Neuraminidase Inhibitor Resistance after Oseltamivir Treatment of Acute Influenza A and B in Children

Iain Stephenson, Jane Democratis, Angie Lackenby, Teresa McNally, James Smith, Manish Pareek, Joanna Ellis, Alison Bermingham, Karl Nicholson, and Maria Zambon

Infectious Diseases Unit, Leicester Royal Infirmary, Leicester, and Health Protection Agency, Colindale, London, United Kingdom; and Pharmaceutical Division, Hoffman LaRoche, Basel, Switzerland

(See the editorial commentary by Monto on pages 397–9)

Background. Oseltamivir, a specific influenza neuraminidase inhibitor, is an effective treatment for seasonal influenza. Emergence of drug-resistant influenza viruses after treatment has been reported, particularly in children in Japan, where the dosing schedule is different from that used throughout the rest of the world. We investigated the emergence of drug-resistant infection in children treated with a tiered weight-based dosing regimen.

Methods. We analyzed sequential clinical nasopharyngeal samples, obtained before and after tiered weight-based oseltamivir therapy, from children with acute influenza during 2005–2007. We isolated viruses, tested for drug resistance with use of a fluorescence-based neuraminidase inhibition assay, performed neuraminidase gene sequencing, and determined quantitative viral loads.

Results. Sixty-four children (34 with influenza A subtype H3N2, 11 with influenza A subtype H1N1, and 19 with influenza B virus) aged 1–12 years (median age, 3 years, 1 month) were enrolled. By days 4–7 after initiation of treatment, of 64 samples tested, 47 (73.4%) and 26 (40.6%) had virus detectable by reverse-transcriptase polymerase chain reaction and culture, respectively. By days 8–12 after initiation of treatment, of 53 samples tested, 18 (33.9%) and 1 (1.8%) had virus detectable by reverse-transcriptase polymerase chain reaction and culture, respectively. We found no statistically significant differences in the reduction of viral shedding or time to clearance of virus between viral subtypes. Antiviral-resistant viruses were recovered from 3 (27.3%) of 11 children with influenza A subtype H1N1, 1 (2.9%) of 34 children with influenza A subtype H3N2, and 0 (0%) of 19 children with influenza B virus, all of whom were treated with oseltamivir (P = .004). There was no evidence of prolonged illness in children infected with drug-resistant virus.

Conclusions. Drug resistance emerges at a higher rate in influenza A subtype H1N1 virus than in influenza A subtype H3N2 or influenza B virus after tiered weight-based oseltamivir therapy. Virological surveillance for patterns of drug resistance is essential for determination of antiviral treatment strategies and for composition of pandemic preparedness stockpiles.

Influenza A and B viruses are responsible for seasonal outbreaks and, in the case of influenza A virus, infrequent pandemics. The M2 ion channel inhibitors amantadine and rimantadine are active against influenza A virus, but their clinical use is limited by inactivity to influenza B virus and the emergence of transmissible drug-resistant strains [1, 2]. The neuraminidase inhibitors oseltamivir and zanamivir were licensed for the treatment and prevention of seasonal influenza during 1999–2000 [1]. These drugs also possess the potential to reduce virus transmission and mortality during the next pandemic [3]. Preparedness plans emphasize early treatment of patients [4], and because oseltamivir is an oral agent suitable for bulk storage, the creation of stockpiles of the drug has been considered.

Large-scale clinical studies of oseltamivir have reported a low frequency of drug resistance of <0.5% and ~4% among posttreatment isolates recovered from adults and children, respectively [1, 5–7]. Smaller studies from Japan that involved oseltamivir treatment for children with influenza A subtype H1N1 and influenza A subtype H3N2 (hereafter referred to as influenza A/H1N1 and influenza A/H3N2) reported drug-resistance.
rates of 16% and 18% in posttreatment isolates, respectively [8, 9]. Persistent viral shedding has been observed in oseltamivir-treated children with influenza B [10, 11]. However, in Japan, oseltamivir is typically administered at a dosage of 2 mg/kg twice daily; this dosage leads to lower plasma drug levels in children, compared with the tiered weight-based dosing regimen that is used in the rest of the world [12]. Because children with influenza exhibit high viral shedding for longer periods than do adults, lower therapeutic drug levels and shortened duration of treatment may favor selection of drug-resistant viruses.

