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Detection of Influenza Viruses Resistant to Neuraminidase Inhibitors in Global Surveillance during the First 3 Years of Their Use

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Emergence of influenza viruses with reduced susceptibility to neuraminidase inhibitors (NAIs) develops at a low level following drug treatment, and person-to-person transmission of resistant virus has not been recognized to date. The Neuraminidase Inhibitor Susceptibility Network (NISN) was established to follow susceptibility of isolates and occurrence of NAI resistance at a population level in various parts of the world. Isolates from the WHO influenza collaborating centers were screened for susceptibilities to oseltamivir and zanamivir by a chemiluminescent enzyme inhibition assay, and those considered potentially resistant were analyzed by sequence analysis of the neuraminidase genes. During the first 3 years of NAI use (1999 to 2002), 2,287 isolates were tested. Among them, eight (0.33%) viruses had a >10-fold decrease in susceptibility to oseltamivir, one (0.22%) in 1999 to 2000, three (0.36%) in 2000 to 2001, and four (0.41%) in 2001 to 2002. Six had unique changes in the neuraminidase gene compared to neuraminidases of the same subtype in the influenza sequence database. Although only one of the mutations had previously been recognized in persons receiving NAIs, none were from patients who were known to have received the drugs. During the 3 years preceding NAI use, no resistant variants were detected among 1,054 viruses. Drug use was relatively stable during the period, except for an approximate 10-fold increase in oseltamivir use in Japan during the third year. The frequency of variants with decreased sensitivity to the NAIs did not increase significantly during this period, but continued surveillance is required, especially in regions with higher NAI use.

The impact of annual influenza outbreaks on morbidity and mortality, especially for populations not previously considered at high risk, has received considerable attention in recent years (14, 24). Prevention and treatment have become an increasing priority in many regions of the world (3). At the same time, the occurrence in humans of avian influenza, particularly type A (H5N1), has sparked fear of a potential pandemic (25). While vaccines remain the major public health strategy for prevention, antivirals could play a particularly important role in response to the early phases of a pandemic, if available in sufficient quantities (5, 22, 32). This is especially true of the neuraminidase inhibitors (NAIs) zanamivir and oseltamivir, which have advantages over the M2 protein inhibitors (adamantanes), amantadine and rimantadine, with respect to antiviral spectrum, efficacy, and resistance patterns. This is especially the case since type A (H5N1) viruses resistant to the M2 inhibitors appeared in 2003 and 2004 (7, 31).

Strains resistant to the M2 inhibitors regularly emerge during treatment of individuals infected with initially susceptible viruses. These resistant strains are transmissible and able to cause disease (9, 19). Resistance to oseltamivir is less frequent than resistance to the M2 inhibitors during therapeutic use, especially in adults. Resistance to zanamivir has been detected only in a virus recovered from an immunocompromised child (7, 8, 30). Still, there is a possibility that, with increasing use of the NAIs, resistance could emerge and prevent their effective use in the future. The Neuraminidase Inhibitor Susceptibility Network (NISN) was established in 1999 to monitor this possibility and to address public health and regulatory concerns about antiviral resistance to NAIs (34). The results of analyzing over 1,000 clinical specimens from the 3 years (1996 to 1999) before introduction of the NAIs anywhere in the world have been reported previously and confirmed the general lack of primary resistance to this class of antivirals (20). We here report on the frequency of detection of viruses with decreased

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susceptibility to the NAIs from 2,287 isolates similarly collected globally during the 3 years after the licensure of zanamivir and oseltamivir.

MATERIALS AND METHODS

Viruses tested. To assure global representation of isolates, viruses from the four WHO collaborating centers responsible for the collection and analysis of human influenza isolates (located in Atlanta, Georgia; London, United Kingdom; Melbourne, Australia; and Tokyo, Japan) were evaluated. The isolates were selected from samples routinely submitted to the centers and not from clinical studies of antiviral use. They were chosen by the centers to represent the types and subtypes circulating in a particular season. The viruses were isolated during three postlicensure periods, 1 September 1999 to 31 August 2000 (year 1), 1 September 2000 to 31 August 2001 (year 2), and 1 September 2001 to 31 August 2002 (year 3). The break at 1 September was chosen to include the transmission season in the southern hemisphere at the end of each northern hemisphere influenza year. All regions were represented throughout the 3 years. However, given the higher usage pattern of NAIs in Japan, the number of isolates from that country was overweighted in year 3.

Clinical isolates of documented NAI-resistant viruses and their corresponding wild-type parental strains were provided by Margaret Tisdale (GlaxoSmithKline, Stevenage, United Kingdom) and Noel Roberts (Roche Products Ltd., Welwyn Garden City, United Kingdom). To represent type B viruses, a B/Memphis/20/96 isolate with an R152K neuraminidase (NA) mutation selected during treatment of an immunocompromised child with zanamivir was included (8). For type A viruses, an A/Texas/36/91 (H1N1) isolate with an H274Y NA mutation, an A/Wuhan/359/95-like N2 isolate with an E119V NA mutation, and an A/Sydney/5/97-like A (H3N2) isolate with an R292K NA mutation selected during oseltamivir treatment were used (15).

