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Influenza Virus Shedding in Laninamivir-Treated Children upon Returning to School

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The current School Health and Safety Act in Japan states that children with influenza infection should stay home until day 6th after symptoms onset. This was an amendment of a previous version recommending school return on day 3 after defervescence. Here, we investigated the duration of fever and virus shedding after laninamivir treatment in 7 children infected with influenza A(H3N2) virus and 21 children with influenza B virus in relation to the school return timing recommended by the School Health and Safety Act during the 2011-2012 influenza season. Nasal discharge was collected on the first, second, and third hospital visits and virus titers were assessed by virus culture and real-time PCR. Duration of fever after laninamivir treatment was 1 day longer for influenza B than for influenza A(H3N2). Virus detection rates with 50% tissue culture infectious dose and viral RNA were highest at the first visit and gradually decreased at subsequent visits. Virus positivity rates were detectable at the time of defervescence in less than half of the enrolled patients (14.3-42.9%). Virus shedding rates were similarly low (0.0-19.0%) on day 3 or later from defervescence and on day 6 or later from fever onset (school return dates per the old and current School Health and Safety Act) regardless of the influenza type. In conclusion, despite the higher efficacy of laninamivir against A(H3N2) viruses than B viruses, viral shedding is low after return to school for both types, regardless of the version of the School Health and Safety Act.

Keywords: influenza; laninamivir; period of school absence; school health; virus shedding
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Introduction

Influenza virus causes acute respiratory infections. Annual epidemics are caused by influenza virus types A and B, while pandemics have been solely associated with influenza A viruses and occur less frequently (Cox and Subbarao 2000; Wright and Webster 2001; Neumann et al. 2009). Neuraminidase inhibitors (NAIs), oseltamivir and zanamivir, have been used for influenza prevention and treatment since 2000 in Japan. In 2010, two new NAIs, laninamivir and peramivir, were approved for prevention and treatment of influenza in Japan (Kawai et al. 2006; Sugaya and Ohashi 2010; Shobugawa et al. 2012; Yates et al. 2013; Hata et al. 2014). Of special interest is laninamivir octanoate hydrate (laninamivir), a long-acting inhaled drug, that is approved for clinical use in Japan only (Sugaya and Ohashi 2010; Watanabe et al. 2010). The clinical efficacy of laninamivir is reported to be similar to that of oseltamivir and zanamivir, although the former has been less frequently studied as it has not been approved in other countries (Sugaya and Ohashi 2010; Watanabe et al. 2010; Shobugawa et al. 2012; Ikematsu et al. 2014a, b, 2015b; Koseki et al. 2014). NAIs have been shown to significantly shorten the duration of fever in children infected with influenza virus, however, virus shedding could still be detected after defervescence (Sugaya et al. 2007; Tamura et al. 2011). Thus, patients might still be infectious and could spread the infection despite the resolution of fever. Further, emergence of NAI-resistant variants during treatment may further limit the effectiveness of NAIs in children (Saito et al. 2010) and

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prolong viral shedding (Kawai et al. 2009).

The recommended duration of school absence for children infected with influenza in Japan was revised by the School Health and Safety Act in 2012 (Ministry of Health, Labour and Welfare 2012). Previously, children were recommended to stay home for the duration of fever plus two days after defervescence (Takayama 2010). The updated guideline for school absence recommends that school children should stay home for a period of five days after the onset of illness regardless of the timing of defervescence.

In this study, we investigated the correlation between the duration of fever and viral shedding in influenza-infected children treated with laninamivir. We also assessed the emergence of drug-resistant viruses among treated patients and examined their viral load throughout the recommended period for school absence.

Patients and Methods

Patient enrollment and ethical statement

Pediatric patients who visited Sado General Hospital in Niigata Prefecture, Japan with influenza-like symptoms such as fever, sore throat, cough, malaise, and rhinorrhea in February or March 2012 were enrolled in the study. Patients were screened with a rapid influenza diagnostic test kit licensed in Japan (Quick-Navi Flu, Denka Seiken Co., Ltd., Tokyo, Japan). Influenza-positive patients or their guardians were informed about the study by clinicians, and those who provided written informed consent were enrolled. This study was approved by the Ethical Committee of the Medical School of Niigata University.

Clinical samples and treatment

Patients were treated with laninamivir, which is approved for treatment of influenza infection in Japan. The decision to use laninamivir was based on the advice of clinicians and/or the preference of the patients or their guardians. Laninamivir was prescribed as a single inhaled dose in accordance with the prescription guidelines by the Ministry of Health, Labor and Welfare in Japan. For children younger than 10 years, the prescribed dose was 20 mg. Children 10 years or older received a dose of 40 mg. All patients were administered the laninamivir under the supervision of a pharmacist within a few hours after receiving the prescription.

Specimen collection and clinical data

Nasal discharge was collected from patients on 3 occasions during the first (before treatment), second (3-5 days after the first visit), and third (5-9 days after the first visit) visits. Clinicians recorded the patients' sex, age, date of clinical examination, date of fever onset, and vaccination status during the first visit. Fever onset was defined as a temperature higher than 37.5°C. Patients or their guardians were asked to fill the patient's body temperature in a daily chart at 9 am, 12 pm, and 8 pm until the third visit day. Patients who did not return the body temperature chart were excluded from the analysis. Samples were kept at the clinic at −20°C until being transport to Niigata University for further analysis.

Virological tests

The specimens were inoculated on Madin-Darby canine kidney (MDCK) cells and monitored for cytopathic effect (CPE). RNA extraction and cDNA synthesis were performed on both the original clinical samples and CPE-positive cultures (Hoffmann et al. 2001; Dapat et al. 2009). Isolates were screened for influenza using a previously reported cycling probe real-time polymerase chain reaction (PCR) method that allows typing and subtyping of influenza (Suzuki et al. 2010, 2011; Dapat et al. 2012). Viral load was determined by using 50% tissue culture infectious dose ($TCID_{50}$) and by quantification of the influenza virus M gene using real-time PCR (Suzuki et al. 2003; Daum et al. 2007).