To investigate whether the use of tiered weight-based oseltamivir therapy for influenza A and B would suppress the emergence of drug resistance, we obtained serial nasopharyngeal specimens before and after treatment to isolate virus, to quantitate virus shedding, and to detect the presence of drug-resistant virus.

PATIENTS AND METHODS

Enrollment. Our study was conducted at Leicester Royal Infirmary Children’s Unit (Leicester, United Kingdom) from January through April during 2006 and 2007. Children aged 1–12 years who presented to the unit within 48 h after onset of febrile symptoms (generally, but not exclusively, fever [temperature, \( \geq 38^\circ C \)), convulsion, cough, and coryza) had a nasal swab sample obtained for rapid antigen testing for influenza virus (Roche Diagnostics). Children with samples positive for influenza virus were offered oseltamivir in pediatric suspension specimens before and after treatment to isolate virus, to quantify virus shedding, and to detect the presence of drug-resistant virus.

Virus isolation and shedding. We aimed to collect nasopharyngeal swab specimens from all children before treatment (day 0), on days 4–7 after the initiation of treatment, on days 8–12 after the initiation of treatment, and, during the second year of the study, on days 13–16 after the initiation of treatment. Clinical symptoms were recorded at each visit. In 2007, all household contacts of the child with influenza had 1 nasopharyngeal swab specimen obtained for rapid antigen testing for influenza virus (Roche Diagnostics). Children with samples positive for influenza virus were offered oseltamivir in pediatric suspension twice daily for 5 days according to the tiered weight-based dosing regimen (60 mg/day was given to children weighing <15 kg, 90 mg/day was given to children weighing 15–23 kg, 120 mg/day was given to children weighing 24–40 kg, and 150 mg/day was given to children weighing >40 kg) licensed by the UK Summary of Product Characteristics. Exclusion criteria were oseltamivir allergy, clinically significant immunosuppression, concomitant treatment requiring oseltamivir dose adjustment, or inability to obtain clinical samples.

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Patients. We obtained at least 2 clinical samples each from 72 children. Eight children were excluded: 2 had mixed influenza A and B infection, and 6 had no recoverable virus from any sample despite having a positive rapid antigen test result. Therefore, the study was composed of 64 children (patients 1–64) with virologically confirmed influenza. Thirty-five children in 2006 (5 children with influenza A/H3N2, 11 with influenza A/H1N1, and 19 with influenza B virus) and 29 in 2007 (all with influenza A/H3N2 virus) were evaluated (table 1). Participants were generally healthy, except for 1 1-year-old girl with myasthenia who had an uncomplicated recovery. The median age of the participants was 3 years, 1 month (range, 1–11 years, 3 months); 69% were aged <5 years. There were no clinically significant differences in demographic characteristics by virus subtypes, except that there were more Asian children in the influenza A/H1N1 group \((P = .009)\). Parents of 10 children (15.6%) reported only partial compliance with oseltamivir treatment; noncompliance was principally attributed to the unpleasant taste of the medication.

Virus isolation and 50% inhibitory concentrations (IC50). Madin-Darby Canine Kidney cells overexpressing \( \beta \)-galactoside-\( \alpha \),2,6-sialyltransferase I gene were used to isolate viruses from clinical samples. The IC50 of virus isolates to oseltamivir carboxylate (Roche) and zanamivir (GlaxoSmithKline) were determined using a fluorescence-based neuraminidase-inhibition assay [13]. The IC50 was determined on the basis of the drug concentration at which there was a 50% reduction in fluorescence; results were reported as the mean of duplicate values.

Influenza subtyping. Multiplex real-time RT-PCR was performed on nucleic acid extracted directly from clinical samples, which were eluted in a volume of 60 μLL, with use of Qiagen columns (Qiagen). Primers and probes targeting conserved regions of the HA gene of H1, H3, and B viruses were used. RT-PCR was performed with a 25-μL reaction mixture with 7.5 μL extracted template; 12.5 μL 2 × RT-PCR reaction mix (SuperScript III Platinum One-Step Quantitative RT-PCR kit; Invitrogen); 900 nmol/L each of H1 and H3 forward and reverse primers; 600 nmol each of B primer; 200 nmol/L H3, 50 nmol/L H1, and 250 nmol/L B TaqMan minor groove binder probes; 0.5 μL SuperScript III RT/Platinum Taq mix; and 0.1 μL ROX Reference Dye. Amplification, detection, and data analysis were performed on an ABI Prism 7500 real-time thermal cycler.

