CENTRAL ASIAN JOURNAL of MEDICAL SCIENCES
CAJMS

Original Article

Cent Asian J Med Sci. 2016 May;2(1):25-31.

Detection of H275Y Mutation of A(H1N1)pdm09 Viruses Using a Real-time RT-PCR Assay in Mongolia

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Submitted: January 30, 2016
Revised: March 25, 2016
Accepted: April 5, 2016

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Objectives: Oseltamivir is recommended as a first-line drug for the prophylaxis and treatment of influenza A(H1N1)pdm09 infection worldwide. However, oseltamivir-resistant influenza A(H1N1)pdm09 viruses have been identified and are mostly associated with an H275Y substitution in the neuraminidase (NA) gene. Careful and rapid laboratory testing for antiviral resistance surveillance of influenza viruses are important to public health and clinical sectors. The aim of our study was to determine oseltamivir-resistant H275Y mutation in clinical specimens by the real-time reverse transcriptase polymerase chain reaction (rtRT-PCR) assay in Mongolia. **Methods:** A total of 215 clinical specimens tested positive for the A(H1N1)pdm09 influenza virus by rtRT-PCR. The specimens were collected between January 2013 and August 2014 from patients who visited influenza surveillance sites. All collected specimens were tested for the presence of the oseltamivir-resistant H275Y mutation by rtRT-PCR and DNA sequencing on the NA gene was performed on four of the clinical samples. **Results:** For all specimens, the H275 (Ct value = 20.4-34.5) was sensitive to oseltamivir by rtRT-PCR assay for detection of the H275Y mutations in the NA gene. Four questionable clinical samples were sequenced for the full NA gene. The data of these samples are available in GISAID influenza virus database with accession numbers EPI533542, EPI462274, EPI462271 and EPI460844. **Conclusion:** The A(H1N1)pdm09 viruses were susceptible to oseltamivir during the study period in Mongolia. The rtRT-PCR assay is useful for detection of the H275Y substitution but other possible mutations in the NA gene of the virus could limit the utility of this technique.

Keywords: Oseltamivir; Influenza, Human; Reverse Transcriptase Polymerase Chain Reaction

Introduction

Oseltamivir has been widely-used for many years as a treatment for influenza virus infection. Until the 2006/2007 influenza season, oseltamivir-resistant influenza viruses were rarely observed worldwide [1]. Oseltamivir resistance in these viruses was due to a point mutation in the neuraminidase (NA) gene that results in a histidine (H) to tyrosine (Y) substitution at the 275th amino acid position (H275Y). During the 2007/2008 influenza season, the presence of oseltamivir-resistant seasonal A(H1N1)

viruses with H275Y mutation was reported, mainly in the European countries [2]. Then during 2008/2009, the infection caused by seasonal A(H1N1) strains with resistant virotype was dominant in many countries with the ability to spread in a very short time [3]. First reports of the A(H1N1)pdm09 virus noted sensitivity to oseltamivir and within a short time since 2009, the oseltamivir-resistant A(H1N1)pdm09 virus has been sporadically detected [4-6]. During 2009/2010, oseltamivir-resistant A(H1N1) pdm09 virus was reported at 0.4% (1/262) in Mongolia [7]. This single patient had no history of using any drug neuraminidase inhibitors (NAI). The infection of oseltamivir-resistant influenza A(H1N1)pdm09 viruses with an H275Y NA substitution has developed due to oseltamivir usage and transmission to others [8]. In some cases, natural genetic variation may have resulted in the change in sensitivity of oseltamivir [9]. In the future, these circulating A(H1N1)pdm09 viruses will have developed complete resistance to oseltamivir, which would make its prevalence and treatment as complicated as the seasonal A(H1N1) virus.

Detection of a resistant virus is usually performed by phenotypic NA inhibition assays or sequencing of viral nucleic acid [10]. These assays are time consuming and are often restricted to reference and research laboratories. In this study, we focused on detecting the oseltamivir resistance of the A(H1N1)pdm09 virus' H275Y mutation, circulating in the 2013 and 2014 cold season, by the real-time reverse transcription polymerase chain reaction (rtRT-PCR) assay in Mongolia.