All cultures were grown in phenol red-free Eagle minimum essential medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO) as well as 1 mM L-glutamine, 1% HEPES, and 1% penicillin-streptomycin (all from Gibco). Medium for growth of viruses was Eagle minimum essential medium supplemented with 0.14% bovine serum albumin fraction V and 2.5 μg of tosylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Biochemical Co., Lakewood, NJ) per ml.

Inhibitors. Zanamivir was provided by Margaret Tisdale (GlaxoSmithKline). Oseltamivir carboxylate (GS4071), the active compound of the ethyl ester prodrug oseltamivir phosphate (GS4104), was supplied by Noel Roberts (Roche Products Ltd.).

NA inhibition assays. Susceptibility to the inhibitors was determined by a previously described chemiluminescent enzyme inhibition assay, using NA-Star (Tropix, Bedford, Mass.) A 1,2-dioxetane derivative of 100 μM sialic acid was used as the substrate (1). The assay was performed at final drug concentrations ranging from 0.028 to 550 nM. Assays were carried out by ViroMed, Minneapolis, MN. The 50% mean inhibitory concentrations (IC_{50}s) were calculated by using Robosage Microsoft Excel software for curve fitting and a process previously described (20).

NAI use. Data on the use of the NAIs during the 3 years were obtained from the manufacturers. The data were available on a global basis and separately from Japan and the United States, the countries with the greatest use. The annual reports of use, starting in October and ending in September, are for each year under study.

Statistical analysis. Values from the susceptibility assay were not normally distributed, which necessitated log transformation for analysis. Statistics are also presented on the original scale. Box-and-whisker plots were used initially to identify extreme IC_{50} values. The box contains 50% of the results, representing the middle two quartiles (25% to 75%). The length of the box represents the interquartile range (IQR). The "whiskers" extend to the largest and smallest values in the central part of the distribution before the region containing outliers is reached. Two types of outliers are defined, the mild (between 1.5 and 3.0 IQR from the 25th and 75th percentiles) and the extreme (more than 3.0 IQR from the 25th and 75th percentiles). If the data followed a normal distribution, about 7 in 1,000 observations would be outliers of one type or another and 2 in 10^6 would be extreme outliers (17). All known resistant variants are extreme outliers, with an IC_{50} value greater than 10-fold that of the corresponding parent virus. In addition, all outliers were examined to determine whether their IC_{50} value was 10-fold or greater than that of the mean for the virus subtype for the relevant year.

NA sequencing. The NAs of all extreme outliers and approximately 80% of the remaining outliers in the upper tails of the IC_{50} distribution were selected for sequencing. Sequencing was carried out at the Health Protection Agency, London, United Kingdom, the WHO laboratory in Tokyo, or the WHO or CSIRO laboratories in Melbourne. Amino acid numbering is based on the N2 sequence throughout. Sequences were initially aligned against both current and earlier NAs of the same subtype in the influenza sequence database. Those showing unique variations were also aligned against the other influenza A subtype or B NAs. This allowed us to identify novel mutations that were distinct from the drift mutations seen between isolates.

RESULTS

Global use of the neuraminidase inhibitors. Following its licensure in some countries in 1999, use of zanamivir has either continued at a low level or declined. Figure 1 shows the changing use of oseltamivir which, outside of use in Japan, has varied little during the 3-year period. During year 2, when it was introduced in that country, 300,000 5-day courses of therapy were distributed. This increased to 2,290,000 courses in year 3, far exceeding use anywhere else in the world.

Viruses tested. A total of 2,287 viruses were evaluated. Table 1 shows the distribution of these isolates by season, type, and subtype. This distribution reflects their global prevalence during each season. Overall, most of the viruses studied were type A (H3N2), with type B and then A (H1N1) following. The total number evaluated per year rose from 465 in 1999 and 2000 to 980 in 2001 and 2002. In the first year, 58% of specimens came from North America, 20% from Asia, and 22% from the rest of the world. In the second year, the distribution was 50%, 30%, and 20%, and in the third year, 22%, 41%, and 37%, respectively. This reflected deliberate oversampling from Japan, the country with the greatest NAI use.

Susceptibility to the neuraminidase inhibitors. Mean IC_{50} values for oseltamivir carboxylate and zanamivir by year and region are shown in Tables 2 and 3, respectively. While there was some variability from year to year, there was no trend toward increasing values. In fact, for type B and oseltamivir carboxylate, the overall values went down in the third year, when the largest number of viruses was studied. Relative susceptibility to the inhibitors was consistent with previously published results (20), with the A/N1 and B viruses being more sensitive to zanamivir and the A/N2 viruses being more sensitive to oseltamivir.