Ten-fold serial dilutions of each culture-positive specimen were prepared and 100 μl of each dilution was inoculated onto three wells of MDCK cells in 96 well plates. The plate was read after 48 hours by checking for CPE in each viral dilution. The $TCID_{50}$ titer was determined using the Reed-Muench method (LaBarre and Lowy 2001). The detection limit of the $TCID_{50}$ assay was 0.5 $TCID_{50}$/100 μl. Positivity rate for infectious titer was determined by proportion of viral strains that yielded measurable $TCID_{50}$ titer above 0.5 log10 $TCID_{50}$/100 μl.

Quantitative real-time PCR using TaqMan probes was performed on cDNA from the original clinical specimens. The type A forward primer (5′-TAACCGAGGTCGAAACGTA-3′), type A reverse primer (5′-GCACGGTGAGCGTGAA-3′), and type A probe (5′-FAM-TCAGGCCCCCTCAAAGC-Eclipse-3′) were designed for the M gene of influenza A. The type B forward primer (5′-GCATCTTTTGTTTTTTATCCATTCC-3′), type B reverse primer (5′-CACAATTGCCTACCTGCTTTCA-3′), and type B probe (5′-FAM-TGCTAGTTCTGCTTTGCCTTCTCCATCTTCT-Eclipse-3′) were designed for the M gene of influenza B. The quantitative real-time PCR conditions were 50 cycles of denaturation at 95°C for 5 seconds and annealing at 60°C for 30 seconds. The target sequence was cloned into a pM20-T vector (Takara Bio Inc., Shiga, Japan) using a Mighty TA cloning kit (Takara Bio Inc.) according to methods described previously (Suzuki et al. 2010). The cloned product was quantified with a spectrophotometer and serial dilution. A standard curve for quantification was prepared. Each sample and quantification control were run in duplicate wells. The resulting viral load was expressed as genome-equivalent copies of viral RNA. A cycle threshold value of < 40 was considered positive. The lower detection limits were 2.86 and 2.92 copies per reaction against type A and type B, respectively.

NAI assay and analysis of 50% inhibitory concentration

Because the emergence of drug resistance can prolong fever, we assessed the susceptibility of all clinical samples isolated during the first, second, and third visits to the 4 approved NAIs (peramivir, oseltamivir, zanamivir, and laninamivir) using a fluorescence-based 50% inhibitory concentration (IC_{50}) method using 2′-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA; Sigma, St. Louis, MO, USA) (Dapat et al. 2013). Laninamivir was provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Oseltamivir was provided by Roche Products Ltd. (Basel, Switzerland). Zanamivir was obtained from GlaxoSmithKline (Brentford, UK). Peramivir was provided by Shionogi & Co., Ltd. (Osaka, Japan). IC_{50} values were calculated using XLfit software (ID Business Solutions Ltd., Surrey, UK). A sample was defined as an outlier if its IC_{50} value was more than 3 times the interquartile range (IQR) from the 75th percentile in a box-and-whisker plot (Monto et al. 2006).

Statistical analyses

First, we assessed clinical demographic information (sex, age, vaccination) and fever duration in relation to virus type/subtype in the laninamivir treatment group. Fever was defined as a temperature higher than 37.5°C. We evaluated the intervals (in days) from fever onset to first visit, from treatment to defervescence, and from fever onset to defervescence in relation to virus type/subtype. Second, virus infectious titer and virus RNA load were compared at the first, second, and third visits. Third, we compared the infectious titers and RNA loads of the A(H3N2) and B viruses after defervescence (< 37.5°C). Finally, to determine the adequacy of the duration of school absence recommended in the Ordinance for Enforcement of the School Health and Safety Act, we compared virus infectious titer and viral load in children on day 3 or later from defervescence (the previous recommendation) and on day 6 or later from fever onset (the current recommendation). Student's t-test was used to compare means, and Fisher's exact test was used to compare percentages. All statistical analysis was performed using Microsoft Excel 2010 software. Statistical significance was defined as a p-value of < 0.05

Results

Patient characteristics

Twenty-eight patients enrolled in the study between February and March 2012. All patients had influenza-like illness and were positive for influenza using the rapid test kit. Subtyping by cycling probe real-time PCR showed that 7 patients were infected with influenza A(H3N2) and 21 patients had influenza B (Table 1). All patients were given laninamivir at the first visit, which they inhaled at local pharmacies within a few hours of receiving the prescription. There were no statistically significant differences in sex, age, vaccination status, or interval from fever onset to first visit between patients infected with influenza A(H3N2) and B subjects (Table 1). The average number of days from treatment to defervescence was significantly longer for patients infected with type B (2.3 ± 1.6 days) than for those infected with type A(H3N2) (1.1 ± 0.5 days) ($p = 0.0005$) (Table 1). Similarly, the average duration from fever onset to defervescence was significantly longer for patients infected with type B (3.5 ± 1.7 days) than for those infected with type A(H3N2) (2.0 ± 1.0 days) ($p = 0.032$).

Virus infection titer and copy number

We then measured viral shedding after laninamivir treatment among children with influenza and compared $TCID_{50}$ titers and virus copy numbers in relation to virus type/subtype at the first (before treatment), second (3-5 days after the first visit), and third (5-9 days after the first visit) visits. In general, viral load determined by infectious titer ($TCID_{50}$) or RNA copies (real-time PCR) steadily decreased after laninamivir treatment for both influenza A(H3N2) and B (Fig. 1). The virus positivity rate by $TCID_{50}$ at the first visit was significantly lower for patients with influenza A(H3N2) (28.6%) than for those with type B (80.9%; $p < 0.01$). However, the detection rates for influenza A(H3N2) and B viruses did not significantly differ at the second (14.3% vs. 23.8%, respectively) or third (0.0% vs 4.8%, respectively) visit. Influenza A(H3N2) and B virus titers and viral RNA copies were highest at the first visit and progressively decreased in subsequent visits (Table 2). One (4.8%) patient with influenza B persistently had high titers (6.3 $TCID_{50}$/100 μl) and viral RNA copies (2.4 $\log_{10}$ copies/μl) at the third visit despite being afebrile as of day 3 after fever onset (Fig. 1).

