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Molecular Characterization and Phylogenetic Analysis of H3N2 Human Influenza A Viruses in Cheongju, South Korea

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To investigate the genetic characteristics of human influenza viruses circulating in Chungbuk province, we tested 510 clinical samples of nasopharyngeal suction from pediatric patients diagnosed with respiratory illness between June 2007 and June 2008. Genetic characterization of the HA genes of H3N2 isolates indicated the relative higher similarity to A/Virginia/04/07 (99.6%) rather than that of A/Wisconsin/67/2005 (98.4%), a Northern Hemisphere 2007~2008 vaccine strain, based on amino acid sequences. We found several altered amino acids at the H3 HA1 antigenic sites compared with the vaccine strain; K140I at site A, K158R at site B, and K173N (H471) or K173Q, and S262N at site E, but there was no antigenic shift among the H3N2 viruses. Interestingly, A/Cheongju/H383/08 and A/Cheongju/H407/08 isolates had single amino acid substitution at D151G on the catalytic site of the N2 NA while A/Cheongju/H412/08 and A/Cheongju/H398/07 isolates had one amino acid deletion at residue 146. Furthermore, we found that 25% (3 out of 12 isolates) of the H3N2 subtype viruses had the amino acid substitution at position 31 on the M2 protein (Aspartic acid to Asparagine) and confirmed their drug-resistance by biological assays. Taken together, the results of this study demonstrated continuous evolutions of human H3N2 viruses by antigenic drift and also highlighted the need to closely monitor antigenic drug resistance in influenza A viruses to aid in the early detection of potentially pandemic strains, as well as underscore the need for new therapeutics.

***Keywords*:** influenza A virus, H3N2, antigenic drift, amantadine-resistant

Influenza virus is a major viral respiratory pathogen that causes yearly epidemics in the temperate climates with epidemic influenza remaining a major cause of morbidity and mortality (Wright and Webster, 2001). There are three different types of influenza viruses (A, B, and C) that cause illness in human population. Type B and C viruses are restricted to humans whereas type A influenza virus is a highly infectious respiratory pathogen of mammals including humans, pigs, horses, whales, and even avian species (Guo *et al*., 1983; Osterhaus *et al*., 2000; Wright and Webster, 2001).

One of the principal biological properties of influenza A viruses is their antigenic variability which takes two forms; antigenic shift and antigenic drift. Antigenic shift is a consequence of the multiple infections of at least two different viruses in one host. The segmented nature of the genome allows for the exchange of genetic information during mixed infection of cells with different viruses. Therefore, genetic reassortment (antigenic shift) has been constantly contributing to the evolution of novel pandemic strains (Webster *et al*., 1975; Kawaoka *et al*., 1989; Webster, 1997). In recent years genetic reassortment has given rise to epidemiologically significant influenza A (H1N2) (Xu *et al*., 2002; Barr *et al*., 2003; Ellis *et al*., 2003) and influenza B viruses (Barr *et al*., 2003; Xu *et al*., 2004). Although these had a widespread distribution, neither had led to unusually large or severe influenza outbreaks. On the other hand, antigenic drift is a consequence of the low fidelity of viral RNA-dependent RNA polymerases (PA, PB1, PB2) combined with selection pressure exercised by neutralizing antibodies directed towards the antigenic sites exhibited on the HA and NA surface antigens (Laver *et al*., 1981). Recurrent epidemics of influenza are due to the frequent emergence of antigenic variants and genetic reassortments (Fitch *et al*., 1997; Lin *et al*., 2004).

The effectiveness of annually applied trivalent influenza vaccines depends on the selection of component strains that offer optimal immunity for the numerous variant in the global influenza virus circulation. Studies based on sequencing analyses of viruses can be utilized as surveillance tools and can contribute to the vaccine selection process when they are combined with classical serological antigenic analysis (Palmer *et al*., 1975). Continuous monitoring of viral genetic changes throughout the year is necessary to develop the ability to precisely define variations in influenza viruses.

Predicting variations of circulating influenza strains for subsequent annual vaccine development has become vital. Furthermore, comparisons between antigenic differences and phylogenetic analyses are necessary to understand further

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the multiple lineages of influenza virus variants. Therefore, to investigate the genetic characteristics of human influenza viruses circulating in Chungbuk province, we collected 510 clinical samples of nasopharyngeal suction from pediatric patients diagnosed with respiratory illness of various hospitals in Cheongju City for influenza A virus detection between June 2007 and June 2008. On all positive samples, we performed sequence analysis to study the antigenic differences and compared them with the vaccine strain. Hemagglutination inhibition (HI) and neuraminidase inhibition (NAI) assays were also done to provide antigenic analysis data and to emphasize on the antigenic difference of the Korean human H3N2 isolates with respect to the reference vaccine strain as what was observed in the genetic characterization.

Materials and Methods

Cells

The Madin-Darby canine kidney (MDCK) cell line was obtained from the American Type Culture Collection (USA). MDCK cells were maintained in minimal essential medium (MEM) with 5% fetal bovine serum and antibiotics antimycotics (antibiotics/antimycotics; 100 U of penicillin, 0.1 mg of streptomycin, and 0.25 μg of amphotericin B per ml) (Sigma, USA). MEM with 0.3% bovine serum albumin, antibiotics/antimycotics, and 1 μg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (USA) per ml was used as an infection medium for MDCK cells.