Figures 2 to 4 show the plots of the log-transformed IC_{50} values for zanamivir and oseltamivir carboxylate for the 3 years of study of isolates possessing A/N1, A/N2 (H1N2 and H3N2), and B neuraminidases. For those viruses identified statistically as outliers, further analysis was undertaken to understand how their susceptibility compared to susceptibilities of known resistant isolates. Results are presented in Table 4 for the viruses used as known resistant standards and the eight viruses identified as having a >10-fold decrease in susceptibility to oseltamivir. Two of these viruses were also >10-fold less sensitive to zanamivir. Overall, the number of extreme outliers with IC_{50} values elevated greater than 10-fold from the mean of the subtype appeared to be relatively stable from year to year: 1 of 465 viruses tested (0.22%) in the first year, 3 of 842 (0.36%) in year 2, and 4 of 980 (0.41%) in year 3.

For A/N1 and oseltamivir, two extreme outliers were detected in year 2 and one in year 3 (Fig. 2 and Table 4). Two of these were also extreme outliers to zanamivir. For A/N2-con-

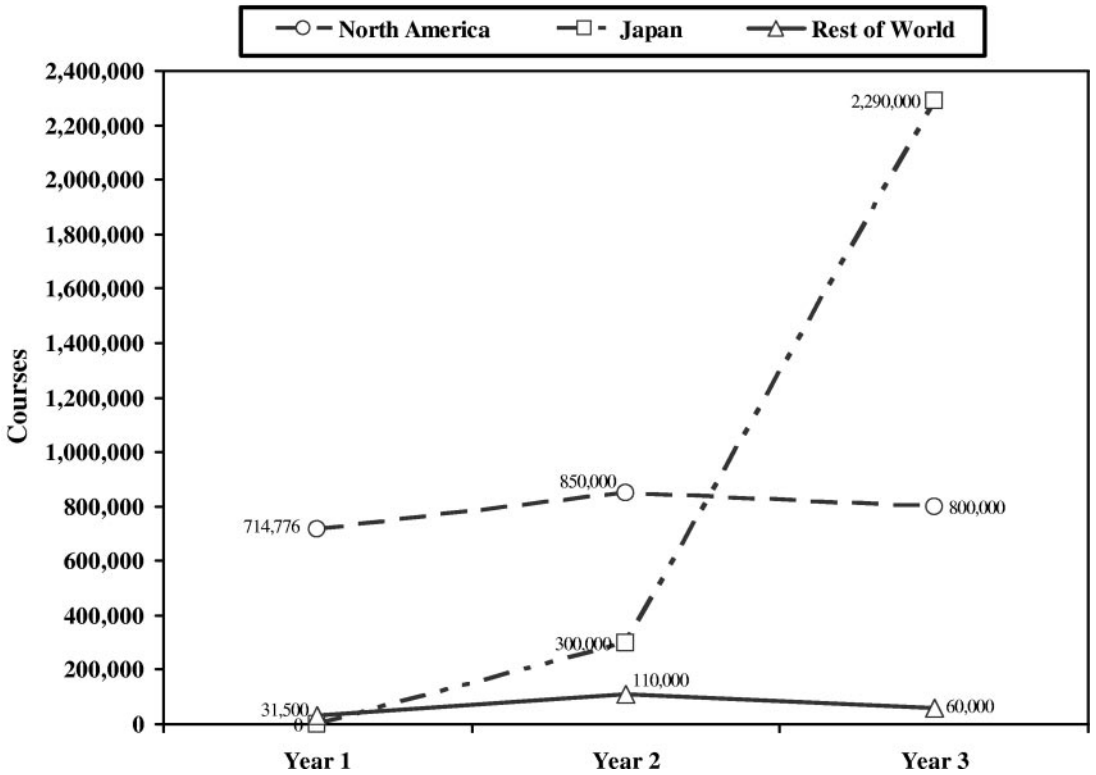

FIG. 1. Estimated numbers of 5-day treatment courses of oseltamivir administered in 1999 to 2000, 2000 to 2001, and 2001 to 2002 (October to September) in various regions of the world.

taining isolates, there was one extreme outlier in year 1 and two in year 3 to oseltamivir (Fig. 3 and Table 4). Although, statistically, only mild outliers to zanamivir were identified in any year for influenza B isolates (Fig. 3), there was one virus in year 3 with a >10-fold decrease in susceptibility to oseltamivir (B/Perth/211/2001). A second virus (B/New York/39/2001) also demonstrated a >10-fold reduction in susceptibility to oseltamivir on initial screening but on repeat testing was found to be a mild outlier to both drugs.