Virus shedding after defervescence

Next, we examined viral shedding patterns after defervescence (body temperature < 37.5°C) in the study patients. Infectious virus shedding, measured by $TCID_{50}$, was detected in one of the seven patients (14.3%) infected with influenza A(H3N2) virus and three of the 21 patients (14.3%) infected with influenza B virus at the visit that immediately followed defervescence (0-4 days after defervescence) (Fig. 2A, B). Viral RNA could be detected in three of the seven influenza A(H3N2) patients (42.9%) and

Table 1. Demographic characteristics of patients infected with influenza A(H3N2) and B treated with laninamivir.

Influenza type/subtype	A(H3N2)	B	p-value
No. of patients	7	21	
Sex, male (%)	6 (85.7%)	10 (47.6%)	0.191
Age, average ± SD (years)	11.0 ± 3.8	10.2 ± 2.1	0.625
Influenza vaccination (%)	4 (57.1%)	12 (51.7%)	1
Duration from fever onset to first visit, Average ± SD (days)	1.0 ± 0.9 (N = 6)[a]	1.1 ± 0.8	0.737
Duration from treatment to defervescence, Average ± SD (days)	1.1 ± 0.5	2.3 ± 1.6	0.005
Duration from fever onset to defervescence, Average ± SD (days)	2.0 ± 1.0 (N = 6)[a]	3.5 ± 1.7	0.032

Student's t-test was used to compare means and Fisher's exact test was used to compare percentages.
SD, standard deviation.
[a]No data on fever onset for 1 patient with influenza A(H3N2) infection.

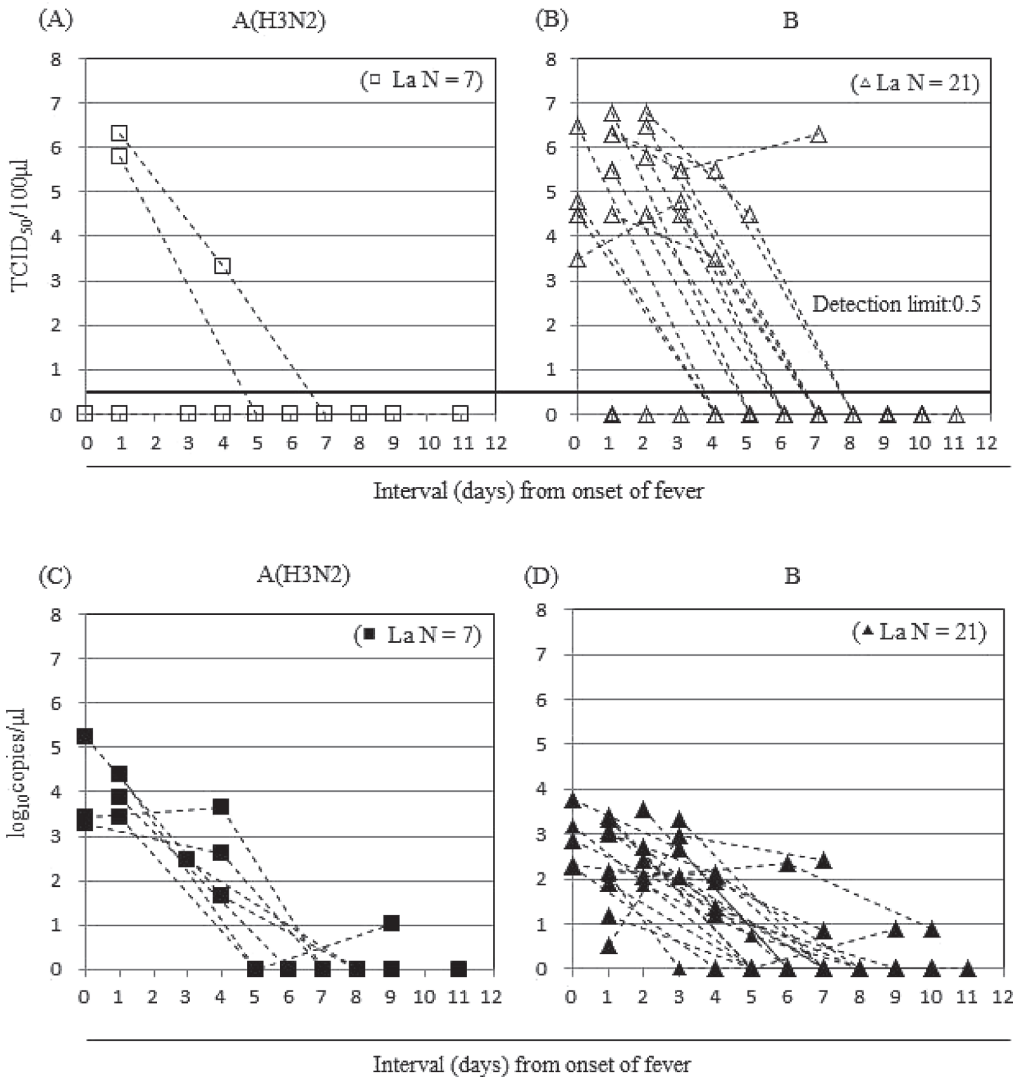

Fig. 1. Change in virus infectious titers and viral RNA copies after laninamivir treatment for patients with influenza A(H3N2) and B. Day 0 indicates fever onset. Panels A and B show virus infectious titers ($TCID_{50}$ titer) for influenza A(H3N2) and B, respectively. Panels C and D show viral RNA copies for influenza A(H3N2) and B, respectively. La, laninamivir.