Sampling and virus isolation

The study protocol was approved by the Ethics Committee, Faculty of Medicine, Chungbuk National University, Cheongju. The parents of all participating children were informed about the study objective and their written consent was obtained. Nasopharyngeal (NP) suction specimens were collected from 510 infants or children (age range: 90 days to 14 years old) suffering from respiratory illness from 1 June, 2007 to 28 June, 2008. All clinical samples were provided by the Park Pediatrics and the Department of Pediatrics of Chungbuk National University Hospital, Cheongju. NP suction samples were collected in transport medium consisting of phosphate-buffered saline with antibiotics (250 U/ml of Penicillin G sodium and 250 μg/ml of Streptomycin sulfate) and stored at -70°C until tested. Suspensions from the NP suction were centrifuged at 3,000 rpm for 20 min, and the supernatants were inoculated in monolayer of MDCK cell lines and 11 day-old embryonated chicken eggs for virus isolation. Virus positive cell culture fluids were harvested and centrifuged for purification. Viruses were passaged in MDCK cells and monitored for virus growth by CPE and hemagglutination

Table 1. List of primers used for amplifying and sequencing of the eight viral gene segments

Primer	Sequences	Position	Polarity
HA 1F	TATTCGTCTCAGGGAGCAAAAGCAGGGG	1-28	Sense
H3 600F	TCACCACCCGGGTACGGACAA	600-620	Sense
H3 1442R	AACCATTGCCCATATCCTCA	1423-1442	Antisense
NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	1744-1778	Antisense
NA 1F	TATTGGTCTCAGGGAGCAAAAGCAGGAGT	1-29	Sense
N2 920F	ATAGGCCCGTTATAGATATA	920-939	Sense
N2 1050R	CCTCTCTCATTATTAGGATC	1031-1050	Antisense
NA 1413R	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	1378-1413	Antisense
M 1F	TATTCGTCTCAGGGAGCAAAAGCAGGTAG	1-29	Sense
M 1027R	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT	992-1027	Antisense
NS 1F	TATTCGTCTCAGGGAGCAAAAGCAGGGTG	1-29	Sense
NS 560R	ATTGCATTTTTGACATCCTC	541-560	Antisense
NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	856-890	Antisense
NP 1F	TATTCGTCTCAGGGAGCAAAAGCAGGGTA	1-29	Sense
NP 1000F	CCAAATGAGAATCCAGCACA	1000-1019	Sense
NP 1132R	GCTCGATCCCATATTATCCATG	1111-1132	Antisense
NP 1565R	ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT	1530-1565	Antisense
PA 1F	TATTCGTCTCAGGGAGCGAAAGCAGGTAC	1-29	Sense
PA 730F	ATTGAGGGCAAGCTTTCTCA	730-749	Sense
PA 1155 R	AGTCTACTTTCTCTGGTGCC	1136-1155	Antisense
PA 2233R	ATATCGTCTCGTATTAGTAGAAACAAGGTACTT	2201-2233	Antisense
PB1 1F	TATTCGTCTCAGGGAGCGAAAGCAGGCA	1-28	Sense
PB1 860F	AGAAGGCTAAATTGGCAAATG	860-880	Sense
PB1 1565R	ACTGTAACACCAATGCTCAT	1546-1565	Antisense
PB1 2341R	ATATCGTCTCGTATTAGTAGAAACAAGGCATTT	2309-2341	Antisense
PB2 1F	TATTGGTCTCAGGGAGCGAAAGCAGGTC	1-28	Sense
PB2 950F	CAAGCTGTGGATATATGCAA	950-969	Sense
PB2 1100 R	TTGTGAACTCCTCATACCCCT	1090-1100	Antisense
PB2 2341R	ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT	2308-2341	Antisense

of chicken erythrocytes. Virus isolates were centrifuged at 500×g to remove cell debris and the supernatants were frozen at -80°C until they were processed for RNA extraction, RT- PCR, and sequencing with specific primers (Table 1).

RNA extraction and diagnosis of influenza virus

Viral RNA was extracted by using the RNeasy mini kit (QIAGEN, Germany) according to the manufacturer's instructions. Briefly, 200 μl of virus was mixed with 550 μl of RLT buffer and incubated for at least 5 min at room temperature. After the addition of 550 μl 100% ethanol, the total mixture was vortexed and applied to a spin-column. After washing and drying steps, RNA was eluted in 40 μl RNase free water. The extracted RNA, primers (random hexamers), and dNTP were incubated at 70°C for 5 min and immediately chilled in ice for at least 3 min. Then RT buffer and Omniscript Reverse Transcriptase (QIAGEN) were added to the chilled mixture and reverse transcribed at 37°C for 60 min. The PCR reaction mixture in 50 μl contained 1 unit of TaKaRa EX *Taq* (TaKaRa BIO INC, Japan), 20 mM Mg^{2+} and 2.5 mM of each dNTP, appropriate concentrations of template DNA, and optimized concentrations of M gene specific primer pairs (Table 1). Each PCR product was amplified by the following conditions: initial denaturation step for 5 min at 94°C, 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 60 sec. The PCR was ended with a final extension step at 72°C for 10 min. The PCR amplified products were analyzed by 0.7% agarose gel electrophoresis, and sequencing of the PCR product was performed at Macrogen (Korea) with an ABI 373 XL DNA sequencer (Applied Biosystems, USA).

Genetic and phylogenetic analyses

Viral gene sequencing and analysis were carried out as described previously (Chang *et al*., 2008). The various primers used for amplification of the eight viral gene segments are listed in Table 1. Briefly, the PCR fragments were extracted and purified with QIAquick Gel Extraction kit (QIAGEN) and sequencing of the template PCR fragment was performed using an ABI 373 XL DNA sequencer (Applied Biosystems, USA). Confirmation of the correct PCR fragment sequences was done utilizing the Lasergene sequence analysis software package (DNASTAR, USA). For comparison, the phylogenetic analysis included sequences from human influenza viruses established in Asia and North America.

Gene sequences determined in this study have been deposited in GenBank under accession numbers FJ009453-FJ009510.