Viral shedding. Clinical samples in which viral RNA was detected were spiked prior to nucleic acid extraction with an exogenous synthetic RNA that acted as an internal control. Standard curves were prepared from A/NewCaledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004 viruses covering a range of viral infectivity of 7–8 log10 plaque-forming units/mL (125–1.25 × 10^8, 105–1.05 × 10^8, 2.8–2.8 × 10^8 plaque-forming units/mL, respectively). Viral loads were ascertained in samples by testing in triplicate and calculating a mean value.

Neuraminidase gene sequencing. Full-length N1 and N2
genes were amplified in 1 step directly from clinical specimens or virus isolates. DNA templates and sequencing primers were prepared for cycle sequencing (GATC). Pyrosequencing was used to detect and quantitate neuraminidase inhibitor–resistance mutations directly from clinical specimens [14]. PCR and biotin-labeled primers were designed from separate consensus sequences from each circulating influenza subtype during 2000–2006 that was generated from the Los Alamos database with use of the Biotage sequence design software (Biotage). The samples were pyrosequenced using the Pyrogold pyrosequencing kit. All primers were purchased from Eurofins (Ebersberg), and biotin-labeled primers were purified using high-performance liquid chromatography. Probes were obtained from Applied Biosystems. All primer and probe sequences and thermocycling conditions for all procedures are available on request.

Statistical methods. We used \( \chi^2 \) tests to compare noncontinuous variables and Fisher’s exact test when the number of expected observations was <5. If the variables were normally distributed, mean values were compared using Student’s \( t \) test for 2 groups or analysis of variance for 3 groups; if the variable were nonnormally distributed, Mann-Whitney \( U \) tests or Kruskal-Wallis tests were used. \( P < .05 \) was considered to be statistically significant (Stata software, version 10; Stata).

Written informed consent from parents was obtained. The study was approved by an independent ethics committee, University Hospitals Leicester, and the Medicines and Healthcare Products Regulatory Agency.

RESULTS

Virus detection before and after oseltamivir treatment. Influenza in sequential clinical samples, obtained before and after oseltamivir treatment, was detected both by RT-PCR for the hemagglutinin gene and virus isolation (figure 1). Among

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**Table 1. Baseline characteristics of children with influenza who received oseltamivir treatment.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Children with virologically confirmed influenza, by virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Influenza A/H1N1 ((n = 11))</td>
</tr>
<tr>
<td>Age</td>
<td>3.31 ± 0.97</td>
</tr>
<tr>
<td>Median years (range)</td>
<td>3.1 (2.1–5.16)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>3 (27)</td>
</tr>
<tr>
<td>Asian</td>
<td>8 (73)</td>
</tr>
<tr>
<td>Afro-Caribbean</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Male sex</td>
<td>4 (36)</td>
</tr>
<tr>
<td>Weight</td>
<td></td>
</tr>
<tr>
<td>Mean kg ± SD</td>
<td>14.5 ± 2.6</td>
</tr>
<tr>
<td>Median kg (range)</td>
<td>15.2 (9.8–18.1)</td>
</tr>
<tr>
<td>Previous influenza vaccination</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Treatment compliance(^a)</td>
<td>9 (82)</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
</tr>
<tr>
<td>Mean °C ± SD</td>
<td>38.2 ± 0.93</td>
</tr>
<tr>
<td>Median °C (range)</td>
<td>38.0 (37.1–40.1)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of patients, unless otherwise indicated.

\(^a\) Treatment received twice daily for 5 days.
of the 64 children who had viral RNA detected by RT-PCR, 58 (90.6%) had virus cultured in pretreatment samples. By days 4–7 (median day, 5) after the initiation of oseltamivir treatment, of the 64 children with samples tested, 47 (73.4%) had virus detectable by RT-PCR, and 26 (40.6%) had virus detectable by culture. By days 8–12 (median 9), of the 53 children with samples tested, 18 (33.9%) had virus detectable by RT-PCR, and 1 (1.9%) had virus detectable by culture. No statistically significant differences in detection rates by RT-PCR or culture between viral subtypes at any posttreatment visit were observed. In 2007, 23 (79.3%) of 29 children, all with influenza A/H3N2 virus, had samples obtained on days 13–16 (median day, 14), and all samples were negative for influenza virus by RT-PCR and culture.