Table 2. Virus infectious titer ($TCID_{50}$ titer) and virus RNA copy number before and after laninamivir treatment.

Influenza type/subtype		A(H3N2)			B		
Number of patients		7			21		
Timing of sample collection		first visit[a]	second visit[b]	third visit[c]	first visit[a]	second visit[b]	third visit[c]
Virus infectious titer	positivity rate (%)	2 (28.6%)	1 (14.3%)	0 (0%)	17 (80.9%)*	5 (23.8%)	1 (4.8%)
	Average ± SD (log10 $TCID_{50}$/100µl)	6.1	3.3	/[d]	5.4 ± 1.0	4.8 ± 0.8	6.3
Virus genome number	positivity rate (%)	7 (100%)	3 (42.9%)	1 (14.3%)	21 (100%)	9 (42.9%)	4 (19.0%)
	Average ± SD (log10 copies/µl)	3.7 ± 0.9	2.6 ± 1.0	1.0	2.5 ± 0.8	1.9 ± 0.7	1.3 ± 0.8

[a]First visit refers to sample collection before laninamivir treatment.
[b]Second visit: Samples were collected within 2-5 days after start of laninamivir treatment.
[c]Third visit: Samples were collected within 5-9 days after start of laninamivir treatment.
[d]Virus culture was negative.
*Positive rate of sample at the first visit was significantly higher for B than for A(H3N2) ($p < 0.005$).

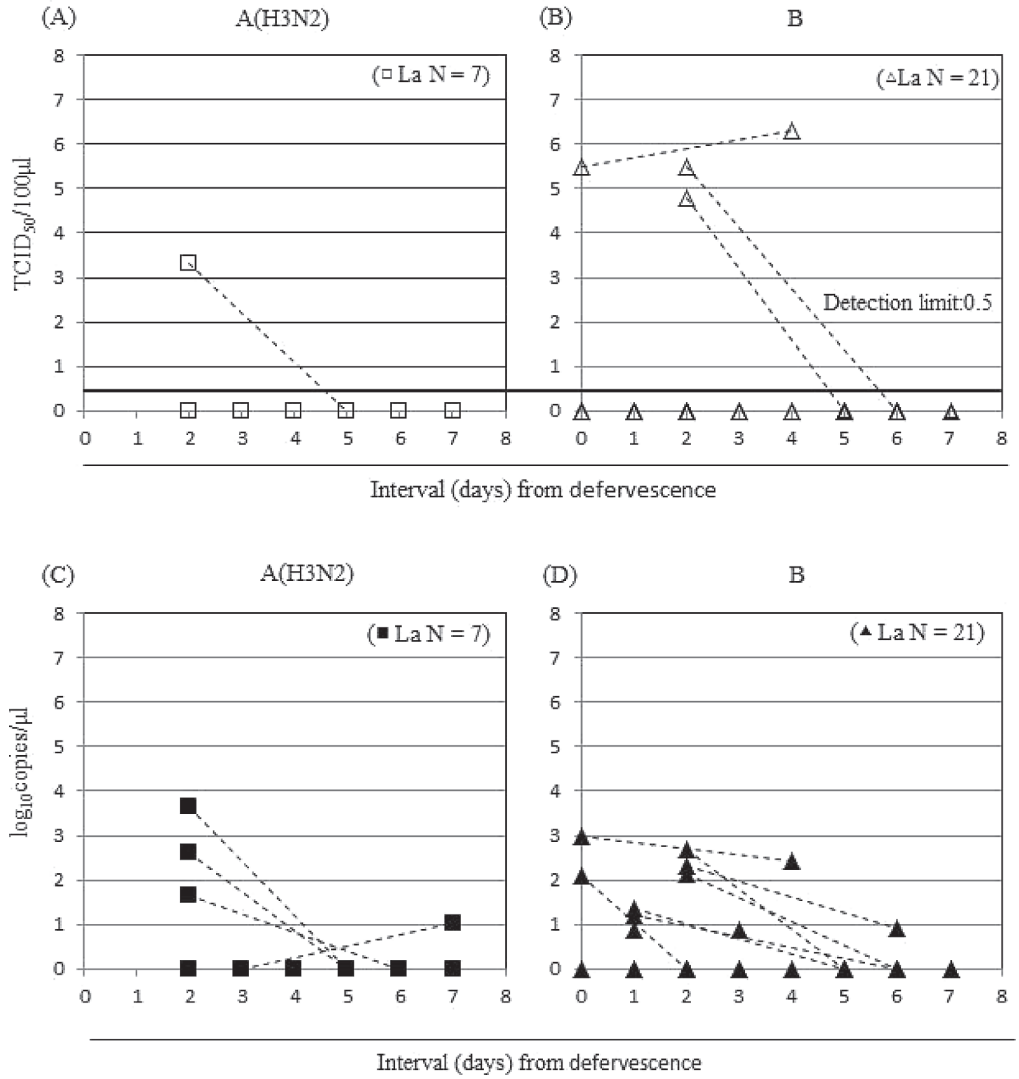

Fig. 2. Viral shedding after defervescence (body temperature < 37.5°C) in influenza A(H3N2) and B infected patients treated with laninamivir. Day 0 indicates defervescence. Panels A and B show virus infectious titers ($TCID_{50}$ titer) for influenza A(H3N2) and B, respectively. Panels C and D show viral RNA copies for influenza A(H3N2) and B, respectively. La, laninamivir.

nine of the 21 influenza B patients (42.9%) at the visit that followed defervescence (Fig. 2C, D).

