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Global update on the susceptibilities of human influenza viruses to neuraminidase inhibitors and the cap-dependent endonuclease inhibitor baloxavir, 2017–2018

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ABSTRACT

The global analysis of neuraminidase inhibitor (NAI) susceptibility of influenza viruses has been conducted since the 2012–13 period. In 2018 a novel cap-dependent endonuclease inhibitor, baloxavir, that targets polymerase acidic subunit (PA) was approved for the treatment of influenza virus infection in Japan and the United States. For this annual report, the susceptibilities of influenza viruses to NAIs and baloxavir were analyzed.

A total of 15409 viruses, collected by World Health Organization (WHO) recognized National Influenza Centers and other laboratories between May 2017 and May 2018, were assessed for phenotypic NAI susceptibility by five WHO Collaborating Centers (CCs). The 50% inhibitory concentration (IC_{50}) was determined for oseltamivir, zanamivir, peramivir and laninamivir. Reduced inhibition (RI) or highly reduced inhibition (HRI) by one or more NAIs was exhibited by 0.8% of viruses tested (n = 122). The frequency of viruses with RI or HRI has remained low since this global analysis began (2012–13: 0.6%; 2013–14: 1.9%; 2014–15: 0.5%; 2015–16: 0.8%; 2016–17: 0.2%). PA gene sequence data, available from public databases (n = 13523), were screened for amino acid substitutions associated with reduced susceptibility to baloxavir (PA E23G/K/R, PA A36V, PA A37T, PA I38F/M/T/L, PA E119D, PA E199G): 11 (0.08%) viruses possessed such substitutions. Five of them were included in phenotypic baloxavir susceptibility analysis by two WHO CCs and IC_{50} values were determined. The PA variant viruses showed 6–17-fold reduced susceptibility to baloxavir. Overall, in the 2017–18 period the frequency of circulating influenza viruses with reduced susceptibility to NAIs or baloxavir was low, but continued monitoring is important.

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1. Introduction

Global influenza surveillance has been conducted through the World Health Organization (WHO) Global Influenza Surveillance and Response System (GISRS) since 1952. Overall, GISRS laboratories process three million or more clinical specimens for detection of influenza viruses each year and isolate influenza viruses for antigenic and antiviral susceptibility characterization. Neuraminidase (NA) inhibitors (NAIs) are the most widely used antiviral drugs for the treatment or prophylaxis of influenza. The WHO GISRS Expert Working Group for Surveillance of Antiviral Susceptibility (WHO-AVWG) has conducted global surveillance to determine the NAI susceptibility of influenza viruses since the 2012–13 period (Gubareva et al., 2017; Hurt et al., 2016; Lackenby et al., 2018; Meijer et al., 2014; Takashita et al., 2015). To standardize interpretation and reporting of susceptibility of influenza viruses to NAIs (oseltamivir, zanamivir, peramivir and laninamivir), criteria were defined by the WHO-AVWG using 50% inhibitory concentration (IC_{50}) fold-change thresholds, compared to the median for viruses of the same type, subtype and lineage showing normal inhibition (NI) (WHO, 2012). Those showing reduced inhibition (RI) are influenza A viruses that have a 10- to 100-fold increase in IC_{50}, or influenza B viruses with a 5- to 50-fold increase in IC_{50}. Viruses showing highly reduced inhibition (HRI) are influenza A viruses with > 100-fold increase in IC_{50} or influenza B viruses with > 50- fold increase in IC_{50}. Since the first global analysis of 2012–13 data, the frequency of influenza viruses with RI or HRI by one or more NAIs has remained low (2012–13: 0.6% (Meijer et al., 2014); 2013–14: 1.9% (Takashita et al., 2015); 2014–15: 0.5% (Hurt et al., 2016); 2015–16: 0.8% (Gubareva et al., 2017); 2016–17: 0.2% (Lackenby et al., 2018)).