NA sequence analysis. The NA sequences of a total of 63 outlier viruses collected over the 3 years were determined. This included all viruses identified as extreme outliers with the required reduction in IC_{50} (Table 4). No history of antiviral use could be obtained in association with collection of any original clinical specimens which yielded these viruses. Only one of them, A/Mississippi/3/2001, had an NA mutation previously recognized by use of clinical studies to confer specific resistance to oseltamivir, an H274Y mutation (21). Among the other A/N1 viruses, A/New York/24/2001 does not appear to have any NA sequence changes identified with known mutations. Although each of the two sequence changes in its NA (G248R and I266V) are seen in other circulating strains, no circulating virus has both changes. A/Hokkaido/15/2002 was found to contain a mixture of sensitive and resistant populations, with a unique Y155H mutation found in the resistant population. Among the A/N2 viruses, the Q226H change seen in A/Belgium/969/2002 and the E41G change in A/Greece/110/2000 are sequence changes which do not appear to be present in circulating viruses. Neither mutation has previously been associated with altered susceptibility to the NA inhibitors. The NA from A/Denmark/25/2002 had no apparent variations compared to the consensus sequence for the corresponding subtype or to sequences for a wide selection of natural isolates available from the WHO collaborating center in London.

Although initial sequencing failed to detect any changes in the B/Perth/211/2001 virus (12), it was subsequently found to contain a mixed population of sensitive and resistant viruses. A novel D198E mutation (D197 B numbering) was found in the resistant population. Sequencing of B/New York/39/2001 revealed an NA I222T mutation. While this variation is seen in several H1N1 NAs, this has not been seen in any B NA. Examination of the sequences of mild outliers indicated that an H1N1 virus, A/Hammamatsu/92/2002, also had a mutation at the same site, but an I222V mutation, which is unique among N1 NAs.

TABLE 1. Numbers of isolates evaluated during the three study years

Yr	No. (%) of isolates of type (subtype)				
	A (H1N1)	A (H1N2)	A (H3N2)	B	Total
1999–2000	54 (12)	1 (0.2)	373 (80)	37 (8)	465
2000–2001	402 (48)	0	100 (12)	340 (40)	842
2001–2002	166 (17)	39 (4)	409 (42)	366 (37)	980
All yr	622 (27)	40 (2)	882 (39)	743 (32)	2,287

TABLE 2. Analysis of IC_{50}s against oseltamivir carboxylate based on influenza type, subtype, and region

Region	Mean IC_{50} (nM)[a] (no. of isolates) (95% confidence limit)								
	N1			N2			B		
	Yr 1	Yr 2	Yr 3	Yr 1	Yr 2	Yr 3	Yr 1	Yr 2	Yr 3
Asia	0.73 (n = 28) (0.61, 0.87)	0.49 (n = 88) (0.42, 0.57)	0.55 (n = 126) (0.49, 0.62)	0.48 (n = 46) (0.41, 0.56)	0.34 (n = 54) (0.28, 0.41)	0.37 (n = 115) (0.34, 0.42)	7.05 (n = 18) (4.72, 10.55)	9.07 (n = 109) (7.93, 10.38)	2.58 (n = 160) (2.31, 2.88)
Australia and Oceania	0.62 (n = 14) (0.54, 0.71)	0.36 (n = 65) (0.31, 0.41)	0.52 (n = 11) (0.40, 0.68)	0.36 (n = 19) (0.29, 0.45)	0.32 (n = 28) (0.25, 0.41)	0.24 (n = 121) (0.22, 0.25)	4.38 (n = 14) (2.55, 7.50)	6.66 (n = 27) (5.97, 9.61)	3.45 (n = 73) (2.85, 4.17)
Africa				0.31 (n = 1) (0.31, 0.31)	0.28 (n = 4) (0.18, 0.42)	0.75 (n = 9) (0.47, 1.20)	9.18 (n = 1) (9.18, 9.18)	5.25 (n = 3) (1.74, 15.84)	2.73 (n = 6) (1.15, 6.45)
Europe	0.46 (n = 9) (0.31, 0.68)	0.41 (n = 20) (0.31, 0.53)	0.60 (n = 27) (0.53, 0.69)	0.54 (n = 43) (0.45, 0.65)	0.45 (n = 6) (0.31, 0.65)	0.56 (n = 74) (0.48, 0.64)	14.33 (n = 4) (12.31, 16.68)	3.91 (n = 18) (2.91, 5.24)	3.51 (n = 44) (2.56, 4.81)
North and South America[b]	0.65 (n = 3) (0.35, 1.21)	0.53 (n = 229) (0.47, 0.58)	0.81 (n = 2) (0.54, 1.22)	0.42 (n = 265) (0.39, 0.45)	0.40 (n = 8) (0.24, 0.65)	0.34 (n = 129) (0.32, 0.36)		7.24 (n = 183) (6.68, 7.83)	4.77 (n = 83) (4.22, 5.39)
Total	0.64 (n = 54) (0.57, 0.73)	0.48 (n = 402) (0.45, 0.52)	0.56 (n = 166) (0.51, 0.62)	0.43 (n = 374) (0.41, 0.46)	0.34 (n = 100) (0.30, 0.39)	0.35 (n = 448) (0.33, 0.37)	6.40 (n = 37) (4.73, 8.67)	7.54 (n = 340) (7.04, 8.07)	3.26 (n = 366) (3.01, 3.54)

[a] Original scale (not log transformed).
[b] One type B isolate from year 2 was from South America.