Duration of viral shedding and the School Health and Safety Act

We next assessed virus isolation and viral RNA detection rates in relation to the previous and current government recommendations (Table 3).

First, we evaluated viral shedding on day 3 or later from defervescence, the recommended school return day in the previous School Health and Safety Act recommendation). None (0.0%) of the 7 influenza A(H3N2) patients had detectable infectious virus shedding. However, viral RNA could still be detected in one (14.3%) of the patients. In case of influenza B, one (4.8%) of the 21 patients had detectable infectious virus shedding and 3 (14.3%) had detectable viral RNA.

Second, we evaluated virus titers for $TCID_{50}$ on day 6 or later from fever onset (the revised school absence period in the School Health and Safety Act recommendation). For influenza A(H3N2), none of the patients (0.0%) had detectable infectious virus shedding and only one (14.3%) had a detectable viral RNA. For influenza B, one (4.8%) patient had detectable infectious virus and four (19.0%) displayed positive viral RNA result (Table 3). The detection rates of influenza A(H3N2) and B were not different.

NAI assay

The IC_{50} values of the four tested NAIs (peramivir, oseltamivir, zanamivir, and laninamivir) were higher for type B influenza than for type A(H3N2) influenza, as was previously reported (Dapat et al. 2013). On the first visit A(H3N2) and B isolates had lower averages of IC_{50} values to peramivir (A(H3N2): 0.16 nM, B 1.17 nM) than other

Table 3. Virus infectious titer ($TCID_{50}$ titer) and virus RNA copy number for A(H3N2) and B viruses collected 3 days after defervescence and 6 days after fever onset.

Influenza type/subtype	A(H3N2)		B	
Number of patients	7		21	
	Day 3 or later from defervescence	Day 6 or later from fever onset	Day 3 or later from defervescence	Day 6 or later from fever onset
Virus infectious titer No. of positives (%)	0 (0.0%)[a]	0 (0.0%)[a]	1 (4.8%)	1 (4.8%)
Virus genome number No. of positives (%)	1 (14.3%)	1 (14.3%)	3 (14.3%)	4 (19.0%)

[a]Virus culture was negative.

Table 4. Susceptibility of isolates to four neuraminidase inhibitors at first, second and third visit after laninamivir treatment for A(H3N2) and B viruses.

		IC_{50} (nM)					
Drug	Type/subtype	First visit		Second visit		Third visit	
		N	Average[a] ± SD	N	Average[a] ± SD	N	Average[a] ± SD
Peramivir trihydrate	A(H3N2)	7	0.16 ± 0.03	2	0.21	0	/ [b]
	B	20	1.17 ± 0.38	15	1.35 ± 0.42	6	1.24 ± 0.31
Oseltamivir carboxylic acid	A(H3N2)	7	0.91 ± 0.21	2	1.24	0	/ [b]
	B	20	39.52 ± 11.03	15	44.60 ± 14.20	6	49.67 ± 11.10
Zanamivir hydrate	A(H3N2)	7	0.64 ± 0.11	2	0.57	0	/ [b]
	B	20	12.97 ± 7.50	15	11.80 ± 6.34	6	10.44 ± 4.00
Laninamivir	A(H3N2)	7	0.56 ± 0.11	2	0.46	0	/ [b]
	B	20	4.42 ± 1.64	15	4.03 ± 1.03	6	4.06 ± 0.98

SD, standard deviation.
[a]Average value was calculated from viruses that showed IC_{50} titer below the cutoff.
[b]Virus culture was negative.

NAIs. The average IC_{50} values for A(H3N2) were 0.56 nM for laninamivir, 0.64 nM for zanamivir, and 0.91 nM for oseltamivir. For influenza B isolates, the average IC_{50} values were 4.42 nM, 12.97 nM, and 39.52 nM for laninamivir, zanamivir, and oseltamivir respectively (Table 4). None of the isolates obtained at the first visit had reduced susceptibility (outlier strains). Additionally, we did not observe a change in the antiviral drug susceptibilities of the influenza A(H3N2) and B isolates at the second and third visits. These findings indicate that no strains with reduced susceptibility or resistant phenotype were induced by laninamivir treatment.

Discussion

Laninamivir is a new long-acting NAI that was approved for influenza treatment in Japan in 2010 (Ikematsu and Kawai 2011). Currently, the drug is only used in Japan. Because of its limited use, less studies have evaluated the clinical efficacy of laninamivir on defervescence and virus shedding compared with those for oseltamivir and zanamivir (Takemoto et al. 2013; Ikematsu et al. 2014a, b, 2015b; Koseki et al. 2014; Mizuno et al. 2014; Sugaya et al. 2015). In this study we examined virus shedding patterns in influenza infected children after defervescence upon laninamivir treatment.

Comparing the duration of fever, laninamivir was less effective in patients infected with influenza B virus than in those infected with A(H3N2), consistent with previous studies (Ikematsu et al. 2014a, b). In the present study, the average number of days from fever onset to defervescence was 1.5 days longer for influenza B than for influenza A(H3N2) upon laninamivir treatment.

Viral RNA shedding after laninamivir treatment as determined by real-time PCR did not significantly differ between influenza A(H3N2) and B. This finding contrasts with previous studies that reported higher rates of viral

shedding in influenza B patients treated with laninamivir (Ikematsu et al. 2014a, b). Nonetheless, a German study reported that the period of viral RNA shedding, as determined by real-time PCR, was around 6-8 days and that there were no significant differences in duration between virus types or subtypes (Suess et al. 2012). In addition, the authors found that the duration of detectable virus shedding was longer for real-time PCR than for virus culture—for both influenza A(H3N2) and B—as was the case in the present study. Further investigation of the relation of viral shedding to influenza type and differences between detection methods is warranted.