The cap-dependent endonuclease inhibitor baloxavir marboxil was approved on 23 February 2018 in Japan and on 24 October 2018 in the United States for the treatment of acute uncomplicated influenza in patients who have been symptomatic for no more than 48 h (Gubareva et al., 2019; Takashita et al., 2018). In Phase II and III clinical trials, I38T, I38F and I38M amino acid substitutions in the polymerase acidic subunit (PA) were detected in A(H1N1)pdm09 and A(H3N2) influenza viruses from baloxavir-treated patients (Hayden et al., 2018; Hirotsu et al., 2019; Omoto et al., 2018; Uehara et al., 2020). Some patients treated with baloxavir, in whom PA variant viruses emerged, exhibited prolonged virus shedding, rebound in virus titers and a delay in symptom alleviation (Hayden et al., 2018; Hirotsu et al., 2019; Uehara et al., 2020). Evidence suggests that the detection of the PA I38T substitution can be considered a laboratory correlate of clinically relevant baloxavir resistance (Gubareva and Fry, 2020). Furthermore, A(H1N1)pdm09 and A(H3N2) viruses with the PA I38T substitution isolated from baloxavir-treated patients showed replicative capacities and pathogenicity similar to those of wild-type isolates in hamsters and transmitted efficiently between ferrets by respiratory droplets (Imai et al., 2020). Since global surveillance of baloxavir resistance is essential, we have initiated global analysis of influenza viruses circulating during the reported period for baloxavir susceptibility.

This is the 6th WHO-AVWG annual review of antiviral susceptibility data generated by four WHO Collaborating Centers for Reference and Research on Influenza and one WHO Collaborating Centre for the Surveillance Epidemiology and Control of Influenza (WHO CCs). The report includes sequence data analysis of NA and PA genes in two public sequence repositories, the Global Initiative on Sharing All Influenza Data (GISAID; www.GISAID.org) and National Centre for Biotechnology Information Influenza Virus resource (NCBI-IVR; www.ncbi.nlm.nih.gov/genomes/FLU/), submitted by the WHO CCs, National Influenza Centers (NICs) and other laboratories.

2. Neuraminidase inhibitors (NAI)

2.1. Overall analysis of phenotypic NAI susceptibility data from WHO CCs

NICs receive and characterize influenza virus-positive clinical specimens in their respective countries. Representative numbers of virus isolates and/or clinical specimens by type, subtype and lineage are forwarded to at least one WHO CC for further characterization, according to the WHO terms of reference and referral guidance for NICs (www.who.int/influenza/gisrs_laboratory/national_influenza_centres/en/). The referral guidance criteria are actioned differently by NICs, dependent on a variety of conditions including but not limited to the local influenza season severity and timing, national testing capability and the level of use of NAIs in the country. Once received at a WHO CC, virus isolation and propagation is performed in MDCK and/or MDCK-SIAT1 cells prior to NAI susceptibility testing. The five WHO CCs perform phenotypic NAI susceptibility analysis on all influenza viruses received or isolated. The fluorescence-based NA inhibition assay with MUNANA (4-(methylumbelliferyl)-N-acetylneuraminic acid) substrate is used for the phenotypic analysis, but with minor modifications in each laboratory. When possible, sequence analysis by Sanger or Next Generation Sequencing (NGS) of paired clinical specimen and virus isolate is performed when a RI/HRI phenotype is identified.

Viruses isolated from specimens collected between week 21/2017 (22 May 2017) and week 20/2018 (20 May 2018) are included in this analysis of phenotypic NAI susceptibility (Fig. 1A). A total of 15409 viruses from 147 countries were tested for susceptibility to oseltamivir and zanamivir by five WHO CCs, while three WHO CCs (Atlanta, United States; Melbourne, Australia; Tokyo, Japan) also tested viruses for susceptibility to peramivir (n = 10084; 65%) and laninamivir (n = 10077; 65%) (Fig. 1B). The majority of isolates tested originate from the WHO regions of Western Pacific (WPRO: 50%), Americas (PAHO: 30%) and Europe (EURO: 12%) (Fig. 1B). Only 8% of viruses tested originate from the Eastern Mediterranean (EMRO: 3%), African (AFRO: 3%) and South-East Asia (SEARO: 2%) regions. FluNet is a global web-based tool for influenza virologic surveillance owned by WHO (https://www.who.int/influenza/gisrs_laboratory/flunet/en/). The virologic data entered into FluNet are critical for tracking the movement of viruses globally and interpreting epidemiologic data. In total, 842143 influenza virus detections, globally, were reported to FluNet in the timeframe of this antiviral analysis. The viruses analyzed for phenotypic NAI susceptibility by the five WHO CCs in this study represent 1.8% of virus detections globally (1.9% of influenza A viruses, 1.7% of influenza B viruses). The proportion of viruses detected globally, that were tested for phenotypic NAI susceptibility in this study varied by WHO region (SEARO: 2.9%; EMRO: 1.8%; AFRO: 6.4%; EURO: 0.7%; PAHO: 1.2%; WPRO: 4.9%). Influenza A(H3N2) viruses prevailed over this time period (5591; 36%) followed by B/Yamagata-lineage (4466; 29%), A(H1N1)pdm09 (3937; 26%) and B/Victoria-lineage (1415; 9%) viruses (Fig. 2A).

