The Journal of Microbiology (2012) Vol. 50, No. 6, pp. 1067–1070
DOI 10.1007/s12275-012-2541-3

NOTE

Susceptibility of Human H3N2 Influenza Virus to Oseltamivir in South Korea, 2009–2011

Sehee Park[1,2†], Jin Il Kim[1,2†], Ilseob Lee[1,2†], Sangmoo Lee[1,2], Min-Woong Hwang[1,2], Joon-Yong Bae[1,2], Jun Heo[1,2], Eun-Joo Lim[3], Won-Seok Seok[3], Hee Jin Cheong[4], Joon Young Song[4], Woo Joo Kim[4], and Man-Seong Park[1,2*]

[1]Department of Microbiology, [2]Center for Medical Science Research, College of Medicine, Hallym University, Chuncheon 200-702, Republic of Korea
[3]Infectious Disease Investigation Division, Gangwon Institute of Health and Environment, Chuncheon 200-820, Republic of Korea
[4]Division of Infectious Diseases, Korea University Guro Hospital, College of Medicine, Korea University, Seoul 152-703, Republic of Korea

(Received October 5, 2012 / Accepted October 31, 2012)

During the 2009–2011 influenza seasons, 10.26% of the specimens isolated from patients in South Korea were subtyped as H3N2 viruses. Some oseltamivir-sensitive H3N2 samples exhibited different plaque morphologies, and were found to have novel mutations in the neuraminidase gene. In a subsequent analysis using NA mutant viruses, viral compensation against oseltamivir treatment was observed only in the N2 mutant virus. All things considered, these novel mutations may account for the exclusive characteristics of selected H3N2 viruses observed in plaque reduction assays.

Keywords: influenza, neuraminidase, oseltamivir

During the 2007–2009 influenza seasons, a markedly increased prevalence of oseltamivir-resistant influenza A H1N1 (ORA/H1N1) viruses, bearing the neuraminidase (NA) H274Y mutation, was reported in European countries and in the United States (CDC, 2008, 2009; Hauge *et al*., 2009; Meijer *et al*., 2009). Since the 2009 outbreak of pandemic H1N1, however, the ORA/H1N1 viruses have been replaced by the oseltamivir-sensitive influenza A H1N1 (OSA/H1N1) pandemic strains (CDC, 2010, 2011).

In South Korea, nasopharyngeal specimens were collected from patients at Korea University Medical Center (KUMC) Guro Hospital and at Gangwon Institute of Health and Environment (GIHE) during the last two consecutive influenza seasons (December 2009–March 2011). After one passage in Madin-Darby canine kidney (MDCK) cells, a total of 380 MDCK-grown influenza viruses were identified by immunofluorescence assay (IFA, 354 isolates) and by sequence analysis (26 isolates). Of the total 380 isolates, 340 (89.47%) were subtyped as 2009 pandemic H1N1 strains, and only one (0.26%) was categorized as an influenza B virus. The remaining 39 (10.26%) isolates were found to be A/ H3N2 strains.

Most influenza A H3N2 viruses are known to be susceptible to oseltamivir (McKimm-Breschkin *et al*., 2003; CDC, 2008, 2009, 2010, 2011; Baek *et al*., 2009). Some exceptional cases of oseltamivir resistance have been reported in H3N2 clinical isolates that bore mutations at residues E119, R292, or N294 of NA (Gubareva, 2004; Kiso *et al*., 2004; Aoki *et al*., 2007; Sheu *et al*., 2008). Given the increasing number of H3N2 cases reported in South Korea (WHO, 2012), we investigated the oseltamivir susceptibility of H3N2 clinical isolates during the 2009–2011 influenza seasons using the 50% inhibitory concentration (IC_{50}) index and plaque reduction assays.