It should be noted that virus isolation rates for influenza A(H3N2) were much lower than influenza B, even at the first visit. A recent study showed that clinical influenza A(H3N2) virus replicates poorly in MDCK cells and rapidly acquires HA or NA mutations when propagated in vitro (Chambers et al. 2014). This may explain the low infectious virus positivity rate in influenza A(H3N2) patients.

The School Health and Safety Act of 2012 is a revision of a law originally enacted in 1958 by the Ministry of Education (currently the Ministry of Education, Science and Technology). Under this law, the recommended school return day for influenza was previously defined as 2 days after defervescence. In 2012 this interval was changed to 5 days after fever onset because of the widespread use of NAIs for influenza infection in Japan (Sugaya 2011). Studies have shown that the duration of fever was 1 to 2 days shorter in patients treated with NAIs than in untreated patients (Saito et al. 2010; Dobson et al. 2015). Thus, treated patients might return to school or work earlier than untreated patients and continue to shed virus. This concern led the Japanese Ministry of Education, Culture, Sports, Science and Technology to change the duration of absence for school children. In the revised act, children are advised to remain at home for 5 days after disease onset, regardless of fever duration. In the present study, virus detection rate was low (0-15%) on day 3 or later from defervescence and similarly low (0-20%) on day 6 or later from fever onset. Therefore, the risk of spreading virus by treated children returning to school in accordance with previous and current recommendations is very limited. The risk of infection spread could be further elucidated in future studies monitoring classmates of index cases after their return to school.

In our study, IC_{50} values for peramivir to influenza A(H3N2) and B were lower than those for other NAIs. It is well documented that IC_{50} values can vary depending on the methods used making it difficult to compare values obtained in different laboratories (Nguyen et al. 2010; Okomo-Adhiambo et al. 2010; Murtaugh et al. 2013). However, the lower IC_{50} values for peramivir compared to the other three NAIs are consistent with past reports (Okomo-Adhiambo et al. 2013; Leang et al. 2014; Farrukee et al. 2015; Ikematsu et al. 2015a; Tamura et al. 2015). It has been shown that antiviral drug resistance can emerge upon treatment with NAIs (Thorlund et al. 2011). In the present study, however, none of the isolates obtained following treatment with laninamivir possessed reduced susceptibility to any of the tested NAIs, consistent with other Japanese studies (Ikematsu et al. 2014a, b). As previously reported, influenza B viruses were less susceptible to NAIs than influenza A viruses (Ikematsu and Kawai 2011). This could explain the higher virus detection rate for influenza B and longer duration of fever in this group of patients compared to influenza A(H3N2) infected patients.

The principal limitations of this study are its small sample size and the fact that only patients treated with laninamivir were enrolled. The original study design included an oseltamivir treatment group, for the purpose of comparison with the laninamivir group. However, only 3 patients received oseltamivir, and the group was thus excluded from the analysis. Future studies should enroll a larger number of patients and investigate other NAIs. In particular, peramivir, oseltamivir, and zanamivir should be evaluated in relation to clinical course and viral shedding.

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Conflict of Interest

None of the first author and co-authors has a conflict of interest with Daiichi Sankyo CO., LTD., other than Reiko Saito in receiving the study funding as a principal investigator. The agency provided the funding but did not interfere with the design, funding, or writing the manuscript.

References

Chambers, B.S., Li, Y., Hodinka, R.L. & Hensley, S.E. (2014) Recent H3N2 influenza virus clinical isolates rapidly acquire hemagglutinin or neuraminidase mutations when propagated for antigenic analyses. *J. Virol.*, **88**, 10986-10989.

Cox, N.J. & Subbarao, K. (2000) Global epidemiology of influenza: past and present. *Annu. Rev. Med.*, **51**, 407-421.

Dapat, C., Kondo, H., Dapat, I.C., Baranovich, T., Suzuki, Y., Shobugawa, Y., Saito, K., Saito, R. & Suzuki, H. (2013) Neuraminidase inhibitor susceptibility profile of pandemic and seasonal influenza viruses during the 2009-2010 and 2010-2011 influenza seasons in Japan. *Antiviral Res.*, **99**, 261-269.

Dapat, C., Saito, R., Kyaw, Y., Naito, M., Hasegawa, G., Suzuki, Y., Dapat, I.C., Zaraket, H., Cho, T.M., Li, D., Oguma, T., Baranovich, T. & Suzuki, H. (2009) Epidemiology of human influenza A and B viruses in Myanmar from 2005 to 2007. *Intervirology*, **52**, 310-320.

Dapat, I.C., Dapat, C., Baranovich, T., Suzuki, Y., Kondo, H., Shobugawa, Y., Saito, R. & Suzuki, H.; Japanese Influenza Collaborative Study Group (2012) Genetic characterization of human influenza viruses in the pandemic (2009-2010) and post-pandemic (2010-2011) periods in Japan. *PLoS One*, **7**,