Plaque assay in MDCK cells

Confluent monolayer of MDCK cells were incubated for 1 h at 37°C with 10-fold serial dilutions of virus in 1 ml of infection medium. The cells were then washed and overlaid with freshly prepared MEM containing 0.3% bovine serum albumin, 0.9% Bacto agar, and 1 μg of TPCK trypsin/ml. The plaques were visualized after incubation at 37°C for 3 days by staining with a 0.1% crystal violet solution containing 10% formaldehyde.

Antigenic analysis

We investigated the cross-reactivity of the isolated viruses by hemagglutinin inhibition (HI) assay, as previously described (Palmer *et al*., 1975), using polyclonal antibodies to H3N2 viruses; A/Wisconsin/67/05 (the vaccine strain in the Northern Hemisphere 2007~2008), A/Cheongju/H383/07, A/Cheongju/H471/08, and A/Cheongju/H412/08 viruses. Because no antiserum to human H3N2 was available, we obtained antisera by vaccinating mice with purified viruses mixed with 5% alum adjuvant.

NA inhibition assays

NA inhibition was assayed with viruses standardized to equivalent NA enzyme activity. Viruses were first incubated with oseltamivir at concentrations of 0.00005 to 10 μM at 37°C for 30 min, and the mixtures were then incubated with MUNANA substrate at a final concentration of 167 μM at 37°C for 30 min. The reaction was stopped by adding 150 μl stop solution (0.014 M NaCl and 0.1 M glycine in 25% ethanol, pH 10.7). The drug concentration that inhibited 50% of the NA enzymatic activity (IC_{50}) was determined from the dose-response curve.

Biological assay for susceptibility testing

A previously reported phenotypic assay for amantadine susceptibility was employed for all influenza A (H1N1) and A (H3N2) isolates (Masuda *et al*., 2000). Two series of 10-fold dilutions of viruses from cytopathic effect (CPE)-positive cultures were plated in triplicate in 96-well microplates with confluent MDCK cells. One dilution series contained a final concentration of 1.0, 2.0, and 20 μg/ml of amantadine in the influenza virus maintenance media, and the other series was free of the drug. After incubation for 48 h at 37°C, virus titers for amantadine-added and -free rows in triplicate were calculated by Reed-Muench method using the wells from the final dilution, for which all cells are infected (Reed and Muench, 1938). The susceptibility test was assessed as interpretable if the virus titer in the amantadine-free rows exceeded 2.5 $\log_{10}$ $TCID_{50}$/0.2 ml. Amantadine-resistant strains were identified when less than a two-fold difference in $\log_{10}$ $TCID_{50}$/0.2 ml titer was observed between series of rows with and without the drug.

Results

Virus isolation

Five hundred and ten nasopharyngeal (NP) suction specimens from infants or children aged between 90 days and 14 years old diagnosed with respiratory illness were tested by RT-PCR for the matrix gene of influenza A virus. Of the 510 NP specimens, 24 samples proved positive for the matrix gene of influenza A virus. Since September 2007, the number of samples collected from patients with respiratory illness had been slightly increasing. It peaked in February 2008 (29.2%) but was on the decrease by June 2008 (Table 2). The average age of patients which tested positive was 2 years 6 months, with a minimum age of 4 months and a maximum age of 14 years. All 24 matrix gene positive samples were inoculated into MDCK cells and 11-day-old embryonated chicken eggs for virus isolation. Twelve of the 24