Materials and Methods

1. Clinical samples

A total of 215 clinical samples (nasal and throat swabs) tested positive for the A(H1N1)pdm09 virus. Samples were collected from patients with influenza-like illness that was defined as the sudden onset of fever of ≥38°C, or with history of fever and cough within the previous three to four days. Sample collection took place at the sentinel surveillance sites in Ulaanbaatar, Mongolia and eight provinces of Mongolia between January 2013 and August 2014. Collected swabs were placed in viral transport medium and kept at -70°C in a deep freezer until performing the rtRT-PCR assay. These samples were pre-tested for detection of influenza A matrix and haemaglutinin genes. A/Denmark/524/2009 with H275 (sensitive) and A/Denmark/528/2009 with 275Y (resistant) viruses were used as reference samples (World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia). The study was approved by the Ethics Committee of the Mongolian Ministry of Health and Sports (ref. N 2013-02).

Table 1. Primers for NA gene sequencing

Primer	Sequence 5'- 3'	Target	Product (bp)
Amplification PCR primers			
NA_F1_0	TGTAAAACGACGGCCAGTAGCAAAAGCAGGAGT	NA	661
NA_R1_661	CAGGAAACAGCTATGACCTCTGTTATTATRCCRTTGTA		
NA_F2_365	TGTAAAACGACGGCCAGTTGTCATBAGRGARCCATTCA	NA	500
NA_R2_865	CAGGAAACAGCTATGACCTAACAGGAGCATTCCTCATA		
NA_F3_525	TGTAAAACGACGGCCAGTCCATACAACTCAAGATTTGAGTCAG	NA	685
NA_R3_1210	CAGGAAACAGCTATGACCCGCTATATCCTGACCACTCATT		
NA_F4_762	TGTAAAACGACGGCCAGTAATGGRCARGCCTCRTACAA	NA	584
NA_R4_1346	CAGGAAACAGCTATGACCGCTGCTYCCRCTAGTCCAGAT		
NA_F5_1080	TGTAAAACGACGGCCAGTGGCAATGGTGTTTGGATAGG	NA	384
NA_R5_1464	CAGGAAACAGCTATGACCCTATGACCAGTAGAAACAAGGAGTTT		
Sequencing primers			
M13F	TGTAAAACGACGGCCAGT	M13	-
M13R	CAGGAAACAGCTATGACC		

2. rtRT-PCR assay for H275Y detection

Viral RNAs were extracted from 140 µL of clinical and reference samples using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) in accordance to the manufacturer's instructions. Ten-fold serial dilutions of extracted RNA from A/Denmark/524/2009 with H275 (sensitive) and A/Denmark/528/2009 with 275Y (resistant) viruses were performed.

The rtRT-PCR of detection for H275Y mutation of the A(H1N1)pdm09 viruses was performed according to the protocol developed by the National Institute of Infectious Diseases (NIID) in Tokyo, Japan [11]. Two probes for detecting oseltamivir-susceptible H275 (VIC-labeled) and oseltamivir-resistant 275Y (FAM-labeled) were used to discriminate nucleotide C and T, respectively, in the NA gene (positioned at 823) of the A(H1N1) pdm09 virus. The rtRT-PCR was held at 60°C for 1 minute, at 50°C for 20 minutes, at 95°C for 5 minutes, cycled for 45 times at 95°C for 15 seconds and at 56°C for 45 seconds and finally held at 60°C for 1 minute in the 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

3. DNA sequencing

The NA gene sequencing of the A(H1N1)pdm09 viruses was performed according to standard methods with an Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems 3130xl Genetic Analyzer using primers which were obtained through the Influenza Reagents Resource, Influenza Division, WHO Collaboration Center, Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA).

At first, the NA gene amplification PCR was performed by five double, reverse and forward (R and F) primers with universal M13 sequence. These primers divide the whole NA gene in each five fragments. Subsequently, the sequencing PCR was performed by universal M13 F and R primers that are designed and based on the universal M13 sequence of the amplified DNA fragments of the NA gene. The used nucleotide sequences of the primers are shown the in Table 1. As a positive control we used reference strains A/Denmark/524/2009 with H275 (sensitive) and A/Denmark/528/2009 with 275Y (resistant). GeneStudio.exe and Mega5.2 software were used to analyze sequences.

Results

Of all the influenza A(H1N1)pdm09 positive samples, 57 were collected from 10 January to 29 April, 2013 and 158 were collected from 8 January to 2 April, 2014. The highest number of collected samples was at the influenza peak season, in January and February of each year (Figure 1).

