a comparison of several RT-PCR techniques. This study revealed that the Roch and Bionote RT-PCRs showed identical results for hemagglutinin gene and matrix gene detection in all cases, except for just one case of matrix gene detection. Real-time RT-PCR has the advantages of high sensitivity and rapid turnaround time but has disadvantages of requiring high technical expertise and specialized equipment. However, the average turnaround time of real-time RT-PCR in our laboratory was not actually short, owing to batch processing and no nighttime availability of testing. In our previous report, the average turnaround times of real-time RT-PCR were 14.9 hours [7]. The Seeplex RT-PCR for the detection of H1N1 2009, which had a sensitivity of 83.7%, had a lower sensitive than the real-time RT-PCR. Although the Seeplex RT-PCR has the advantages of simultaneous detection of influenza A, influenza B, seasonal H1, seasonal H3, and H1N1 2009, results were not immediately available because of its requiring longer amplification times than the real-time RT-PCR and its additional electrophoresis step.

Because of rapid results (within 15–30 min), easy availability, and low costs, the influenza RAT is used for the rapid diagnosis of influenza in a variety of clinical settings, such as emergency departments and outpatient clinics [7, 25]. The RAT has revealed a broad range of sensitivities and specificities depending on the virus types or test kits used. Sensitivities of the RAT for the detection of seasonal influenza A and H1N1 2009 were reported as ranging from 10–95% and 9.6–69%, respectively, with specificities reported as 76–100% and 80–100%, respectively [4, 12, 14, 15, 21]. Sensitivities of the Binax RAT and SD RAT for the detection of seasonal influenza A were reported as 47.2–80% and 61.9%, respectively, with specificities reported as 98–100% and 96.8%, respectively [2, 20, 22, 29]. Moreover, the sensitivity of the Binax RAT for H1N1 2009 was reported as ranging from 9.6% to 60.3% [4, 11, 12]. In this study, there were no significant

differences between the sensitivities of the Binax RAT and SD RAT for H1N1 2009 (64.5% by Binax and 69.5% by SD), and the specificities of both were relatively high (94.7% and 100%). Our results showed that the sensitivity of the Binax RAT for the detection of H1N1 2009 was higher than those reported by other authors. We presume that the higher sensitivity in our study was due to a higher quality of clinical specimens (collected with flocked swabs by well-trained nurses) and differences in the sample types and patient populations (*e.g.*, age distribution and duration from onset of illness). Diagnostic tests to differentiate between seasonal influenza A virus subtypes may be important because of resistance of current seasonal H1 strains to oseltamivir [26]. However, most of currently available RATs cannot distinguish between seasonal influenza viruses and pandemic influenza A/H1N1 2009 virus. Recently, a new RAT for the specific detection of the H1N1 2009 virus was developed [7]. There has been much debate as to the role of the RAT in the diagnosis of H1N1 2009 [26]. Although some have reported that the RAT is unsuitable for virologic diagnoses owing to its lower sensitivity, others have deemed the RAT valuable in countries with lack of resources, owing to its ease of performance and rapid turnaround time [8, 25].

The sensitivities of RATs for seasonal influenza in adult and senior patients have been reported to be lower than those in children (70–80% in children and <40–60% in adults), as a reason of children shedding larger quantities of virus for longer periods than adults [13, 23]. Our RAT results in adult were similar to those of other studies showing lower sensitivities than in children (47.4% by Binax, 57.9% by SD in the over 21-year-old groups). According to symptom duration, the sensitivities of RATs on the day of initial onset of symptoms were mostly low, and those on day 2 were mostly high. These findings suggest that the clinical usefulness of the RAT may be limited when it is used during the very early phases of illness.

In conclusion, the multiplex RT-PCR and RAT for the detection of H1N1 2009 were significantly less sensitive than the real-time RT-PCR. Moreover, a negative RAT may require more sensitive confirmatory assays, because it cannot be ruled out from influenza infection.

Acknowledgments

We sincerely thank Youn Kyoung Oh and Sun Nyeo Jeong, BioNote, Inc., for cloning and sequencing of RT-PCR products. We also thank Sun Hwa Lee, Tae Young Lee, Young Ho Kim, and Young Il Park for excellent technical assistance, and Eun Joo Lee and Min Joo Yoo for specimen collection.

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