e36455.
Daum, L.T., Canas, L.C., Arulanandam, B.P., Niemeyer, D., Valdes, J.J. & Chambers, J.P. (2007) Real-time RT-PCR assays for type and subtype detection of influenza A and B viruses. *Influenza Other Respir. Viruses*, **1**, 167-175.
Dobson, J., Whitley, R.J., Pocock, S. & Monto, A.S. (2015) Oseltamivir treatment for influenza in adults: a meta-analysis of randomised controlled trials. *Lancet*, **385**, 1729-1737.
Farrukee, R., Leang, S.K., Butler, J., Lee, R.T., Maurer-Stroh, S., Tilmanis, D., Sullivan, S., Mosse, J., Barr, I.G. & Hurt, A.C. (2015) Influenza viruses with B/Yamagata- and B/Victoria-like neuraminidases are differentially affected by mutations that alter antiviral susceptibility. *J. Antimicrob. Chemother.*, **70**, 2004-2012.
Hata, A., Akashi-Ueda, R., Takamatsu, K. & Matsumura, T. (2014) Safety and efficacy of peramivir for influenza treatment. *Drug Des. Devel. Ther.*, **8**, 2017-2038.
Hoffmann, E., Stech, J., Guan, Y., Webster, R.G. & Perez, D.R. (2001) Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol.*, **146**, 2275-2289.
Ikematsu, H. & Kawai, N. (2011) Laninamivir octanoate: a new long-acting neuraminidase inhibitor for the treatment of influenza. *Expert Rev. Anti Infect. Ther.*, **9**, 851-857.
Ikematsu, H., Kawai, N., Iwaki, N. & Kashiwagi, S. (2014a) Continued effectiveness of laninamivir octanate hydrate for influenza treatment in Japan: comparison between the 2011-2012 and 2012-2013 influenza seasons. *J. Infect. Chemother.*, **20**, 799-803.
Ikematsu, H., Kawai, N., Iwaki, N. & Kashiwagi, S. (2014b) The duration of fever and other symptoms after the initiation of laninamivir octanoate hydrate in the Japanese 2011-2012 influenza season. *J. Infect. Chemother.*, **20**, 81-85.
Ikematsu, H., Kawai, N., Iwaki, N. & Kashiwagi, S. (2015a) In vitro neuraminidase inhibitory activity of four neuraminidase inhibitors against clinical isolates of influenza virus in the Japanese 2012-2013 season. *J. Infect. Chemother.*, **21**, 39-42.
Ikematsu, H., Kawai, N., Iwaki, N. & Kashiwagi, S. (2015b) Clinical outcome of laninamivir octanoate hydrate for influenza in the 2013-2014 Japanese season. *J. Infect. Chemother.*, **21**, 802-807.
Kawai, N., Ikematsu, H., Iwaki, N., Kondou, K., Hirotsu, N., Kawashima, T., Maeda, T., Tanaka, O., Doniwa, K. & Kashiwagi, S. (2009) Clinical effectiveness of oseltamivir for influenza A(H1N1) virus with H274Y neuraminidase mutation. *J. Infect.*, **59**, 207-212.
Kawai, N., Ikematsu, H., Iwaki, N., Maeda, T., Satoh, I., Hirotsu, N. & Kashiwagi, S. (2006) A comparison of the effectiveness of oseltamivir for the treatment of influenza A and influenza B: a Japanese multicenter study of the 2003-2004 and 2004-2005 influenza seasons. *Clin. Infect. Dis.*, **43**, 439-444.
Koseki, N., Kaiho, M., Kikuta, H., Oba, K., Togashi, T., Ariga, T. & Ishiguro, N. (2014) Comparison of the clinical effectiveness of zanamivir and laninamivir octanoate for children with influenza A(H3N2) and B in the 2011-2012 season. *Influenza Other Respir. Viruses*, **8**, 151-158.
LaBarre, D.D. & Lowy, R.J. (2001) Improvements in methods for calculating virus titer estimates from TCID50 and plaque assays. *J. Virol. Methods*, **96**, 107-126.
Leang, S.K., Kwok, S., Sullivan, S.G., Maurer-Stroh, S., Kelso, A., Barr, I.G. & Hurt, A.C. (2014) Peramivir and laninamivir susceptibility of circulating influenza A and B viruses. *Influenza Other Respir. Viruses*, **8**, 135-139.
Ministry of Health, Labour and Welfare (2012) Infection Control Guidelines for nursery. Revised version in 2012. http://www.mhlw.go.jp/bunya/kodomo/pdf/hoiku02.pdf [*Accessed*: December 8, 2015].
Mizuno, T., Mizuno, S. & Kanda, T. (2014) Effects of vaccination and the new neuraminidase inhibitor, laninamivir, on influenza infection. *PLoS One*, **9**, e92601.
Monto, A.S., McKimm-Breschkin, J.L., Macken, C., Hampson, A.W., Hay, A., Klimov, A., Tashiro, M., Webster, R.G., Aymard, M., Hayden, F.G. & Zambon, M. (2006) Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. *Antimicrob. Agents Chemother.*, **50**, 2395-2402.
Murtaugh, W., Mahaman, L., Healey, B., Peters, H., Anderson, B., Tran, M., Ziese, M. & Carlos, M.P. (2013) Evaluation of three influenza neuraminidase inhibition assays for use in a public health laboratory setting during the 2011-2012 influenza season. *Public Health Rep.*, **128** Suppl 2, 75-87.
Neumann, G., Noda, T. & Kawaoka, Y. (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature*, **459**, 931-939.
Nguyen, H.T., Sheu, T.G., Mishin, V.P., Klimov, A.I. & Gubareva, L.V. (2010) Assessment of pandemic and seasonal influenza A (H1N1) virus susceptibility to neuraminidase inhibitors in three enzyme activity inhibition assays. *Antimicrob. Agents Chemother.*, **54**, 3671-3677.
Okomo-Adhiambo, M., Sleeman, K., Ballenger, K., Nguyen, H.T., Mishin, V.P., Sheu, T.G., Smagala, J., Li, Y., Klimov, A.I. & Gubareva, L.V. (2010) Neuraminidase inhibitor susceptibility testing in human influenza viruses: a laboratory surveillance perspective. *Viruses*, **2**, 2269-2289.
Okomo-Adhiambo, M., Sleeman, K., Lysen, C., Nguyen, H.T., Xu, X., Li, Y., Klimov, A.I. & Gubareva, L.V. (2013) Neuraminidase inhibitor susceptibility surveillance of influenza viruses circulating worldwide during the 2011 Southern Hemisphere season. *Influenza Other Respir. Viruses*, **7**, 645-658.
Saito, R., Sato, I., Suzuki, Y., Baranovich, T., Matsuda, R., Ishitani, N., Dapat, C., Dapat, I.C., Zaraket, H., Oguma, T. & Suzuki, H. (2010) Reduced effectiveness of oseltamivir in children infected with oseltamivir-resistant influenza A (H1N1) viruses with His275Tyr mutation. *Pediatr. Infect. Dis. J.*, **29**, 898-904.
Shobugawa, Y., Saito, R., Sato, I., Kawashima, T., Dapat, C., Dapat, I.C., Kondo, H., Suzuki, Y., Saito, K. & Suzuki, H. (2012) Clinical effectiveness of neuraminidase inhibitors–oseltamivir, zanamivir, laninamivir, and peramivir–for treatment of influenza A(H3N2) and A(H1N1)pdm09 infection: an observational study in the 2010-2011 influenza season in Japan. *J. Infect. Chemother.*, **18**, 858-864.
Suess, T., Remschmidt, C., Schink, S.B., Schweiger, B., Heider, A., Milde, J., Nitsche, A., Schroeder, K., Doellinger, J., Braun, C., Haas, W., Krause, G. & Buchholz, U. (2012) Comparison of shedding characteristics of seasonal influenza virus (sub)types and influenza A(H1N1)pdm09; Germany, 2007-2011. *PLoS One*, **7**, e51653.
Sugaya, N. (2011) Widespread use of neuraminidase inhibitors in Japan. *J. Infect. Chemother.*, **17**, 595-601.
Sugaya, N., Mitamura, K., Yamazaki, M., Tamura, D., Ichikawa, M., Kimura, K., Kawakami, C., Kiso, M., Ito, M., Hatakeyama, S. & Kawaoka, Y. (2007) Lower clinical effectiveness of oseltamivir against influenza B contrasted with influenza A infection in children. *Clin. Infect. Dis.*, **44**, 197-202.
Sugaya, N. & Ohashi, Y. (2010) Long-acting neuraminidase inhibitor laninamivir octanoate (CS-8958) versus oseltamivir as treatment for children with influenza virus infection. *Antimicrob. Agents Chemother.*, **54**, 2575-2582.
Sugaya, N., Sakai-Tagawa, Y., Bamba, M., Yasuhara, R., Yamazaki, M., Kawakami, C., Yamaguchi, Y., Ide, Y., Ichikawa, M., Mitamura, K. & Kawaoka, Y. (2015) Comparison between virus shedding and fever duration after treating children with pandemic A H1N1/09 and children with A H3N2 with a neuraminidase inhibitor. *Antivir. Ther.*, **20**, 49-55.
Suzuki, H., Saito, R., Masuda, H., Oshitani, H., Sato, M. & Sato, I. (2003) Emergence of amantadine-resistant influenza A viruses: epidemiological study. *J. Infect. Chemother.*, **9**, 195-200.