According to the Neuraminidase Inhibitor Susceptibility Network (NISN), the IC_{50} value is a key index commonly used to determine whether an influenza virus is susceptible to NA inhibitors (NIs) (NISN, 2012). Compared to that of sensitive strains, the IC_{50} of an oseltamivir-resistant virus is more than 10-fold higher (Okomo-Adhiambo *et al*., 2010). As shown in Table 1, three selected H3N2 viruses (H3N2/16, 17, and 50) were susceptible to oseltamivir (Toronto Research Chemicals, Canada), resulting in a range of IC_{50} values of 2.25–9.26 μM, which was similar to that for two H3N2 oseltamivir-sensitive control viruses, A/Brisbane/10/2007 (BR10) and A/Perth/16/2009 (PE16). In a plaque reduction assay, which was reported as a useful tool for investigating the antiviral susceptibility in influenza viruses (Tisdale, 2000), MDCK cells were infected with approximately 200 plaque-forming units (PFU) of H3N2/16, 17, and 50 viruses, respectively, overlaid with 2% agar media containing 0.0625–1 mM of oseltamivir, and the plaque morphology was examined after 72 h of incubation. The plaque sizes of the oseltamivir-treated H3N2/16, 17, and 50 viruses were almost the same as those of untreated viruses, whereas the plaques of oseltamivir-treated BR10 and PE16 viruses were significantly decreased in size (Fig. 1).

In MDCK cells, H3N2/16, 17, and 50 viruses produced plaques of varying sizes. These viruses were then plaque-purified and subjected to reverse-transcriptase PCR (RT-PCR)

†These authors contributed equally to this work.
*For correspondence. E-mail: ms0392@hallym.ac.kr; manseong.park@gmail.com; Tel.: +82-33-248-2632; Fax: +82-33-252-2843

Table 1. Susceptibility of human H3N2 clinical samples to oseltamivir

	Virus		Symptom	Treatment	IC_{50} value to oseltamivir (μM)
Control viruses	H1N1	rK09[a]	-	-	10.64 (9.069–12.49)
		rK09/NA:Y275[b]	-	-	416.1 (197.8–875.3)
	H3N2	BR10	-	-	0.69 (0.31–1.06)
		PE16	-	-	3.382 (0.6419–17.82)
Clinical isolates	H3N2	16	cough, fever	Tamiflu, 5 days	2.25 (1.01–3.49)
		17	cough, fever, sputum, rhinorrhea, myalgia	Tamiflu, 5 days	9.26 (1.81–16.70)
		50	fever, myalgia	Tamiflu, 5 days	3.74 (1.29–6.19)

[a] 2009 pandemic H1N1 K09 virus that was rescued by reverse genetics
[b] 2009 pandemic H1N1 K09 virus having an H274Y mutation in the NA gene that was rescued by reverse genetics.