The Ct values were detected between the 20th and 30th cycles of the serial diluted A/Denmark/524/2009 and A/Denmark/528/2009 reference viruses as shown in the result of amplification plot (Figure 2).

All specimens were sensitive (Ct value = 20.4-34.5) to oseltamivir by rtRT-PCR assay for detection of the H275Y mutation in the NA gene. Generally, the resultant data of the viruses belonged to the zone of the oseltamivir-sensitive positive controls (along Allele X(H275), allelic discrimination plots, Figure 3). Four

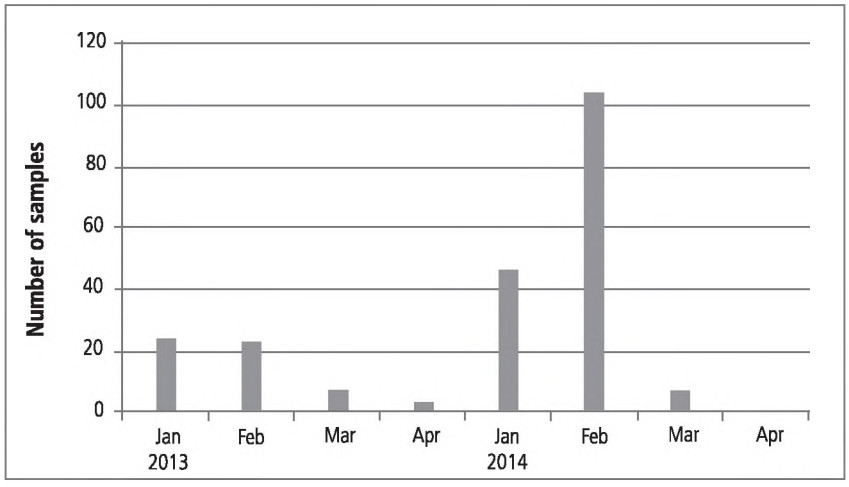

Figure 1. Monthly distribution of samples tested by rtRT-PCR for detection of the H275Y mutation in 2013 and 2014 in Mongolia.

clinical specimens (A/Ulaanbaatar/567/2013(H1N1)pdm09, A/Arvaikheer/684/2013(H1N1)pdm09, A/Ulaanbaatar/1397/2013(H1N1)pdm09, A/Ulaanbaatar/739/2014(H1N1)pdm09) were above the group along the Allele X(H275) line. These four samples are circled in Figures 3A and 3B may be a mixture of viruses.

To clarify the four questionable clinical samples, full NA gene sequencing was performed on them. This NA gene sequencing data was submitted to GISAID influenza virus database and it is available now with accession numbers EPI533542, EPI462274, EPI462271 and EPI460844. Also, the A/Denmark/528/2009(Y275) and A/Denmark/524/2009(H275) reference strains were sequenced for comparison.

The sequencing results confirmed the clinical specimens had no changes of nucleotides C to T at the position 823(C823T) in the NA gene as compared with the resistant and sensitive reference strains. There are not any double peaks of the C and T nucleotides at position 823 on the electropherograms (Figure 4). Therefore, these four clinical specimens were confirmed to not have any mixture of amino acids H275 and Y275 caused by