DISCUSSION

NISN was established to monitor antiviral susceptibility and the potential public health impact of clinical use of the NAIs (34). To establish a baseline level of susceptibility of circulating wild-type strains and the incidence of primary occurrence of resistance during the 3 years before introduction of the drugs, 1,054 viruses representing the 1996 to 1999 influenza seasons were studied for susceptibility to zanamivir and oseltamivir; no extreme outliers were identified (20). Furthermore, none of the sequenced mild-outlier viruses was found to contain NA substitutions known to confer resistance (20). The present study used similar methods applied to viruses collected during the first 3-year period of increasing use of the drugs in certain parts of the world following licensure. A significant decrease in susceptibility (>10-fold) to the NAIs, particularly oseltamivir, was detected in eight viruses during screening by chemiluminescence assay of 2,287 unselected clinical isolates, none of which were obtained from individuals known to have had treatment with NAIs. These isolates included those collected from Japan and the United States, the two countries with greatest per capita NAI use during these years. Such observations are consistent with findings that selection of oseltamivir- or zanamivir-resistant mutants is generally slow in the laboratory and that they are often less infectious and transmissible in experimental animal models (2, 10, 13).

On sequencing, the NA of only one virus, A/Mississippi/3/2001 (H1N1), had an H274Y mutation previously associated with resistance after oseltamivir treatment (13). All other viruses had changes not previously seen in isolates from clinical studies or had no sequence changes from the consensus sequence of circulating wild-type strains. Both B/Perth/211/2001 and A/Hokkaido/15/2002 had mixed populations of resistant and sensitive viruses, thus confirming the role of their mutations in resistance. The B/Perth/211/2001 virus had a D198E (D197 B numbering) NA mutation. A mutation at this residue, the D198N mutation, has also been seen in an influenza B virus isolated from an immunocompromised patient treated with oseltamivir (6). Structurally, residue 198 is adjacent to R152 in the enzyme active site. An R152K mutation is known to confer resistance to both zanamivir and oseltamivir in influenza B NAs (6). Hence, it is plausible that the D198E mutation may

TABLE 3. Analysis of IC_{50}s against zanamivir based on influenza type, subtype, and region

Region	Mean IC_{50} (nM)[a] (no. of isolates) (95% confidence limit)								
	N1			N2			B		
	Yr 1	Yr 2	Yr 3	Yr 1	Yr 2	Yr 3	Yr 1	Yr 2	Yr 3
Asia	0.55 (n = 28) (0.47, 0.63)	0.43 (n = 88) (0.38, 0.47)	0.25 (n = 126) (0.22, 0.28)	1.72 (n = 46) (1.53, 1.93)	1.06 (n = 54) (0.84, 1.35)	0.95 (n = 115) (0.86, 1.05)	2.16 (n = 18) (1.73, 2.68)	2.19 (n = 109) (1.90, 2.52)	1.03 (n = 160) (0.96, 1.11)
Australia and Oceania	0.56 (n = 14) (0.48, 0.64)	0.41 (n = 65) (0.36, 0.46)	0.39 (n = 11) (0.29, 0.50)	1.40 (n = 19) (1.07, 1.83)	1.95 (n = 28) (1.47, 2.59)	1.50 (n = 121) (1.36, 1.64)	1.48 (n = 14) (1.15, 1.91)	2.55 (n = 27) (2.06, 3.15)	0.85 (n = 73) (0.74, 0.98)
Africa				1.20 (n = 1) (1.20, 1.20)	0.53 (n = 4) (0.24, 1.19)	1.30 (n = 9) (0.91, 1.86)	2.09 (n = 1) (2.09, 2.09)	2.49 (n = 3) (2.17, 2.86)	2.09 (n = 6) (1.29, 3.38)
Europe	0.55 (n = 9) (0.44, 0.68)	0.33 (n = 20) (0.29, 0.38)	0.32 (n = 27) (0.28, 0.37)	1.30 (n = 43) (1.07, 1.58)	1.41 (n = 6) (0.85, 2.32)	1.46 (n = 74) (1.28, 1.66)	1.86 (n = 4) (0.84, 4.13)	1.68 (n = 18) (1.40, 2.02)	2.07 (n = 44) (1.63, 2.61)
North and South America[b]	0.37 (n = 3) (0.31, 0.46)	0.47 (n = 229) (0.44, 0.51)	0.39 (n = 2) (0.39, 0.39)	1.58 (n = 265) (1.46, 1.71)	2.51 (n = 8) (1.48, 4.26)	1.22 (n = 129) (1.13, 1.31)		3.04 (n = 183) (2.79, 3.31)	1.35 (n = 83) (1.21, 1.51)
Total	0.54 (n = 54) (0.49, 0.59)	0.44 (n = 402) (0.42, 0.47)	0.27 (n = 166) (0.24, 0.29)	1.55 (n = 374) (1.45, 1.66)	1.34 (n = 100) (1.12, 1.59)	1.25 (n = 448) (119, 1.31)	1.84 (n = 37) (1.55, 2.18)	2.61 (n = 340) (2.43, 2.80)	1.16 (n = 366) (1.09, 1.24)

[a] Original scale (not log transformed).
[b] One type B isolate from year 2 was from South America.