2.2. A(H1N1)pdm09 viruses showing RI or HRI

Of the 3937 A(H1N1)pdm09 viruses tested, 58 (1.5%) exhibited RI or HRI by one or more NAIs (Fig. 2B; Fig. 3A). The majority of viruses exhibiting RI/HRI contained the NA H275Y amino acid substitution (n = 48) (Table 1). Seven of the 48 H275Y variants harboured a mixed population of H275Y variant virus and H275 wild type virus and showed 24- to 506-fold higher oseltamivir IC_{50} values and 8.6- to 136-fold higher peramivir IC_{50} values, depending on the proportion of the H275Y variant in the mixed population (Table S1). Viruses with pure H275Y substitution exhibited HRI by oseltamivir (260–2078 fold-increase in IC_{50}) and peramivir (172–495 fold-increase in IC_{50}). These H275Y variants were isolated from 11 countries; Australia (1), China (2), France (1), India (1), Japan (24), Kuwait (1), Mexico (1), Russian Federation (3), Spain (1), United Arab Emirates (2) and United States (11) (Table S1). In 37 of the 48 cases, the H275Y substitution was confirmed in corresponding clinical specimens, the remaining 11 cases did not have clinical specimens available (Table 1; Table S1). In the 30 cases with data available, there were 23 outpatients and 7 hospitalized patients, of which one patient was immuno-compromised (Table 1;