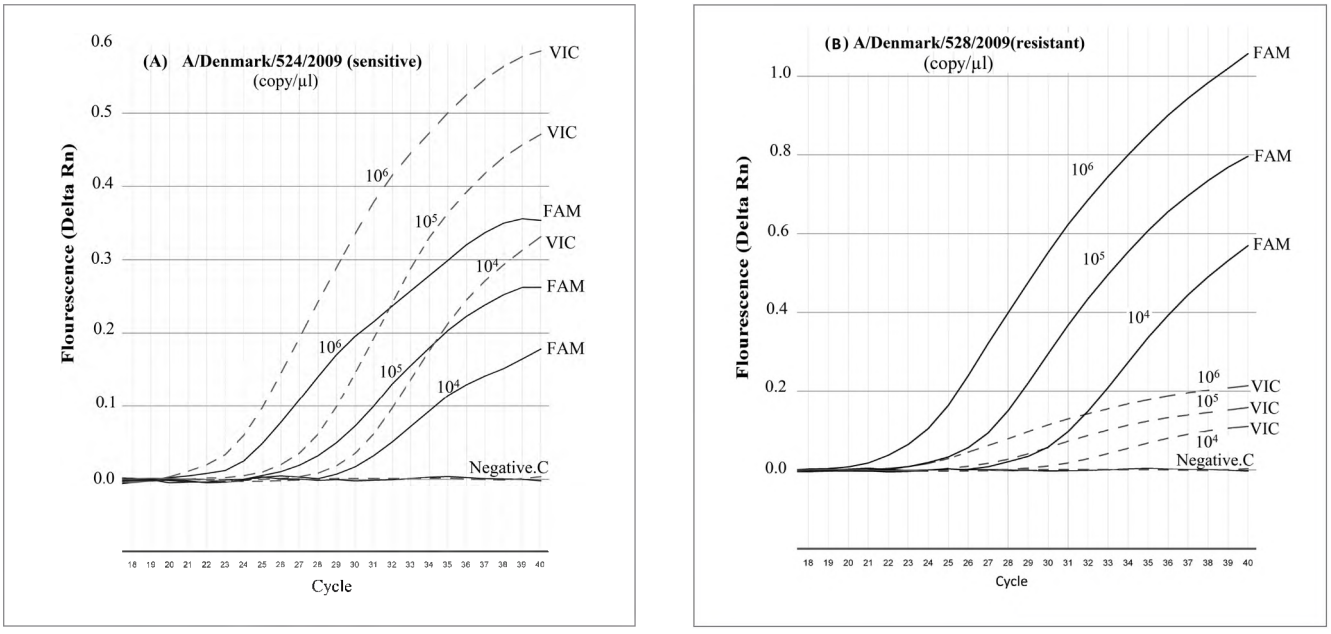

Figure 2. Result of amplification plot obtained from the rtRT-PCR assay for detection of oseltamivir-sensitive and resistant positive controls of (A) A/Denmark/524/2009(H275) and (B) A/Denmark/528/2009(Y275).

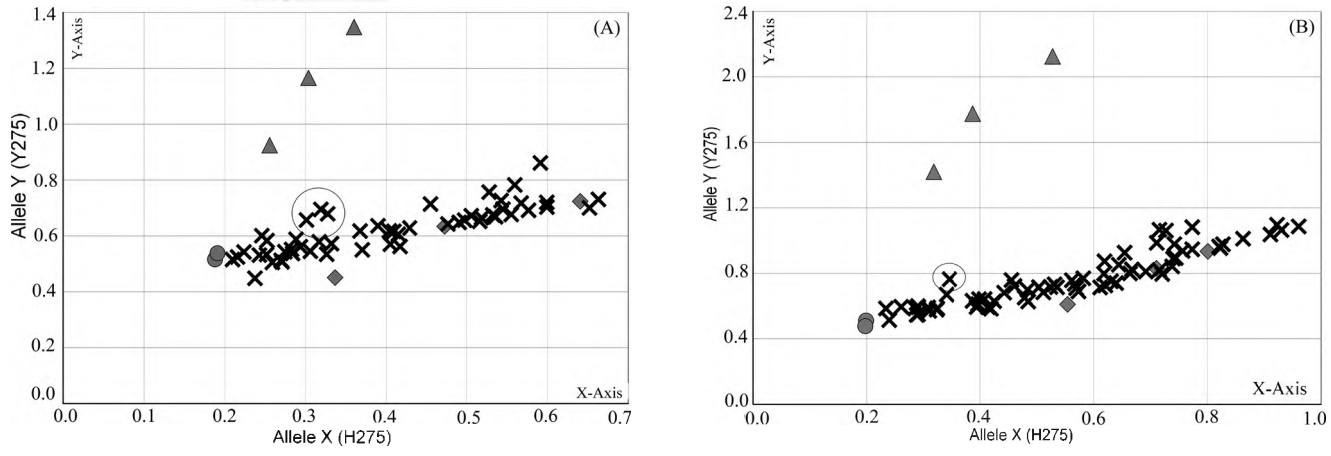

Figure 3. Result of allelic discrimination plot obtained from rtRT-PCR assay detection of H275Y mutation in clinical samples (● negative control, ▲ A/Denmark/528/2009(Y275), ◆ A/Denmark/524/2009(H275) and × clinical samples, four circled samples were used for sequencing).

substitution of nucleotide C to T at position 823 of the NA gene. That the nucleotide C was detected always at position 823 shows that these clinical specimens were sensitive to oseltamivir.