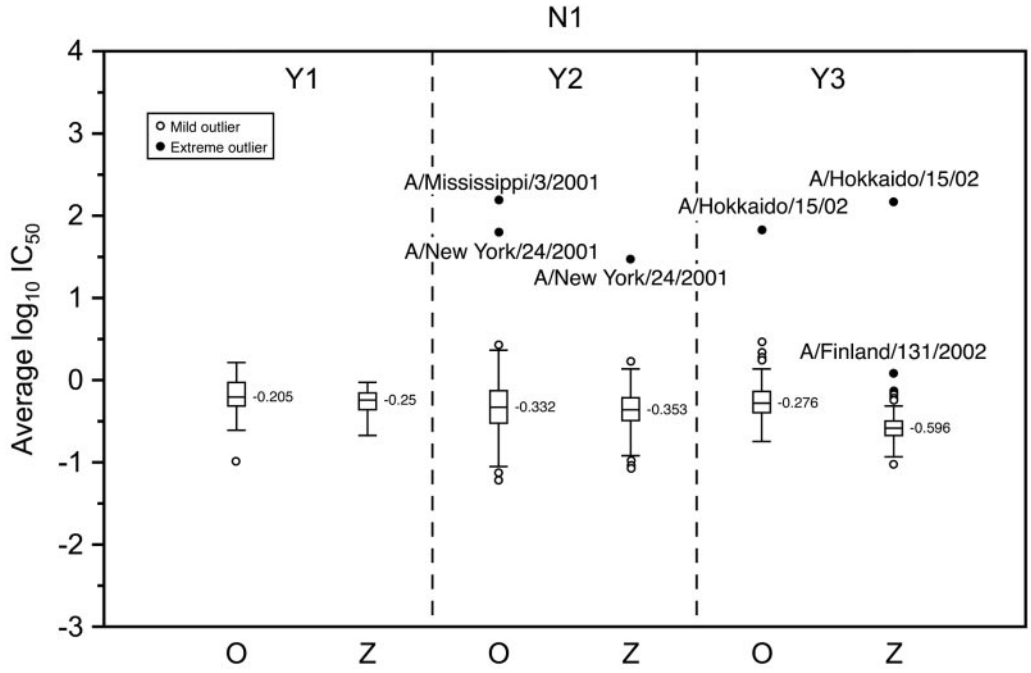

FIG. 2. Box plots of IC_{50} values (nM), log transformed, for zanamivir (Z) and oseltamivir carboxylate (O) for A/N1 viruses during years 1, 2, and 3 (Y1, Y2, and Y3). Boxes represent the 25th to 75th percentiles, and horizontal lines within the boxes represent the median values.

disrupt the interaction of R152 in the active site, leading to altered binding of the inhibitors.

Although the Y155H mutation confers resistance in the A/Hokkaido/15/2002 virus and Y155 is conserved in all human N1 viruses, H155 is also found in some swine and avian N1 viruses and in some earlier N2 viruses. In fact, the NA of reassortant A/NWS/Tokyo/67 (H1N2), on which the design of zanamivir was based, has H155 yet is clearly sensitive to the inhibitors (27). Interestingly, the NAs of the highly pathogenic avian influenza viruses circulating in Asia also have H155 and