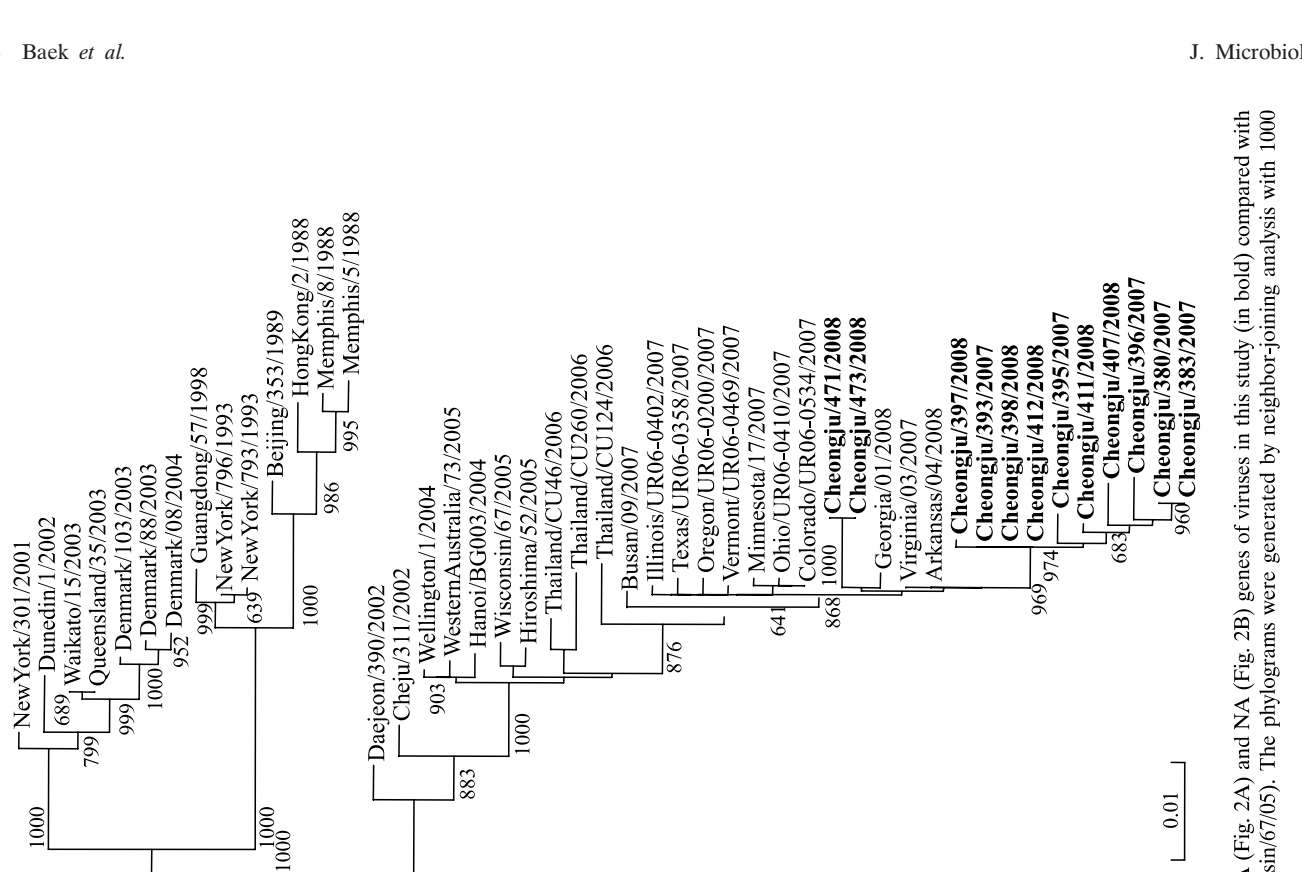

Fig. 1. Phylogenetic analysis of the surface genes. Phylogenetic trees of the nucleotide sequences for the HA (Fig. 2A) and NA (Fig. 2B) genes of viruses in this study (in bold) compared with those from selected human influenza viruses available in GenBank including the vaccine strain (A/Wisconsin/67/05). The phylograms were generated by neighbor-joining analysis with 1000 bootstrapped replicates. The percent bootstrap values for each node are shown in each tree.

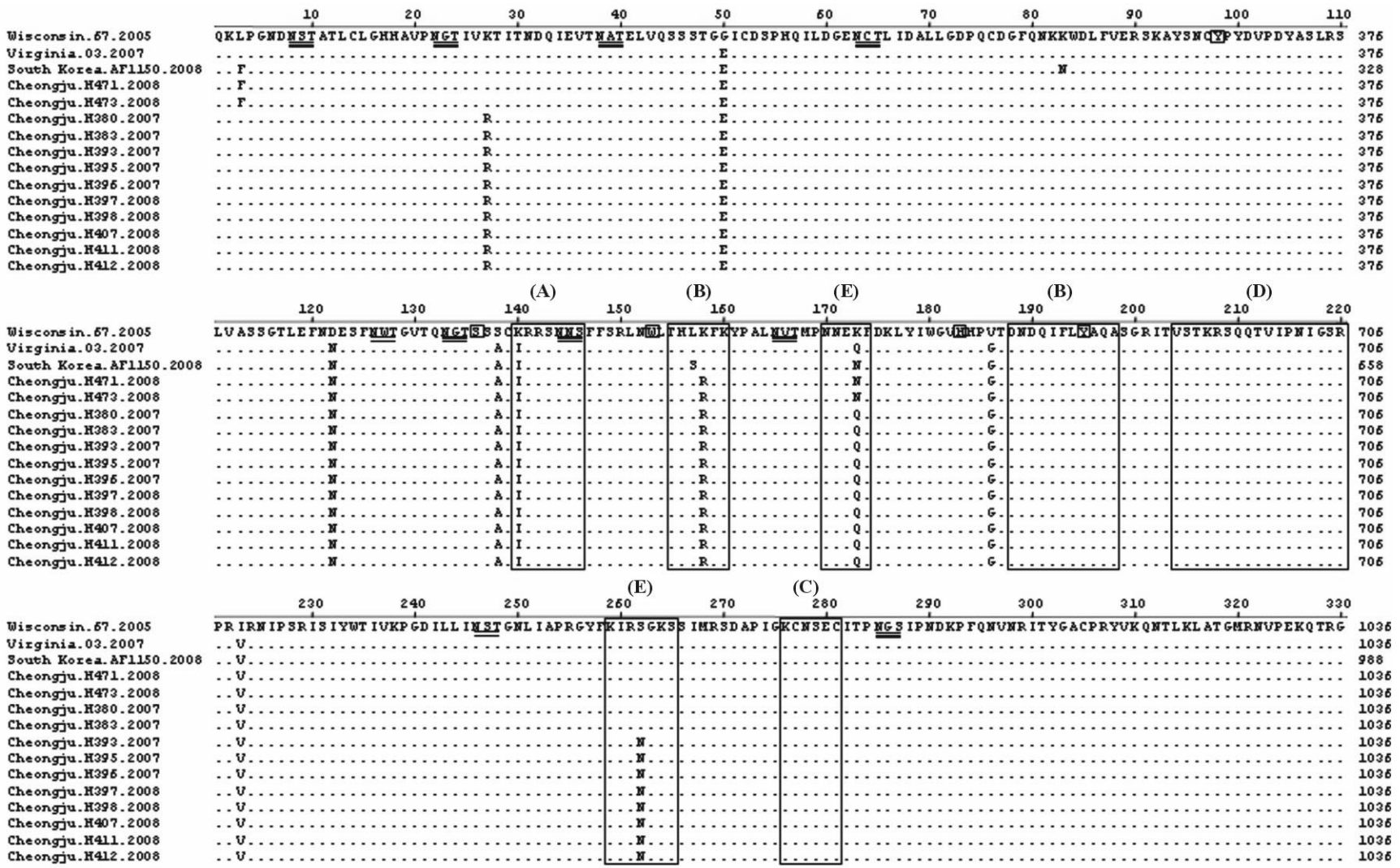

Fig. 2. Amino acid comparison between the HA1 domains of H3N2 isolates and the vaccine strain A/Wisconsin/67/05. Dots represent amino acids similar to the consensus. The conserved amino acid residues at the receptor-binding sites are shown as small rectangles. Alternative amino acids for sialic acid linkages of HA are underlined. The amino acids for sialic acid residues mapped at previously defined antigenic sites A-E are shown as large rectangles. All potential N-linked glycosylation sequons (NXS/T) are double-underlined.

positive samples were isolated from MDCK cell culture. All twelve isolates were subtyped as type A H3N2 by multiplex RT-PCR and nucleotide sequencing. No type A or B viruses were isolated from embryonated chicken eggs.