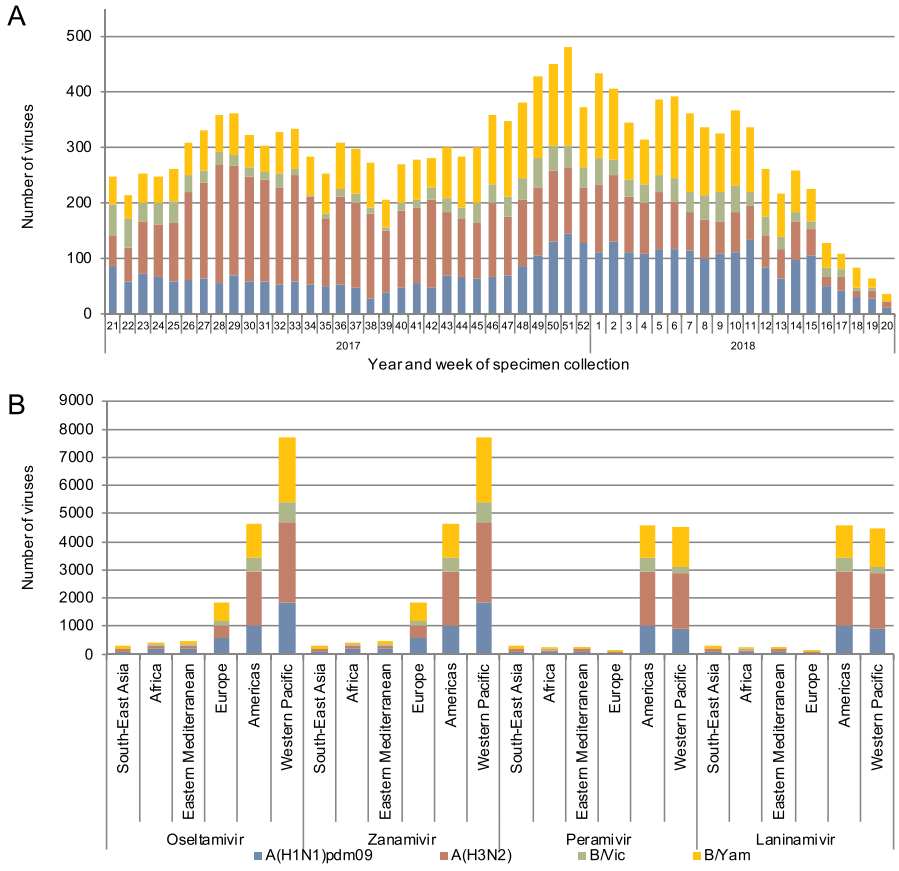


Fig. 1. Influenza viruses collected and tested for phenotypic neuraminidase inhibitor (NAI) susceptibility during 2017–2018. A) Week of specimen collection and virus type/subtype/lineage; for specimens tested, peaks in specimen collection during the Southern Hemisphere winter and during the Northern Hemisphere winter were observed. B) Number of viruses tested for phenotypic susceptibility to the four NAIs by World Health Organization region.

Table S1). Treatment history was available for 30 of the 48 H275Y cases; 7 had received oseltamivir treatment, one had received peramivir treatment, one had received laninamivir treatment, one had received oseltamivir and peramivir treatment, one had received peramivir and laninamivir treatment, while 14 from Japan and 5 from the United States had not received any NAIs prior to specimen collection (Table 1; Table S1).

Data pertaining to other A(H1N1)pdm09 virus variants is presented in Tables 1 and S1. NA I223R and NA I223T variants exhibiting RI by oseltamivir were isolated from hospitalized patients but their treatment histories were unknown. A virus with I223R substitution, isolated from an outpatient who had not received NAI treatment, showed RI by oseltamivir and borderline RI by zanamivir. NA Q136R and NA Q136K substitutions, which typically occur during propagation of influenza viruses in cell culture (Little et al., 2015), were detected in three viruses; Q136R substitution was not detected in the corresponding clinical specimens. One virus, with NA D199Y substitution detected in both clinical specimen and virus isolate, exhibited RI by oseltamivir and zanamivir. NA T148A and NA I427T variants showed borderline RI by zanamivir or RI by oseltamivir and zanamivir and borderline RI by laninamivir, respectively, but patient information was unavailable.

2.3. A(H3N2) viruses showing RI or HRI

Of the 5591 A(H3N2) viruses tested, 21 (0.4%) exhibited RI or HRI by one or more NAIs (Fig. 2B; Fig. 3B). One virus with NA R292K substitution was isolated from a hospitalized patient in Australia and showed remarkable HRI, 7735-fold increase in IC_{50}, by oseltamivir and RI by peramivir (Table 1; Table S1). Data pertaining to other A(H3N2) virus variants is presented in Tables 1 and S1. One virus with NA E119V substitution showed HRI by oseltamivir but had no effect on susceptibility to the other NAIs. Another virus, containing NA E119V/E and NA R292K/R polymorphisms and a 4-amino acid deletion (Del 245–248), showed HRI by oseltamivir and RI by zanamivir. This variant came from an immuno-compromised patient who had received oseltamivir treatment. NA S333G and NA S334R substitutions conferred RI by oseltamivir and zanamivir or RI by oseltamivir, respectively. However, other S333G and S334R variants showed NI by four NAIs (Table S3); these variants had IC_{50} values that were close to the intersect between NI and RI categories. One virus with NA Q136K/Q polymorphism, with Q136K typically emerging during propagation of influenza viruses in cell culture (Little et al., 2015), was detected in a hospitalized patient. NA G320R, NA N329R and NA N342K substitutions exhibiting RI by oseltamivir and/or zanamivir were detected in both clinical specimens and corresponding virus isolates but patient treatment histories were unavailable. Other NA substitutions (V143M, D161N, V303I, V313A, S315R, N342K and D463N) were detected in viruses with borderline RI by one or two NAIs but patient information was unavailable.