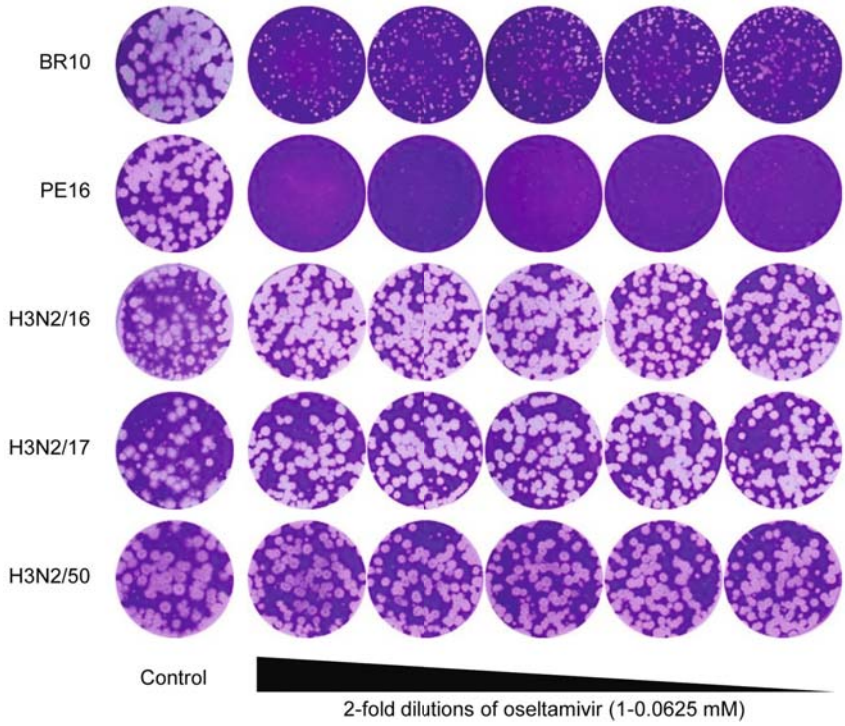

Fig. 1. Plaque reduction assay of H3N2 viruses treated with oseltamivir. For the plaque reduction assay, MDCK cells were inoculated with 200 pfu of H3N2/16, 17, or 50 viruses. After one hour of inoculation, MDCK cells were overlaid with 2% agar medium supplemented with 1–0.0625 mM of oseltamivir and incubated for 72 h. The control well was treated with phosphate buffered saline (PBS). A/Brisbane/10/2007 (BR10) and A/Perth/16/2009 (PE16) viruses were used as oseltamivir-sensitive controls.

Table 2. Sequence alignment of HA and NA genes of human H3N2 clinical samples

		Alignment of sequences															
		HA[a]										NA[b]					
		97	160	174	178	187	189	205	210	230	237	127	151	177	307	338	342
	Virus	N[c]	K	N	S	N	Q	K	L	I	P	D[c]	D	**A**	I	L	N
	BR10		N	K	P		K	N	P								
	PE16				P					S							
H3N2	16-2	D										N			M	F	D
	16-3	D										N			M	F	D
	17-3	D										N	E		M	F	D
	17-5	D										N	E	**T**	M	F	D
	17-6	D										N	E		M	F	D
	50-3					K					L	N			M	F	D
	50-4					K						N			M	F	D

[a] HA sequences in the H3 numbering system
[b] NA sequences in the N2 numbering system
[c] Consensus sequences of selected H3N2 viruses from 2000-2009, available from National Center for Biotechnology Information (NCBI)

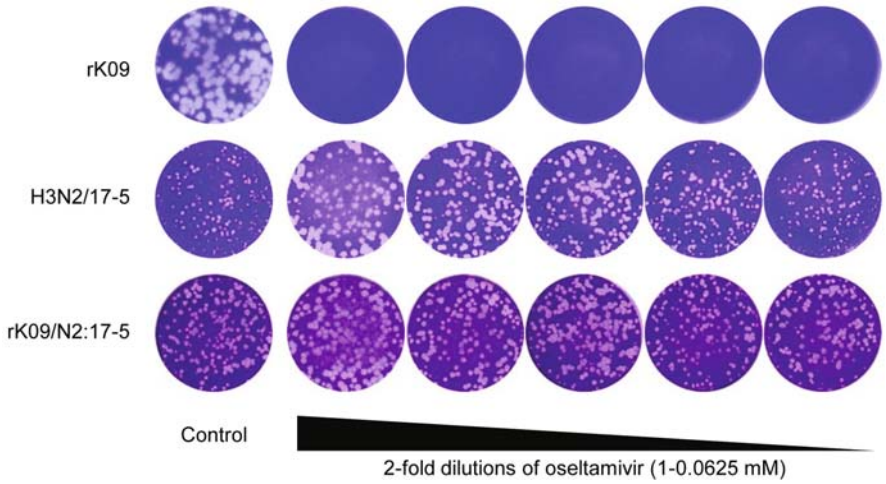

Fig. 2. Plaque reduction assay of the H1N2 recombinant virus treated with oseltamivir. After having been rescued by reverse genetics, the 7:1 recombinant rK09/N2:17-5 (NA gene from the H3N2/17-5 virus and the remaining seven genes from the 2009 pandemic H1N1 A/Korea/01/2009 virus, K09) virus was used in a plaque reduction assay to investigate the effects of NA on the oseltamivir susceptibility. rK09 (K09 virus rescued by reverse genetics) and H3N2/17-5 viruses were used as the controls.