Genetic characterization of H3N2 viruses

Genetic relationships of the HA1 region and NA genes of the H3N2 isolates together with vaccine strains and other influenza viruses were constructed by Neighbor-Joining analysis with 1000 bootstrapped replicates. Comparison of respective surface genes both showed continuous evolution and the isolates were relevant to the recent vaccine strain (Fig. 1).

To investigate the detailed genetic characteristics of our isolates, the nucleotide and deduced amino acid sequences of the hemagglutinin 1 (HA1) from the 12 isolated samples were compared against virus isolates available in GenBank and a current human vaccine strain (Fig. 1 and 2). For the H3N2 subtype, A/Wisconsin/67/05 was used as the vaccine strain in the Northern Hemisphere 2007∼2008, including Korea. The twelve H3N2 isolates indicated a relatively low percent similarity to the reference vaccine strain with 97.5∼97.6% based on nucleotide and 98.1∼98.4% based on amino acid sequences. However, the average percent nucleotide and amino acid similarities of the twelve H3N2 isolates in this study to A/Virginia/04/07 were 99.1∼99.59% and 98.6∼99.6%, to A/South Korea/AF1150/08 were 98.0% and 98.8∼99.0% and to A/Thailand/CU124/06 were 98.4∼98.6% and 98.2∼98.5%, respectively. HA constitutes the receptor-binding and membrane fusion glycoprotein of influenza virus. Alignment of the terminal sialic acid (SA) residues of glycoproteins and glycolipids representing the cellular receptors for influenza virus, which are the targets for neutralizing antibodies, and the N-linked glycosylation sites are shown in Fig. 2. Five conserved amino acid residues, Tyr(Y)-98, Ser(S)-136, Trp(W)-153, His(H)-183, and Tyr(Y)-195 (numbering according to H3 structure) at the HA receptor-binding site (RBS) were observed in all our human H3N2 isolates as was previously been described by Daniels *et al*. (1984) in H3 influenza A virus. All other proposed RBS are also conserved except for one apparent substitution at residue S138A. The alanine at 138 is highly conserved in avian viruses (Matrosovich *et al*., 2000). The residues mainly responsible for NeuAc α-2,6Gal linkage of H3 are Leu(L)-226 and Ser(S)-228 (Vines *et al*., 1998); however, the amino acids at the terminal SA of all our H3 isolates were Ile(I)-226 and Ser(S)-228 which is the evolutionary characteristic of recent human H3N2 influenza A isolates from Japan and China (Lindstrom *et al*., 1996).

The HA1 domain of HA, the major antigenic protein of influenza A viruses, contains all the antigenic sites of HA and is under continual immune-driven selection. For H3N2 viruses, the antigenic sites A∼E have been described by Wiley *et al.* (1981). We found several altered amino acids in three of the five sites at the HA1 region of our H3N2 isolates; K140I at site A, K158R at site B, K173N (H471) or K173Q and S262N at site E (Fig. 2). N-linked glycosylation is commonly found in the HA of influenza A viruses with (Asn-*X*-Ser/Thr) as the specific polypeptide for glycosylation where *X* can be any amino acid except for aspartic acid or proline. These sites may contribute to antigenic conservation or variation since carbohydrate side-chains can mask antigenic sites sterically (Air and Laver, 1986). There were 11 potential glycosylation sites on H3 HA1 (one site greater than the vaccine strain) and one more at the HA2 region. There were also several differences which occurred in regions other than the proposed antigenic sites.

Table 2. Number of influenza virus isolates, 2007.6∼2008.6

Date	Subtype of Influenza virus		
	No. of samples (%)[a]	No. of H3 isolates (%)[b]	No. of isolates/ No. of samples
07. Jun	23(4.5)	0(0.0)	0/23
Jul	25(4.9)	0(0.0)	0/25
Aug	21(4.1)	1(4.2)	1/21
Sep	24(4.7)	2(8.3)	2/24
Oct	65(12.7)	3(12.5)	3/65
Nov	56(11.0)	5(20.83)	5/56
Dec	52(10.2)	2(8.3)	2/52
08. Jan	86(16.9)	3(12.5)	3/86
Feb	49(9.6)	7(29.2)	7/49
Mar	25(4.9)	0(0.0)	0/25
Apr	39(7.6)	1(4.2)	1/39
May	32(6.3)	0(0.0)	0/32
Jun	13(2.5)	0(0.0)	0/13
Total	510	24	24/510

[a] Percentage distribution of monthly sample over the total number of samples collected in the period covered.
[b] Percentage distribution of monthly H3 positive isolates over the total number of H3 viruses detected by RT-PCR and sequence analysis.