**Determination of ICso of neuraminidase inhibitors.**

ICso of oseltamivir (and zanamivir [data not shown]) for all 58 pretreatment and 27 posttreatment virus isolates obtained were compared with those for 662 UK community-acquired influenza isolates from influenza seasons 2005–2006 and 2006–2007 (figure 2). No pretreatment study viruses exhibited reduced susceptibility to neuraminidase inhibitors; the mean ICso of oseltamivir was 1.5 nmol/L (95% CI, 1.2–1.8 nmol/L) for influenza A/H1N1, 0.4 nmol/L (95% CI, 0.4–0.5 nmol/L) for influenza A/H3N2, and 23.5 nmol/L (95% CI, 21.3–25.7 nmol/L) for influenza B virus. There was no statistically significant change in the mean ICso of oseltamivir for influenza B virus isolates recovered after treatment (mean oseltamivir ICso 22.3 nmol/L; 95% CI, 15.6–29.1 nmol/L). However, by days 5–6, virus isolates from 2 (18.2%) of 11 children with influenza A/H1N1 (patients 34 and 37) and 1 (2.9%) of 34 children with influenza A/H3N2 virus (patient 106) displayed reductions in oseltamivir susceptibility of >100-fold, compared with their parent viruses (figure 3 and table 2). In these patients, virus was undetectable by RT-PCR or culture on day 9, which indicated clearance of the drug-resistant virus. However, 1 additional influenza A/H1N1 virus, isolated on day 9 from a different child (patient 37), showed a >100-fold reduction in oseltamivir susceptibility. The influenza A/H3N2 isolate exhibited a >10-fold reduction in susceptibility to zanamivir, in contrast with the influenza A/H1N1 isolates, which retained zanamivir susceptibility. Parents indicated full compliance with treatment for every child from whom a drug-resistant virus was isolated. Overall, antiviral-resistant viruses were recovered from 3 (27.3%) of 11 children with influenza A/H1N1, 1 (2.9%) of 34 children with influenza A/H3N2, and 0 (0%) of 19 children with influenza B virus, all of whom were treated with oseltamivir ($P = .004$, for influenza A/H1N1 vs. influenza A/H3N2 virus).

**Neuraminidase sequencing.** Neuraminidase gene pyrosequencing was performed on the nucleic acid material extracted directly from clinical specimens; this identified the presence of mutations known to confer resistance to oseltamivir in isolates with reduced drug susceptibility: H274Y in N1 and R292K in N2. These mutations were confirmed by full sequence analysis of the neuraminidase genes of all viruses.

**Quantitative viral load and shedding.** Viral titers in sequential clinical samples were measured directly by quantitative PCR (figure 3). By days 4–7, 1 (9.1%) of 11 children with influenza A/H1N1, 2 (5.9%) of 34 children with influenza A/H3N2, and 0 (0) of 19 children with influenza B virus had increased viral titers (cultivable isolates were oseltamivir susceptible; $P = .68$); at this time, 3 (27.3%), 7 (20.6%), and 7 (36.8%) children, respectively, had cleared the infecting virus ($P = .58$). By days 8–12, the proportions of oseltamivir-treated children with influenza A/H1N1, influenza A/H3N2, or influenza B who cleared virus increased to 6 (60%) of 10, 18 (62.1%) of 29, and 11 (78.6%) of 14, respectively ($P = .89$). Three children with influenza A/H1N1 and 1 child with influenza B virus showed an increase in viral load by PCR during days 4–7 and 8–12; although no cultivable viruses were isolated, all 4 children had fully recovered and were asymptomatic at the time when samples were obtained.