2.4. B/Victoria-lineage viruses showing RI or HRI

Of the 1415 B/Victoria-lineage viruses tested, 16 (1.1%) exhibited RI or HRI by one or more NAIs (Fig. 2B; Fig. 3C). Six viruses contained NA D197N substitution; four showed RI by zanamivir, one showed RI by zanamivir and borderline RI by peramivir and one showed borderline RI by oseltamivir (Table 2; Table S2). In our previous reports, the D197N substitution conferred NI or borderline RI by oseltamivir (3.6–4.7-fold) and zanamivir (5.0-fold) but phenotypic NAI susceptibility for peramivir and laninamivir has not been analyzed (Lackenby et al., 2018; Takashita et al., 2015). The D197N substitution was detected in the clinical specimen from an outpatient who had not received NAI treatment but patient information for the other five was unavailable. These D197N variants were isolated from four countries; Japan (1), Madagascar (2), Ukraine (2) and United States (1) (Table S2). NA

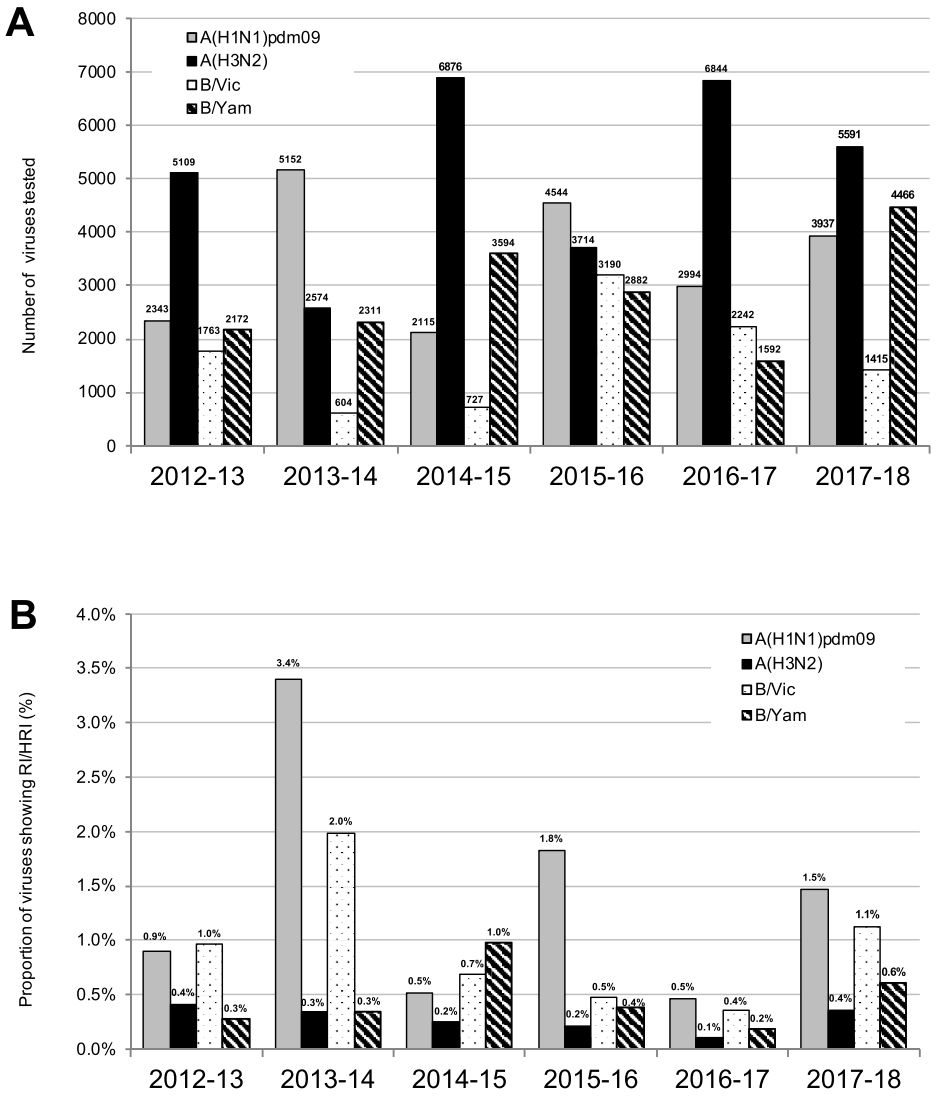


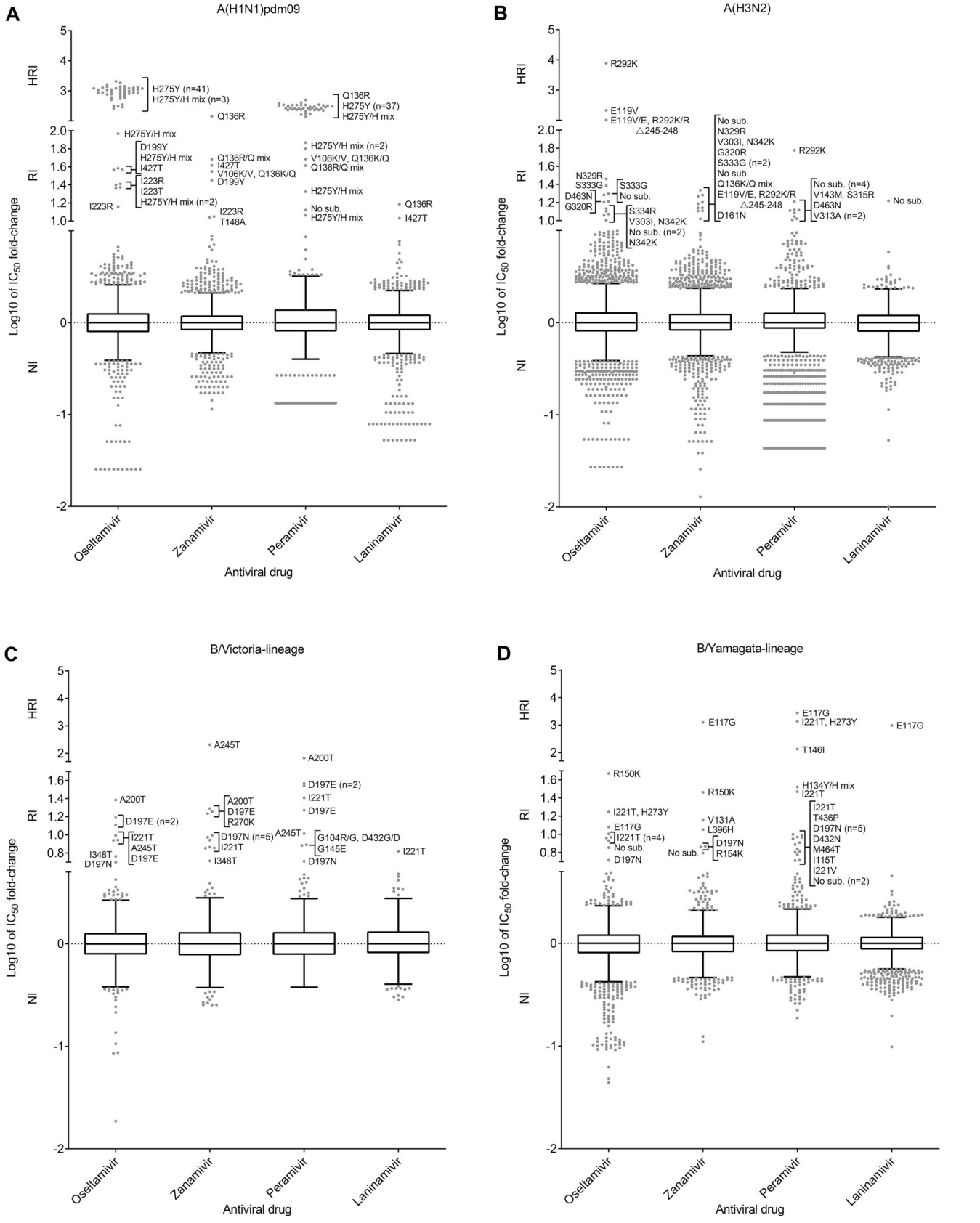
Fig. 2. Comparison of neuraminidase inhibitor (NAI) susceptibility surveillance over six seasons. A) Number of viruses tested in neuraminidase inhibition assays over the 2012–2018 period. B) Proportion of viruses showing reduced inhibition (RI) or highly reduced inhibition (HRI) by NAIs over the 2012–2018 period. Data compiled from the global studies reporting on viruses isolated during 2012-13 (Meijer et al., 2014), 2013–14 (Takashita et al., 2015), 2014–15 (Hurt et al., 2016), 2015–16 (Gubareva et al., 2017), 2016–17 (Lackenby et al., 2018) and 2017–18 (current study).