for sequence analysis. The hemagglutinin (HA) and NA sequences of each purified virus were compared with those of BR10 and PE16 viruses, and no consistent mutations were detected around the receptor binding domain of HA (Table 2). However, the NA genes of all the purified H3N2/16, 17, and 50 viruses bore four mutations (D127N, I307M, L338F, and N342D; N2 numbering) (Table 2). In addition to these four mutations, the H3N2/17 virus also had a D151E mutation in the NA gene. Interestingly, the H3N2/17-5 virus, which was one of the purified clones from the parental H3N2/17 virus, also had an additional A177T mutation in the NA gene (Table 2), and its plaques were the smallest when compared with those of other purified viruses (data not shown). In the subsequent plaque reduction assay, unusual oseltamivir susceptibility was observed only with the H3N2/17-5 virus. Rather than being decreased or unchanged, the plaque size of the H3N2/17-5 virus increased proportionally with increasing concentrations of oseltamivir (Fig. 2).

Cell-based assays may occasionally be considered as inadequate procedures for assessing the susceptibility of influenza viruses to NIs due to the HA binding property (Tisdale, 2000; Abed *et al*., 2002; Okomo-Adhiambo *et al*., 2010). However, we previously reported NI-mediated replication inhibition of a recombinant OSA/H1N1 virus in MDCK cells (Kim *et al*., 2012) and found no reliable amino acid mutations around the HA receptor binding domain of H3N2/16, 17, and 50 viruses (Table 2). Therefore, we speculated that the plaque morphology in the presence of an NI among the tested H3N2 viruses primarily resulted from NA mutations (Fig. 1). To address whether the NA protein governed the plaque morphology in a plaque reduction assay, the NA of the H3N2/17-5 virus was used to generate a 7:1 reassortant virus (H1N2, rK09/N2:17-5) by reverse genetics (Fodor *et al*., 1999) in the context of a 2009 pandemic H1N1 strain, A/Korea/01/2009 (rK09), which had been characterized previously by *in vitro* and *in vivo* analyses (Kwon *et al*., 2010). While the parental rK09 formed large plaques, the plaque size of rK09/N2:17-5 virus was greatly reduced in MDCK cells (Fig. 2). This observation implies that the H3N2/17-5 virus has reduced NA activity as compared to the rK09 virus. However, surprisingly, the plaque size of rK09/N2:17-5 virus increased proportionally with increasing concentrations of oseltamivir, as was observed with the H3N2/17-5 virus (Fig. 2). This result suggests that the N2 NA protein containing the A177T mutation has this unusual property, irrespective of HA subtype. Furthermore, this mutation may lead to maximal viral fitness during antiviral drug therapy. Therefore, it will be very interesting to further elucidate an unusual oseltamivir susceptibility triggered by the novel A177T mutation using not only *in vitro* but also *in vivo* models in the future.

This study was supported by grants from the Korea healthcare technology R&D project of the Ministry of Health & Welfare, Republic of Korea (Grants No. A103001, A084411), Korea Centers for Disease Control and Prevention (2010-E43002-00) and Hallym University, Specialization Fund (HRF-S-41). We would like to thank Saem Shin and Sulhwa Jung for technical assistance.

References

Abed, Y., Bourgault, A.M., Fenton, R.J., Morley, P.J., Gower, D., Owens, I.J., Tisdale, M., and Boivin, G. 2002. Characterization of 2 influenza a(h3n2) clinical isolates with reduced susceptibility to neuraminidase inhibitors due to mutations in the hemagglutinin gene. *J. Infect. Dis.* **186**, 1074–1080.

Aoki, F.Y., Boivin, G., and Roberts, N. 2007. Influenza virus susceptibility and resistance to oseltamivir. *Antivir. Ther.* **12**, 603–616.

Baek, Y.H., Park, J.H., Song, Y.J., Song, M.S., Pascua, P.N., Hahn, Y.S., Han, H.S., Lee, O.J., Kim, K.S., Kang, C., and *et al.* 2009. Molecular characterization and phylogenetic analysis of h3n2 human influenza a viruses in cheongju, south Korea. *J. Microbiol.* **47**, 91–100.

CDC. 2008. Influenza activity – united states and worldwide, 2007–08 season. *MMWR Morb. Mortal Wkly Rep.* **57**, 692–697.