**Figure 2.** Box plot of 50% inhibitory concentrations (IC50) of oseltamivir in influenza A/H1N1 (a), influenza A/H3N2 (b), and influenza B (c) viruses in our study, compared those for with UK community-acquired influenza virus isolates from influenza seasons 2005–2006 and 2006–2007 (mean values and 95% CIs are shown). Drug-resistant virus in patients 34, 37, and 46 (influenza A/H1N1) and patient 106 (influenza A/H3N2) are represented by stars. Drug-susceptible virus isolates are represented by black circles. The mean IC50 of UK isolates is represented by vertical black bars.
Oseltamivir Resistance in Influenza

Clinical symptoms. Children from whom antiviral-resistant viruses were recovered were treated as outpatients; they were afebrile by days 5–6 and fully recovered by days 8–10 (table 2). No prolonged or increased symptoms were seen in the children infected with antiviral-resistant viruses, compared with children from whom antiviral-susceptible viruses were recovered. The number of symptomatic children on days 4–7 who had antiviral-susceptible influenza A/H1N1, influenza A/H3N2, and influenza B virus were 5 (62.5%) of 8, 22 (66.7%) of 33, and 12 (63.2%) of 19, respectively. By days 8–12, the number of symptomatic children had decreased to 2 (25%) of 8, 8 (28.6%) of 28, and 0 (0%) of 14, respectively. Of 17 children with a detectable viral load on days 8–12, (4 with influenza A/H1N1, 10 with influenza A/H3N2, and 3 with influenza B virus), all were afebrile, and only 3 (17.6%; all with influenza A/H3N2 virus) reported minor symptoms. During the study, 3 children (4.7%) required intensive care for systemic sepsis or respiratory failure. All were infected with fully drug-susceptible influenza A/H3N2 viruses, had no bacterial organisms isolated, and fully recovered.

Household transmission. Nasal samples from 85 contacts (53 were adults aged ≥16 years) from 27 different households of oseltamivir-treated children with influenza A/H3N2 virus during the second year (2007) were obtained on days 5–16 (median day, 9), regardless of whether the contacts had symptoms. Fourteen samples (16%) were positive for influenza A/H3N2 virus by RT-PCR, and 6 (7.1%) were positive for influenza A/H3N2 virus by culture. No isolates showed reduced IC50 drug susceptibility, and neuraminidase gene pyrosequencing did not reveal any mutations known to be associated with antiviral resistance.

DISCUSSION

We evaluated drug resistance and viral shedding in children who received oseltamivir for acute influenza. Drug-resistant viruses were isolated from 4 children in the present study; 3 (27.3%) were infected with influenza A/H1N1 virus, 1 (2.9%) was infected with influenza A/H3N2 virus, and none were infected with influenza B virus. Although small numbers of drug-
resistant viruses were observed, these results have implications for management of seasonal influenza and the development of antiviral drug stockpiles.

High-level oseltamivir resistance in influenza A/H1N1 virus is caused principally by a single-point substitution of histidine by tyrosine at position 274 (H274Y) of the neuraminidase gene [5]. The frequency of oseltamivir resistance in oseltamivir-treated children with influenza A/H1N1 virus in our study was consistent with that in a previous report in Japan in which 7 (16.3%) of 43 treated children had recoverable oseltamivir-resistant influenza A/H1N1 virus [9]. Furthermore, oseltamivir-resistant H274Y strains were isolated from 2 (25.0%) of 8 oseltamivir-treated patients with influenza A subtype H5N1 virus in Vietnam in 2005 [15]. For these individuals, there was concern regarding the efficiency of oral absorption or delays in therapy being contributory to the emergence of drug resistance; there have been no subsequent reports, despite additional treated patients.

Our results for oseltamivir-treated children with influenza A/H3N2 and influenza B virus contrast with those of Japanese studies, in which 9 (18.0%) of 50 treated children with influenza A/H3N2 virus were reported to have drug-resistant virus [8]; prolonged virus shedding with reduced clinical effectiveness in young children with influenza B was also reported [10, 11]. We did not observe any differences in viral shedding or time to clearance of virus between viral subtypes. In Japan, oseltamivir is generally prescribed at a dosage of 2 mg/kg twice daily, rather than on the basis of tiered weight-based dosing, which is recommended elsewhere. Pharmacokinetic studies have revealed that Japanese dosing results in ~20% lower plasma levels of oseltamivir carboxylate (the active drug), compared with tiered weight-based dosing, particularly in children aged <5 years [12]. Young children, often with a first influenza virus infection, typically shed higher virus titers for a longer duration than do adults; this phenomenon is likely to be representative of pandemic influenza. Relative underdosing of oseltamivir may contribute to posttreatment resistance to N2 and B viruses seen in Japan. Although antiviral therapy compliance during a pandemic should be strongly encouraged, because suboptimal dosing (e.g., premature treatment withdrawal after symptom resolution, as often occurs with antibiotics) may favor posttreatment drug resistance; it is notable that patients with drug-resistant viruses were fully compliant to treatment in our study.