D197E substitution was detected in three viruses; two showed RI by oseltamivir and peramivir and one showed RI by oseltamivir, zanamivir and peramivir (Table 2; Table S2). These are the first D197E substitutions detected in viruses of the B/Victoria lineage. The three viruses were collected over a 1-month period from two children and one young adult residing in the same state and county of the United States. The three patients had not received antiviral treatment prior to specimen collection; they had fever for several days, but were not hospitalized. The genomes of all three viruses shared a high degree of similarity. Taken together, these data indicate possible local circulation of B/Victoria-lineage viruses displaying RI by NAIs.

Single virus variants, carrying a range of NA amino acid substitutions and showing RI/HRI by one or more NAIs, were detected (Table 2; Table S2): NA I221T in an immuno-compromised patient without NAI treatment showed RI by four NAIs; NA A200T showed HRI by peramivir and RI by oseltamivir and zanamivir with the substitution being detected in the clinical specimen; NA A245T showed HRI by zanamivir and RI by oseltamivir and peramivir but patient information was unavailable; NA I348T substitution was detected in a hospitalized patient and showed borderline RI by oseltamivir and zanamivir; NA G145E showed RI by peramivir and the substitution was detected in the clinical specimen. Two further variants displaying two polymorphisms (NA G104R/G and NA D432G/D) or NA R270K substitution showed RI by peramivir and zanamivir, respectively, and polymorphism/substitution was not detected in the clinical specimens.

2.5. B/Yamagata-lineage viruses showing RI or HRI

Of 4466 B/Yamagata-lineage viruses tested, 27 (0.6%) showed RI or HRI by one or more NAIs (Fig. 2B; Fig. 3D). Virologic and patient details are shown in Tables 2 and S2. Six viruses contained NA D197N substitution; four showed RI by peramivir, one showed borderline RI by oseltamivir and RI by peramivir and one showed RI by zanamivir. The D197N substitution was detected in available clinical specimens from four patients. These D197N variants were isolated from three countries; Japan (1), Russian Federation (2) and United States (3). However, two additional D197N variants isolated from Japan and the United States showed NI by all four NAIs (Table S3). In our previous reports, the



(caption on next page)

Fig. 3. Column-scatter plots of log-transformed 50% inhibitory concentration (IC_{50}) fold-change values. Data are presented by virus subtype or lineage [A) A(H1N1) pdm09; B) A(H3N2); C) B/Victoria-lineage; and D) B/Yamagata-lineage] and neuraminidase (NA) inhibitor (NAI) (labelled on the X-axis: oseltamivir, zanamivir, peramivir, laninamivir). The boxes indicate the 25–75 percentile and the whiskers stretch to the lowest and highest values within 1.5 times of the interquartile region (IQR) value from both the 25 and 75 percentile values respectively (Tukey's definition). The Y-axes have been split into 3 compartments according to the thresholds recommended by the World Health Organization Global Influenza Surveillance and Response System Expert Working Group for Surveillance of Antiviral Susceptibility (WHO-AVWG) for normal inhibition (NI) (type A viruses < 10-fold; type B viruses < 5-fold), reduced inhibition (RI) (type A viruses 10- to 100-fold; type B viruses 5- to 50-fold), and highly reduced inhibition (HRI) (type A viruses > 100-fold; type B viruses > 50-fold). NA amino acid substitutions are shown for viruses displaying RI or HRI that have been sequenced. Amino acid position numbering is A subtype- and B lineage-specific. The vast majority of viruses were tested for susceptibility to oseltamivir and zanamivir but only a subset was tested against peramivir and laninamivir. Viruses tested by the one concentration qualitative NA inhibition test are not included in these plots. No sub. indicates 'No amino acid substitution identified explaining the RI or HRI phenotype'.