Suzuki, Y., Saito, R., Sato, I., Zaraket, H., Nishikawa, M., Tamura, T., Dapat, C., Caperig-Dapat, I., Baranovich, T., Suzuki, T. & Suzuki, H. (2011) Identification of oseltamivir resistance among pandemic and seasonal influenza A (H1N1) viruses by an His275Tyr genotyping assay using the cycling probe method. *J. Clin. Microbiol.*, **49**, 125-130.

Suzuki, Y., Saito, R., Zaraket, H., Dapat, C., Caperig-Dapat, I. & Suzuki, H. (2010) Rapid and specific detection of amantadine-resistant influenza A viruses with a Ser31Asn mutation by the cycling probe method. *J. Clin. Microbiol.*, **48**, 57-63.

Takayama, K. (2010) Recent tasks of school health administration in Japan. *JMAJ*, **53**, 144-147.

Takemoto, Y., Asai, T., Ikezoe, I., Yano, T., Ichikawa, M., Miyagawa, S. & Matsumoto, J. (2013) Clinical effects of oseltamivir, zanamivir, laninamivir and peramivir on seasonal influenza infection in outpatients in Japan during the winter of 2012-2013. *Chemotherapy*, **59**, 373-378.

Tamura, D., Okomo-Adhiambo, M., Mishin, V.P., Guo, Z., Xu, X., Villanueva, J., Fry, A.M., Stevens, J. & Gubareva, L.V. (2015) Application of a seven-target pyrosequencing assay to improve the detection of neuraminidase inhibitor-resistant Influenza A(H3N2) viruses. *Antimicrob. Agents Chemother.*, **59**, 2374-2379.

Tamura, D., Sugaya, N., Ozawa, M., Takano, R., Ichikawa, M., Yamazaki, M., Kawakami, C., Shimizu, H., Uehara, R., Kiso, M., Kawakami, E., Mitamura, K. & Kawaoka, Y. (2011) Frequency of drug-resistant viruses and virus shedding in pediatric influenza patients treated with neuraminidase inhibitors. *Clin. Infect. Dis.*, **52**, 432-437.

Thorlund, K., Awad, T., Boivin, G. & Thabane, L. (2011) Systematic review of influenza resistance to the neuraminidase inhibitors. *BMC Infect. Dis.*, **11**, 134.

Watanabe, A., Chang, S.C., Kim, M.J., Chu, D.W. & Ohashi, Y.; MARVEL Study Group (2010) Long-acting neuraminidase inhibitor laninamivir octanoate versus oseltamivir for treatment of influenza: A double-blind, randomized, noninferiority clinical trial. *Clin. Infect. Dis.*, **51**, 1167-1175.

Wright, P.F. & Webster, R.G. (2001) Orthomyxoviridae. Chapter 47. In *Fields virology*, 4th ed. edited by Knipe, D.M. & Howley, P.M. Philadelphia, pp. 1562.

Yates, P.J., Mehta, N., Horton, J. & Tisdale, M. (2013) Virus susceptibility analyses from a phase IV clinical trial of inhaled zanamivir treatment in children infected with influenza. *Antimicrob. Agents. Chemother.*, **57**, 1677-1684.