Influenza neuraminidase is essential for release and spread of new virions from infected cells. Nine subtypes of influenza A neuraminidase (N1–N9) virus are identified in nature; they are classified into groups on the basis of structural characteristics, as follows: group 1 (N1, N4, N5, and N8), group 2 (N2, N3, N6, N7, and N9), and B neuraminidase [16, 17]. The N1 and N2 groups differ with regard to the structure of a pocket adjacent to the active site of the neuraminidase enzyme. Current inhibitors were designed from structural analysis of the N2 subtype, with the aim of activity against all influenza A and B neuraminidases [18]. Oseltamivir binds to the active site pocket, but the presence of H274Y in group 1 (N1) but not group 2 (N2) neuraminidase disrupts the binding site and reduces drug susceptibility [16]. Other single amino acid changes in or around the active site, principally R292K or E119V in N2 and R152K or D198N in B neuraminidase, also reduce virus susceptibility to oseltamivir [19, 20]. Zanamivir binding is generally unaffected by conformational changes conferred by H274Y; therefore, oseltamivir-resistant N1 strains remain susceptible to zanamivir. However, cross-resistance with the N2 R292K mutation, as was observed in our study, is recognized [19, 20].

As in previous reports involving children from whose samples posttreatment drug-resistant viruses were isolated, we found no evidence of a prolonged illness [8–11]. The 2 patients infected with H274Y-carrying influenza A/H1N1 viruses who continued to have detectable virus shedding after day 7 were both asymptomatic. Cultivable virus was detected in a sample from only 1 of these patients; this indicated clearance of viable virus from 75% of children in our study who developed in-
fection with drug-resistant strains. The ability of neuraminidase inhibitor–resistant viruses to infect and transmit in animal models varies on the basis of the mutation. The replicative fitness of some clinical isolates of H274Y-carrying influenza A/H1N1 virus appear to be compromised, and other isolates and recombinant H274Y-carrying influenza A/H5N1 viruses remain as pathogenic and transmissible as wild-type strains, which suggests that background genes also determine fitness [21–23].

After the introduction of neuraminidase inhibitors to clinical practice, the Neuraminidase Inhibitor Susceptibility Network was established to monitor global influenza isolates. Before 2007, annual surveillance identified low levels of naturally occurring neuraminidase inhibitor–resistance of <1% among community-acquired influenza A and B virus strains [24–27]. Of note, in Japan, where is the highest frequency of use of prescribed oseltamivir per capita, no accumulation of drug resistance in community-acquired influenza A and B virus strains [24–27]. Isolates were identified in Europe, North America, and Asia; the proportions of oseltamivir-resistant isolates were ~10% in the United States, the United Kingdom, and Hong Kong; ~20% in Canada and Germany; and ~70% in Norway. These proportions were in contrast with that observed in Japan (~2%). The source of these H274Y-carrying influenza A/H1N1 viruses with high-level resistance to oseltamivir [27, 28]. Isolates were identified in Europe, North America, and Asia; the proportions of oseltamivir-resistant isolates were ~10% in the United States, the United Kingdom, and Hong Kong; ~20% in Canada and Germany; and ~70% in Norway. These proportions were in contrast with that observed in Japan (~2%). The source of these H274Y-carrying influenza A/H1N1 viruses with high-level resistance to oseltamivir [27, 28]. Isolates were identified in Europe, North America, and Asia; the proportions of oseltamivir-resistant isolates were ~10% in the United States, the United Kingdom, and Hong Kong; ~20% in Canada and Germany; and ~70% in Norway. These proportions were in contrast with that observed in Japan (~2%). The source of these H274Y-carrying influenza A/H1N1 viruses with high-level resistance to oseltamivir [27, 28].

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