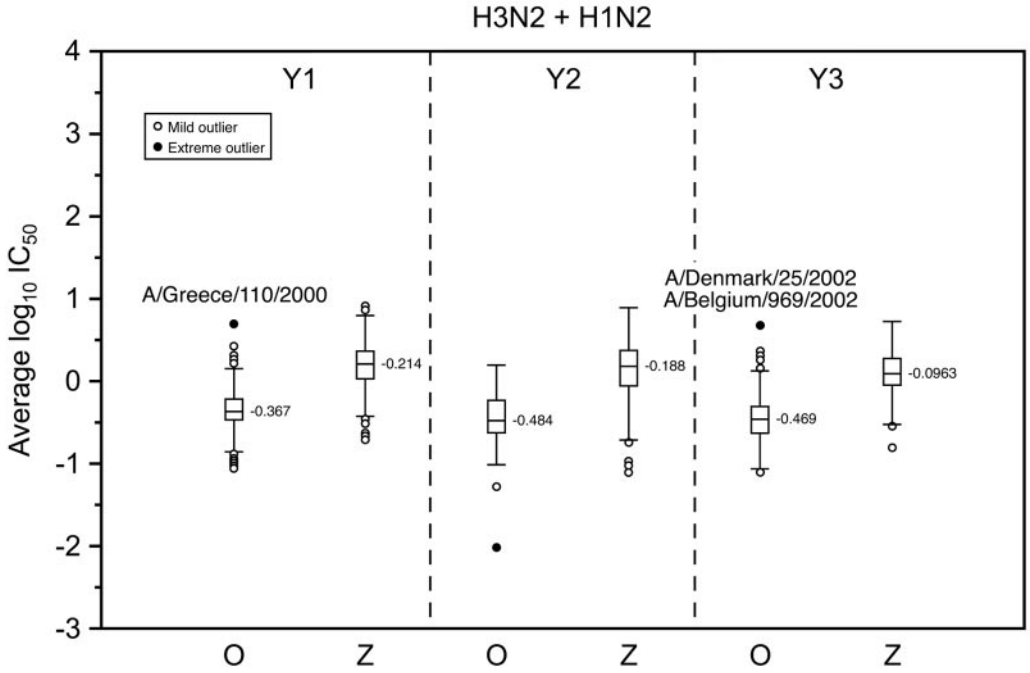

FIG. 3. Box plots of IC_{50} values (nM), log transformed, for zanamivir (Z) and oseltamivir carboxylate (O) for A/N2 viruses during years 1, 2, and 3 (Y1, Y2, and Y3). Boxes represent the 25th to 75th percentiles, and horizontal lines within the boxes represent the median values.

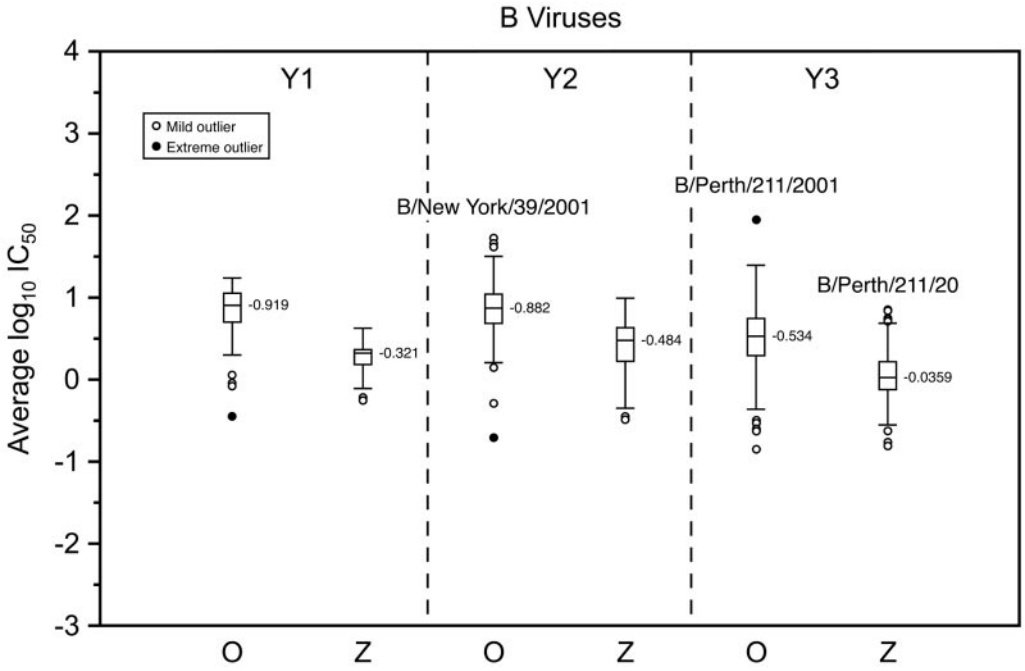

FIG. 4. Box plots of IC_{50} values (nM), log transformed, for zanamivir (Z) and oseltamivir carboxylate (O) for B viruses during years 1, 2, and 3 (Y1, Y2, and Y3). Boxes represent the 25th to 75th percentiles, and horizontal lines within the boxes represent the median values.

yet are also sensitive to the inhibitors (18). Such a finding reinforces the conclusion from earlier work that resistance motifs are likely to vary between different influenza NA subtypes and even within subtypes.

Three viruses with decreased susceptibility to oseltamivir had mutations in the NA in the highly conserved sequence ILRTQES (residues 222 to 228): B/New York/39/2001 had an I222T mutation, A/Hamamatsu/92/2002 had an I222V mutation, and A/Belgium/969/2002 had a Q226H mutation. A role for these mutations in altered binding of the NAIs is supported by the findings of others where viruses with I222T and I222V NA mutations were generated in N1 and N2 viruses, respectively, after passage in oseltamivir in vitro (Gilead Sciences, data on file). These led to two- and fourfold decreases in sensitivity, respectively. I222 is located in the substrate binding pocket, near the *N*-acetyl and glycerol side chains of the ligand. It is also near R224, which is important in the formation of a salt link to E276, which creates the hydrophobic pocket necessary to accommodate the side chain of oseltamivir (26). While Q226 is below the active site, it is also located under

TABLE 4. Sensitivities of H1N1, H3N2, H1N2, and B extreme outliers to both inhibitors