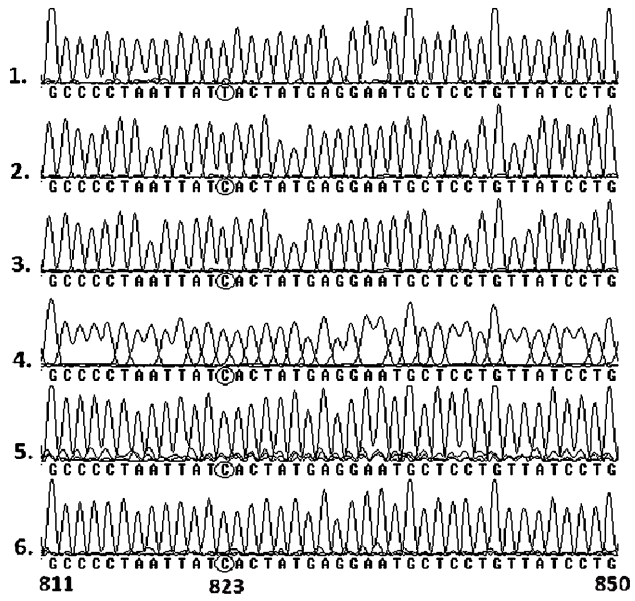

Figure 4. Electropherograms of the NA gene sequences that compared four clinical specimens with reference strains. Sequences between the 811th and 850th nucleotides of the NA gene are shown and the 823rd nucleotides are circled. Samples numbered are:
(1) A/Denmark/528/2009(Y275), (2) A/Denmark/524/2009(H275),
(3) A/Ulaanbaatar/567/2013(H1N1)pdm09,
(4) A/Arvaikheer/684/2013(H1N1)pdm09,
(5) A/Ulaanbaatar/1397/2013(H1N1)pdm09, and
(6) A/Ulaanbaatar/739/2014(H1N1)pdm09.

Discussion

The H275Y amino acid substitution of the NA gene is the most common mutation conferring oseltamivir resistance of the influenza virus. In 2009, WHO described the first A(H1N1)pdm09 virus as naturally resistant to adamantanes but susceptible to the NAIs oseltamivir and zanamivir [12, 13]. In recent years, the most cases of oseltamivir resistance in A(H1N1)pdm09 strains have been associated with the H275Y mutation of NA gene [14]. Oseltamivir-resistant A(H1N1)pdm09 influenza viruses were not identified by rtRT-PCR detection of H275Y mutation in this study. The overall level of oseltamivir resistance has remained relatively low among A(H1N1)pdm09 viruses in the world. However, the sensitivity of rtRT-PCR assay allows it to be performed directly on clinical specimens because it can detect resistant virus at a low level and likely reflects an early warning of developing resistance to oseltamivir.

During the 2013/2014 influenza season, the prevalence of oseltamivir-resistant A(H1N1)pdm09 viruses with H275Y mutation was 1.2% in United States [15, 16], 1.3% in Europe [17], and 2.14% in China [18]. The WHO reported that out of 5152 A(H1N1)pdm09 viruses tested, 175 (3%) showed resistance to one or more of the NA inhibitor drugs, of which the NA amino acid substitution H275Y was present in 169 viruses in the 2013/2014 season [19]. In many countries in the world, the oseltamivir-resistant H275Y mutation development in the NA gene of the influenza viruses depends on usage of oseltamivir and transmission to others. Japan has the highest annual usage of oseltamivir in the world [20] and prevalence was 4.1% oseltamivir-resistant A(H1N1)pdm09 virus with H275Y mutation in the 2013/2014 cold seasons [21].

In Mongolia generally, the availability of oseltamivir and the usage is rare since the first emergence of A(H1N1)pdm09 virus in 2009. For several years, the Mongolian Minister of Health and Sports has made an arrangement for a limited supply of oseltamivir for Mongolian hospitals. In 2013/2014, the oseltamivir usage was also very limited in Mongolia. Therefore, we predicted and showed through our study that the prevalence of oseltamivir-resistant A(H1N1)pdm09 viruses is lower in Mongolia (0%) as compared with developed countries with higher usages of oseltamivir.