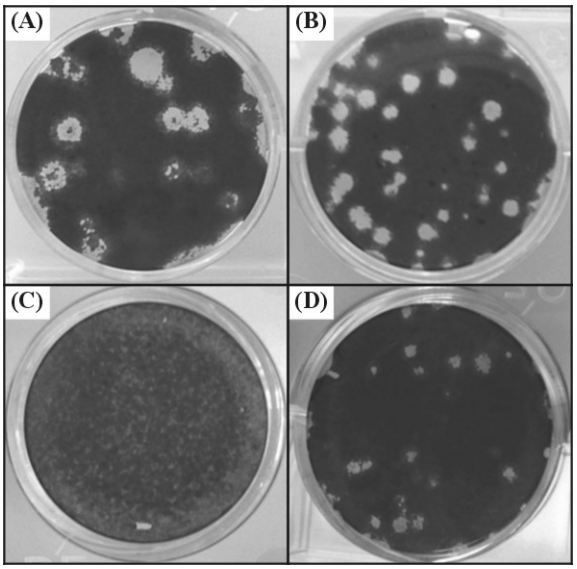

Fig. 3. Plaque phenotypes of various human influenza viruses containing the mutation on neuramindase activity site or amino acid deletion on NA gene in MDCK cells. After titration of viruses, cells were stained and fixed with 0.1% crystal violet solution containing 10% formaldehyde. (A) A/PR/8/34 (H1N1), (B) A/Cheongju/H471/08 (H3N2), (C) A/Cheongju/H383/08 (H3N2) D151G mutation, (D) A/Cheongju/H412/08 (H3N2) N146 Deletion.

Table 3. Amino acid substitutions in the neuraminidase genes of influenza A viruses subtype H3N2 isolated in Cheongju compared with vaccine strains

Virus strain H3N2	Amino acid position of NA gene																										
	2	3	4	15	16	19	45	50	83	93	146	147	149	150	151	194	204	208	214	215	223	249	285	310	370	372	387
A/Wisconsin/67/05[a]	N	P	N	L	T	T	P	V	E	A	N	D	V	R	D	V	S	N	S	I	L	K	L	Y	L	S	N
A/Virginia/03/07	·	·	·	·	·	·	·	·	·	D	·	·	·	·	N	I	·	·	·	V	·		P	H	S	L	K
A/Cheongju/H380/07	D	·	I	·	·	·	·	M	·	D	·	·	A	·	G	I	·	D	·	V	·	E	P	H	S	L	K
A/Cheongju/H383/07	D	·	I	·	·	·	·	M	·	D	·	·	A	·	G	I	·	D	·	V	·	E	P	H	S	L	K
A/Cheongju/H396/07	D	S	·	·	·	·	·	M	·	D	·	·	A	G	G	I	·	D	G	V	·	E	P	H	S	L	K
A/Cheongju/H407/08	·	·	·	F	I	S	·	M	·	D	·	·	A	·	G	I	·	D	·	V	·	E	P	H	S	L	K
A/Cheongju/H411/08	·	·	·	·	·	·	·	M	K	D	·	·	A	·	G	I	·	·	·	V	·	E	P	H	S	L	K
A/Cheongju/H471/08	·	·	·	·	·	·	S	·		D	·	N	·	·	·	I	N	·	·	·	F	·	P	H	S	·	K
A/Cheongju/H473/08	·	·	·	·	·	·	S	·		D	·	N	·	·	·	I	N	·	·	·	F	·	P	H	S	·	K
A/Cheongju/H393/07	·	·	·	·	·	·	·	M	·	D	-	·	A	·	·	I	·	·	·	V	·	E	P	H	S	L	K
A/Cheongju/H395/07	D	·	·	·	·	·	·	M	·	D	-	·	A	·	·	I	·	·	·	V	·	E	P	H	S	L	K
A/Cheongju/H397/08	·	·	I	·	·	·	·	M	·	D	-	·	A	·	·	I	·	·	·	V	·	E	P	H	S	L	K
A/Cheongju/H398/08	·	·	·	·	·	·	·	M	·	D	-	·	A	·	·	I	·	·	·	V	·	E	P	H	S	L	K
A/Cheongju/H412/08	·	·	·	·	·	·	·	M	·	D	-	·	A	·	·	I	·	·	·	V	·	E	P	H	S	L	K

[a] The vaccine strain (A/Wisconsin/67/05) was used as a reference for comparison. The amino acid substitution in different sites is shown. Dots represent amino acid similar to the reference strain while the dash denotes deletion.

We also compared the nucleotide and deduced amino acid sequences of the NA from 12 isolated samples with the vaccine strain. NA genes of the 12 isolates showed a higher average of percent nucleotide and amino acid similarity to A/Virginia/04/07 (98.3% to 98.7%), A/Illinois/UR06-0402/07 (98.3% to 98.99%), than to the vaccine strain A/Wisconsin/67/2005 (96.4 to 97.6%). Furthermore, analysis of the N2 protein demonstrated conserved residues including the catalytic sites (R118, D151, R152, R224, E276, R292, R371, and Y406) (N2 numbering) that directly interact with the substrate and the framework sites supporting the catalytic residues (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, and E425) in most of the H3N2 isolates. However, five of twelve isolates (A/Cheongju/H383/08, A/Cheongju/H407/08, A/Cheongju/H380/08, A/Cheongju/H396/08, and A/Cheongju/H411/08) had a single amino acid substitution at D151G on the catalytic site (Table 3). This residue was thought to be highly conserved, acting as a proton donor in the catalytic reaction (Varghese and Colman, 1991). It is also noteworthy that A/Cheongju/H412/08, A/Cheongju/H398/07, A/Cheongju/H393/08, A/Cheongju/H395/08, and A/Cheongju/ H397/08 isolates have one amino acid deletion at site 146, a positional residue far from the NA stalk region. We also observed amino acid substitutions in the neuraminidase gene of the H3N2 isolates not related to catalytic or framework sites as shown in Table 3. Only A/Cheongju/H471/07 and A/Cheongju/H473/08 did not contain the amino acid deletion (residue 146) or mutation (residue 151) in N2 NA.