Virus subtype and strain	Sequence change(s)	Oseltamivir IC_{50} (nM)[a]		Fold difference	Zanamivir IC_{50} (nM)[a]		Fold difference
		Wild type	Variant		Wild type	Variant	
H1N1 outliers							
Reference strain A/Texas/36/91[b]	H274Y	0.4	253.9	632	0.75	0.7	1
A/Mississippi/3/2001	H274Y	0.48	**157**	327	0.44	0.47	1
A/New York/24/2001	G248R I266V	0.48	**64**	133	0.44	**30**	68
A/Hokkaido/15/2002	Y155H	0.56	**69**	123	0.27	**150**	555
H3N2/H1N2 outliers							
Reference strain A/Wuhan/359/95[b]	E119V	0.3	15.6	52	0.7	1.3	2
Reference strain A/Sydney/5/97[b]	R292K	0.4	3,877	9,692	1.8	6.7	3.7
A/Greece/110/2000	E41G	0.43	**5.07**	11.7	1.55	1.67	1.1
A/Denmark/25/2002	Drift only	0.35	**4.91**	14.0	1.26	1.76	1.39
A/Belgium/969/2002	Q226H	0.35	**4.74**	13.5	1.26	0.96	0.76
B outliers							
Reference strain B/Memphis/20/96[b]	R152K	6.7	509.2	76	3.5	33.7	9.6
B/New York/39/2001[c]	I222T	7.44	**100** (55)	13.4 (7.3)	2.6	**17** (6)	6.5 (2.3)
B/Perth/211/2001	D198E	3.26	**90**	26.3	1.16	**7.36**	6.7

[a] Wild-type values are the means for virus type/subtype and drug for the same year. Boldface type indicates the value on which outlier status was based.
[b] Values for reference strains were taken from reference 29.
[c] Results for B/New York/39/2001 in parentheses indicate results from repeat testing.

R224. Therefore, both I222 and Q226 may play an important structural role, being needed for the proper orientation of R224 for oseltamivir binding. Hence, mutations at these sites could impact oseltamivir sensitivity.

The A/New York/24/2001 NA differed by two amino acids from NAs of other viruses currently circulating, G248R and I266V. Other viruses in the database have either one of these variations but are sensitive to the inhibitors. Molecular modeling shows that these amino acids are more than 20 Å away from each other. Hence, it is unclear how these variations could have a synergistic effect. However, the role of at least the G248R mutation in altered susceptibility cannot be discounted, since it is adjacent to H274 and an H274Y mutation confers oseltamivir-specific resistance.

Overall, the present results also indicate that resistance may involve amino acid substitutions not previously observed in clinical trials. However, further detailed characterization of plaque progeny of the other extreme-outlier viruses is needed to confirm the role of potential mutations in altered drug sensitivity, and this emphasizes the current level of uncertainty in linking phenotypic data with genotypic information when screening unselected isolates for altered susceptibility to NAIs.

Evidence suggests that the viruses with decreased NAI susceptibility in vitro detected in the current study came from untreated individuals; several were from parts of the world with little use of NAIs. Therefore, a low level of naturally occurring resistant variants may be present. Transmission from an NAI-treated patient remains a possibility especially for the isolate with the known oseltamivir-resistant H274Y mutation. However, recent studies of ferrets indicate that the most commonly observed mutant viruses from clinical trials are less infectious and also may transmit less readily than their wild-type parents (10, 11). This lack of fitness in some but not all of the known resistant viruses is in sharp contrast to the transmissible nature of viruses resistant to the M2 inhibitors (9, 19).

Evaluation of resistance has been an integral part of the prelicensure and other studies of the NAIs. Resistance was not encountered in clinical trials of zanamivir, and the only mutant resistant to that drug was identified in a virus recovered from a highly immunosuppressed child (8). Variants resistant to oseltamivir have only rarely (<0.5%) been detected in placebo-controlled treatment studies of immunocompetent adults but have been detected in published studies of treatment of children up to a frequency of 4% (28, 30). This probably reflects the higher level of replication and more prolonged shedding of virus in children experiencing a primary or second infection. Recently, a higher frequency (18%) of detection of oseltamivir-resistant viruses from young children has been reported, when highly sensitive detection techniques were employed (16). In contrast, similar techniques have identified amantadine-resistant variants in 80% of treated children (23).

In much of the world, the NAIs, particularly zanamivir, were little used during this survey. They were introduced into Japan, the country with the current greatest per capita use, only in 2000 to 2001. Questions about the extent of selective pressure on virus evolution towards altered drug susceptibility can be answered only by continued monitoring of viruses obtained mainly from surveillance in parts of the world where usage of the drugs has increased versus parts of the world where it has not. A clear answer has become even more critical with the discovery that the recent type A (H5N1) avian viruses, a potential source of the next pandemic strain, are susceptible only to the NAIs; preliminary data indicating the detection of reduced oseltamivir susceptibility in an H5N1 isolate due to the recognized H274Y substitution recovered from treated human cases of H5N1 emphasize the importance of this work (4, 33). Future NISN work will concentrate on surveillance for drug resistance in clinical isolates of influenza obtained in areas of the world with high drug usage and attempt to better understand the relationship between phenotype and genotype with respect to altered susceptibility to NAIs.

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