Laboratory testing for NAI resistance of influenza viruses can be performed by phenotypic and/or genotypic assays. Among phenotypic assays, the chemiluminescence and fluorescence assays are the most commonly used [22]. Since 2010, the Virology Laboratory at the National Influenza Center in Mongolia started using the chemiluminescence assay to test for NAI resistance of influenza viruses. The phenotypic assays required virus isolation in cell culture. This virus isolation is not only time consuming, but it may also lead to cell-selected NA mutation during virus propagation in vitro. In 2012/2013, 3532 clinical samples were tested by rtRT-PCR for detecting influenza viruses. A total of 462 (13%) were positive and among the positive samples, 99 of them (21.4%) were A(H1N1)pdm09. A total of 20 (20.2%) A(H1N1)pdm09 viruses were isolated by the MDCK cell line [23]. In 2013/2014, 64 A(H1N1)pdm09 viruses were isolated and the isolation rate was 30.3% [24]. The low rate of isolation may be dependent upon delayed arrival of samples and inadequate transportation systems in the country.

The rtRT-PCR detection for oseltamivir-resistant H275Y mutation is performed directly on clinical specimens (such as

nasal or nasopharyngeal swabs). Timely detection of resistance has been useful especially for severe cases because it has provided critical guides on clinical decisions regarding drug usage. An advantage of the rtRT-PCR assay is that it has an increased throughput for the detection of H275Y mutation when compared with phenotypic assays. We suggest that this method might be suitable for active surveillance to monitor the oseltamivir-resistant A(H1N1)pdm09 viruses without virus isolation and phenotypic assays in low income countries like Mongolia. However, the rtRT-PCR assay can only detect the presence of substitution H275Y in NA. Numerous novel NA mutations have been detected such as S247N [25] and I223R [26] using phenotypic assays in viruses isolated from NAI-treated patients. Alternately other rtRT-PCR methods could be developed for detecting them. However, the rtRT-PCR assay does not recognize all possible substitutions or uncharacterized substitutions that effect NAI susceptibility and cannot replace phenotypic assays. The combination of applied and genotypic methods would be the most informative and would provide the optimal testing method for newly emerging oseltamivir-resistant strains.

Genotypic assays are useful in the clinical setting but NAIs are required and NA full-length sequencing is further required in some cases such as for patients with severe immune suppression who may have prolonged virus harboring Because of the high amount of isolates from the phenotypic NAI assay, it is a useful method for monitoring other drug-resistant mutations besides the H275Y mutation in influenza viruses. This research team successfully established the rtRT-PCR assay for detection of H275Y in the laboratory. This assay is important for public health services and clinical management for oseltamivir- resistance detection.

This study has several limitations which need to be addressed. First of all, enough virus-positive samples were not tested due to limited supplies of the kits. Second, there was a limited supply of test kits and primer probes for detecting other possible mutations besides H275Y. However, it might be possible to see other available drug resistant mutations. Third, due to limited time, the details of patient cases such as previous oseltamivir treatment or hospital service on account of influenza virus infection were not studied. Therefore, the probability of screening for antiviral resistance was reduced and only a retrospective study focused on monitoring general prevalence of oseltamivir resistance with H275Y mutation was performed.

In conclusion, the A(H1N1)pdm09 viruses were susceptible to oseltamivir during the study period in Mongolia. The rtRT-PCR assay was shown to be useful for detecting H275Y substitution but other possible mutations in the NA gene of the virus could limit the utility of this technique.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

The authors would like to thank the doctors and nurses at the influenza sentinel surveillance sites in the eight provinces and in Ulaanbaatar, Mongolia for collecting samples. We thank the staff at the Influenza Virus Research Center, National Institute of Infectious Diseases in Tokyo, Japan, especially Mina Nakauchi. We are grateful to the staff, especially Aeron Hurt, of the WHO Collaborating Center for Reference and Research on Influenza in Melbourne, Australia for providing reference control strains and for their laboratory technical support. We also express our gratitude to the US/ Mongolia Cooperative Agreement Project "Development of Influenza Surveillance Network" U50/ CCU024411 supported by CDC in Atlanta, GA, USA

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