All internal genes clustered together with A/Virginia/UR06-0580/07 virus or the vaccine strain A/Wisconsin/67/05 viruses (data not shown). These results suggested that all H3N2 viruses originated from the same lineage (strictly humans) without any reassortment events with other subtypes of influenza viruses but rather evolved by antigenic drift in the antigenic sites of their respective HA and NA glycoproteins.

Growth in MDCK cells

To assay differences in growth properties of the isolates with variable NA genes (deletion and substitution) *in vitro*, we conducted five times serial passage of representative virus and parallel plaque assays with MDCK cells including the reference human virus A/Puerto Rico/8/34 (H1N1). A/PR/8/

Table 4. Antigenic analysis and NAI index of H3N2 influenza viruses isolated in Cheongju City during 2007~2008

Virus	Antiserum against selected H3N2 strains					
	Wiscosin/67/05	Cheongju/H471/07	Cheongju/H383/08	Cheongju/H412/08	PR/8/34	IC_{50} (nM)[b]
H3N2						
Wiscosin/67/05	1280[a]	160	80	80	<20	NT
Cheongju/H471/07	160	1280	320	640	<20	1.382
Cheongju/H383/08	80	160	1,280	640	<20	7.10
Cheongju/H412/08	80	320	1,280	1,280	<20	3.13
H1N1						
PR/8/34	<20	<20	<20	<20	2560	4.2

[a] The HI titer of each serum that reacted with the virus listed at the top of the column. The highest HI titers are in bold face.
[b] The IC_{50} values for sensitive and resistant strain to Oseltamvir in the NI assay
NT, not tested

34 and A/Cheongju/H471/08 showed large and stable plaques and were relatively grown well with $2\sim6\times10^6$ plaque forming units per unit volume of media (PFU/ml) (Fig. 3A and B). However, A/Cheongju/H383/08 isolate containing the single amino acid substitution at D151G on the catalytic site maintained the small-plaque phenotype in the fourth passage and was not able to grow to a titer of $>10^5$ PFU/ml. A mixture of small and large plaques was observed at the fifth passage (Fig. 3C). The A/Cheongju/H412/08 (H3N2) containing the N146 deletion on NA gene showed relatively large and stable plaques during the five passages but viral titer was relatively low at 2.5×10^5 PFU/ml compared with A/Cheongju/H412/08 isolate (Fig. 3D).

Antigenic analysis

Genetic characterization of the 12 human H3N2 isolates indicated antigenic differences in their respective surface glycoproteins. To investigate the antigenic properties of the H3N2 viruses isolated in this study and to emphasize that such differences reflect antigenic variability, we tested them by HI assay for cross-reactivity with polyclonal antisera against A/Wisconsin/67/05. All of the H3N2 viruses isolated during this study reacted weakly (HI160 to 80) with antisera to A/Wisconsin/67/05 but reacted relatively strongly with antisera to their homologues (Table 4). Therefore, most of the H3N2 viruses circulating in Cheongju, South Korea during 2007∼2008 were indeed antigenically distinct from the A/Wisconsin/67/05 vaccine strain.

Biological assays for amantadine-resistance and NA inhibition

For influenza viruses, the amino acid residue at position D151 of the NA segment is proposed to act as the acid catalyst in the initial step of the sialyl-enzyme formation due to its hydrogen linkage with the glycosidic oxygen (Ghate and Air, 1998). However, natural variations (G/V/N/E) for the Asp (D) at residue 151 have been identified in N1 and N2 influenza A NA in a large-scale influenza virus NAI susceptibility screening. Therefore we evaluated the role of residue D151G mutation in NAI resistance. D151G was only slightly resistant to Oseltamivir (IC_{50}7.10) (Table 4), but this value is within the normal sensitive NAI ranges (0.001 nM∼15 nM). These data suggested that all isolates tested in this study are sensitive to the neuraminidase inhibitor Oseltamivir (Table 4).

We also analyzed the deduced amino acid sequences of M2 proteins to determine any point mutation in regions implicated as hot spots for amantadine-resistance. Three out of twelve H3N2 human virus isolates (A/Cheongju/H395/08, A/Cheongju/H471/08, and A/Cheongju/H473/08) tested in this study have an amino acid substitution (Asp31Asn) in the M2 protein (Table 5). To evaluate whether the observed aspartic acid to asparagine substitution would consequently result in an amantadine-resistant phenotype, a drug-resistance assay was performed on a blind subset of 12 H3N2 isolates (9 sensitive and 3 resistant isolates). The results showed that there were no growth differences between the viruses in the media without the antiviral drug; however, three H3N2 isolates containing the substitution (Asp31Asn) had a 2.5-fold higher $\log_{10}$ $TCID_{50}$/0.2 ml titer compared with the wild type viruses having in the media containing 20 μg/ml of amantadine (Table 5). Therefore, amantadine resistance correlated 100% with the presence of the mutation that was obtained by sequencing.