CDC. 2009. Update: Influenza activity – united states, September 28, 2008–January 31, 2009. *MMWR Morb. Mortal Wkly Rep.* **58**, 115–119.

CDC. 2010. Update: Influenza activity – united states, 2009–10 season. *MMWR Morb. Mortal Wkly Rep.* **59**, 901–908.

CDC. 2011. Update: Influenza activity – united states, 2010–11 season, and composition of the 2011–12 influenza vaccine.

MMWR Morb. Mortal Wkly Rep. **60**, 705–712.

Fodor, E., Devenish, L., Engelhardt, O.G., Palese, P., Brownlee, G.G., and Garcia-Sastre, A. 1999. Rescue of influenza a virus from recombinant DNA. *J. Virol.* **73**, 9679–9682.

Gubareva, L.V. 2004. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. *Virus Res.* **103**, 199–203.

Hauge, S.H., Dudman, S., Borgen, K., Lackenby, A., and Hungnes, O. 2009. Oseltamivir-resistant influenza viruses a (h1n1), norway, 2007–08. *Emerg. Infect. Dis.* **15**, 155–162.

Kim, J.I., Park, S., Lee, I., Lee, S., Shin, S., Won, Y., Hwang, M.W., Bae, J.Y., Heo, J., Hyun, H.E., and *et al.* 2012. Gfp-expressing influenza a virus for evaluation of the efficacy of antiviral agents. *J. Microbiol.* **50**, 359–362.

Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F.G., Sugaya, N., and Kawaoka, Y. 2004. Resistant influenza a viruses in children treated with oseltamivir: Descriptive study. *Lancet.* **364**, 759–765.

Kwon, D., Shin, K., Kim, S., Ha, Y., Choi, J.H., Yang, J.S., Lee, J.Y., Chae, C., Oh, H.B., and Kang, C. 2010. Replication and pathogenesis of the pandemic (h1n1) 2009 influenza virus in mammalian models. *J. Microbiol.* **48**, 657–662.

McKimm-Breschkin, J., Trivedi, T., Hampson, A., Hay, A., Klimov, A., Tashiro, M., Hayden, F., and Zambon, M. 2003. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. *Antimicrob. Agents Chemother.* **47**, 2264–2272.

Meijer, A., Lackenby, A., Hungnes, O., Lina, B., van-der-Werf, S., Schweiger, B., Opp, M., Paget, J., van-de-Kassteele, J., Hay, A., and *et al.* 2009. Oseltamivir-resistant influenza virus a (h1n1), europe, 2007-08 season. *Emerg. Infect. Dis.* **15**, 552–560.

NISN. 2012. Neuraminidase inhibitor susuceptibility network. Virological assay - laboratory tests of neuraminidase inhibition. (http://www.nisn.org/v_laboratory_tests.html , accessed on october 3, 2012).

Okomo-Adhiambo, M., Sleeman, K., Ballenger, K., Nguyen, H.T., Mishin, V.P., Sheu, T.G., Smagala, J., Li, Y., Klimov, A.I., and Gubareva, L.V. 2010. Neuraminidase inhibitor susceptibility testing in human influenza viruses: A laboratory surveillance perspective. *Viruses* **2**, 2269–2289.

Sheu, T.G., Deyde, V.M., Okomo-Adhiambo, M., Garten, R.J., Xu, X., Bright, R.A., Butler, E.N., Wallis, T.R., Klimov, A.I., and Gubareva, L.V. 2008. Surveillance for neuraminidase inhibitor resistance among human influenza a and b viruses circulating worldwide from 2004 to 2008. *Antimicrob. Agents Chemother.* **52**, 3284–3292.

Tisdale, M. 2000. Monitoring of viral susceptibility: New challenges with the development of influenza NA inhibitors. *Rev. Med. Virol.* **10**, 45–55.

WHO. 2012. Influenza update (update number 154): Countries in the temperate zone of the nothern hemisphere (www.who.int/influenza/surveillance_monitoring/updates/2012_03_02_gip_surveillance/en/index.html#northern).