Discussion

From June 2007 to June 2008, we collected nasopharyngeal suction samples by inserting a nasal catheter into the posterior nasopharynx of pediatric patients diagnosed with respiratory tract illness, and placed them in virus transport medium. Based on our results, the H3N2 subtype was the only influenza A virus in Cheongju during this study. Each year, WHO recommends the most suitable composition of influenza vaccine strains for both the Northern and Southern Hemispheres, respectively. Comparison of the nucleotide

Table 5. Comparative alignment of the deduced amino acids of the M2 protein

Virus strain	No. of strains with the amino acid substitutions in the M2 gene at position				
H3N2	26	27	30	31	
	CTT : Leucine	GTT : Valine	GCG : Alanine	AAT : Asparagine GAT : Aspartic acid	$\log_{10}$ $TCID_{50}$/0.2 ml in 20 μg/ml of amantadine media
A/Wisconsin/67/05	L	V	A	N	NT[a]
A/Cheongju/H395/07	L	V	A	N	4.7
A/Cheongju/H471/08	L	V	A	N	4.5
A/Cheongju/H473/08	L	V	A	N	4.7
A/Cheongju/H380/07	L	V	A	D	1.3
A/Cheongju/H383/07	L	V	A	D	0.7
A/Cheongju/H393/07	L	V	A	D	1.3
A/Cheongju/H396/07	L	V	A	D	0.7
A/Cheongju/H397/08	L	V	A	D	1.3
A/Cheongju/H398/08	L	V	A	D	0.7
A/Cheongju/H407/08	L	V	A	D	1.3
A/Cheongju/H411/08	L	V	A	D	1.3
A/Cheongju/H412/08	L	V	A	D	1.7

[a] NT, not tested

and amino acid sequences of the HA1 region and NA genes of our isolates and recent human isolates including a vaccine strain showed that most of Cheongju isolates (H3N2 subtype) were more closely related to A/Virginia/04/07 (99.1∼99.5% nucleotide similarity) than to the vaccine strain (A/Wisconsin/67/05) recommended for the Northern Hemisphere 2007∼2008 (97.5∼97.6% nucleotide similarity). Furthermore, the comparison of antigenic sites of HA1 regions revealed that the H3N2 viruses isolated in Cheongju significantly evolved by antigenic drift of their HA genes (site A: K140I; site B: K158R, and site E: K173N (H471) or K173Q, and S262N). However, the residues within the receptor-binding sites are relatively conserved except for the residue Ile226, mainly responsible for NeuAca2,6Gal linkage specific for the H3 subtype, as previously reported (Skehel and Wiley, 2000; Parrish and Kawaoka, 2005). Because of the similar neutral non-polar amino acids of Leucine, Isoleucine, and Valine, the intermolecular interactions of the binding pocket remains hydrophobic and proper conformation is maintained regardless of any substitution among these three amino acid bases at this site.

The N2 genes of the 12 isolates were also more closely related to A/Virginia/03/07 (98.3 to 98.7%) or A/Illinois/UR06-0402/07 (98.3% to 98.99%) than to the vaccine strain A/Wisconsin/67/05 (96.4 to 97.6%), as observed in HA genes. Although most of N2 genes had conserved bases at the catalytic or framework sites of the neuraminidase enzyme, five isolates (A/Cheongju/H383/08, A/Cheongju/H407/08, A/Cheongju/H380/08, A/Cheongju/H396/08, and A/Cheongju/H411/08) had single amino acid substitution D151G on the catalytic site which was originally reported as conserved. However, natural variations (G/V/N/E) at residue D151 have been identified in N1, N2, and influenza B NA in a large-scale influenza virus NAI susceptibility screening suggesting that D151 may not be as conserved as previously thought (Varghese and Colman, 1991). However, the Asp (D)151 to Gly (G) did not alter the overall sensitivity of the viruses to Oseltamivir where treatment of patients with such antiviral drug may still potentially inhibit virus propagation and spread. Of worth noting is the single amino acid deletion at residue 146 of A/Cheongju/H412/08, A/Cheongju/H398/07, A/Cheongju/H393/08, A/Cheongju/H395/08, and A/Cheongju/H397/08 isolates. There are many reports regarding the deletion of stalk regions on various NA subtypes (Castrucci *et al*., 1993; Mitnaul *et al*., 2000). However, this is the first report of amino acid deletion on this site of the N2 subtype. Our initial investigation of these human N2 variants with respect to virus kinetics demonstrated that the viruses which contained the deletion produced large plaques but relatively low viral titer whereas viruses which contained the substitution produced small plaques and low viral titer potentially indicating differences in neuraminidase activity. To further elucidate the roles of these characteristic sites in NA protein, more studies are needed. Overall, these changes in the surface (HA and NA) genes are indicating that the viruses isolated herewith had accumulated mutations which could present potential problems in vaccine administration due to antigenic mismatch.

From 1991 to 1995, surveillance for amantadine resistance among A (H3N2) viruses revealed the global frequency of resistance to be as low as 0.8%. However, recent studies show that the incidence of resistance had reached 96%, 72%, 65.3%, and 14.5% in China, South Korea, Japan, and United States, respectively. A substantially rising percentage of amantadine-resistant H3N2 viruses from specific countries in Asia, including China and Hong Kong, Taiwan, and South Korea during 1995∼2004 were one of the concerns for treatment of patients. In this study, we found that 25% (3 out of 12 isolates) of the H3N2 subtype viruses have the amino acid substitution on residue 31 of the M2 protein from Aspartic acid to Asparagine and confirmed their drug-resistance by a biological assay.

In conclusion, we isolated twelve H3N2 isolates in Cheongju, South Korea, and genetic characterization revealed that they are closely related to each other. Phylogenetic analysis also showed close similarities to current Northern Hemisphere isolates including the vaccine strain of this region 2007∼2008 (A/Wisconsin/67/2005). The present study has confirmed conserved sequences as well as discovered variations due to amino acid substitutions at the receptor-binding site and the antigenic site including the potential N-linked glycosylation sites of HA, at the catalytic and framework sites of NA, and in the M2 genes of H3N2 isolates from Cheongju, South Korea. The results of this study highlight the need to closely monitor antigenic drift and drug resistance in influenza A viruses to aid in the early detection of potentially pandemic strains, as well as underscore the need for new therapeutics.

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