D197N substitution conferred NI or RI by oseltamivir (4.4–11-fold), zanamivir (2.2–32-fold), peramivir (5.4–29-fold) and laninamivir (1.6–9.0-fold) (Gubareva et al., 2017; Hurt et al., 2016; Lackenby et al., 2018; Takashita et al., 2015). A previous study reported that the IC_{50} variation in the D197N variant was highly dependent on assay conditions such as the choice of buffer in the fluorescence-based NA inhibition assay (Hurt et al., 2004). NA I221T substitution variants were detected in four patients; two patients were hospitalized, one was an outpatient without NAI treatment and information on the fourth patient was unavailable. One virus containing dual substitution (NA I221T and NA H273Y) showed remarkable HRI, 1360-fold increase in IC_{50}, by peramivir and RI by oseltamivir was isolated from a patient treated with oseltamivir in the United States. Both I221T and H273Y substitutions were detected in the clinical specimen. One virus with NA E117G substitution, showing HRI with high fold increases in IC_{50} values for zanamivir (1250-fold), peramivir (2778-fold) and laninamivir (971-fold), and RI by oseltamivir, was isolated from a patient in Canada but patient information was unavailable. Individual viruses carrying single NA amino acid substitution or polymorphism (I115T, V131A, H134Y/H, T146I, R150K, R154K, I221V, L396H, D432N, T436P or M464T) were detected with all but one, NA T146I showing HRI by peramivir, having RI by oseltamivir, zanamivir or peramivir. The H134Y, T146I and T436P substitutions were not detected in corresponding clinical specimens, while the I115T, I221V, D432N and M464T substitutions were. The H134Y/H, R154K, I221V and M464T variants were isolated from patients that had not received NAI treatment.

2.6. Frequency of NA amino acid substitutions associated with RI/HRI by NAIs in sequence databases

We analyzed NA sequences from viruses collected between weeks 21/2017 and 20/2018 that had been deposited in either GISAID or NCBI-IVR databases. Deduplication by strain name was performed, with preference given to sequence from the original clinical specimen, where available. The WHO-AVWG has published a summary table of NA amino acid substitutions associated with reduced susceptibility to NAIs (www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/NAI_Reduced_Susceptibility_Marker_Table_WHO.pdf). Sequences were screened for these amino acid substitutions but cell culture-selected substitutions, such as NA E119K, NA Q136X and NA D151X for A(H1N1)pdm09, were excluded.

A total of 21992 sequences were analyzed, 11050 (50.2%) of which were from viruses not phenotypically tested by WHO CCs. Screening of the additional NA sequences (2547 A(H1N1)pdm09, 4529 A(H3N2), 563 B/Victoria-lineage and 3411 B/Yamagata-lineage) identified a further 32 (0.3%) viruses with NA substitutions associated with HRI (Table S4). Of the 2547 A(H1N1)pdm09 sequences, 23 (0.9%) contained NA H275Y substitution, associated with HRI by oseltamivir and peramivir. One of the 23 H275Y variants possessed NA H275Y and NA I223R dual substitutions. H275Y/I223R variant viruses, showing HRI with very high increases in oseltamivir and peramivir IC_{50} values and RI by zanamivir and laninamivir, have been detected previously from patients treated with oseltamivir or peramivir (Nguyen et al., 2010; Takashita et al., 2015). Of the 4529 A(H3N2) sequences, 5 (0.1%) contained NA substitutions associated with HRI; one had NA E119V, one had NA E119V and a 4-amino acid deletion (Del 245–248), two had 4-amino acid deletion (Del 245–248) and one had NA R292K/R polymorphism. A(H3N2) viruses with E119V substitution, E119V substitution and a 4-amino acid deletion (Del 245–248) or R292K substitution were analyzed for phenotypic NAI susceptibility in this study and showed HRI by oseltamivir (Table 1, Table S1). Four-amino acid deletion (Del 245–248) variants detected previously showed HRI by oseltamivir (Abed et al., 2009; Tamura et al., 2015). A further 50 of the 4529 A(H3N2) sequences contained NA substitutions associated with HRI or RI but the viruses showed NI by all four NAIs (Table S3); many of these substitutions are in the 331–334 region of NA, notably NA S331R, with some variants carrying this substitution having IC_{50} values that were close to the intersect between NI and RI categories (Lackenby et al., 2018; Takashita et al., 2015). Similar positioning of variants with NA S333G or NA S334R are seen here (Fig. 3B). No NA substitutions associated with HRI were detected among 563 B/Victoria-lineage sequences. Four (0.1%) of the 3411 B/Yamagata-lineage NA sequences had substitutions associated with HRI; one each had NA E105K/E polymorphism, NA I221T and NA H273Y, NA H273Y or NA R374K, respectively (Table S4). The E105K substitution has been detected previously and showed HRI by peramivir (Fujisaki et al., 2012). A I221T with H273Y variant was analyzed for phenotypic NAI susceptibility in this study and showed HRI by peramivir and RI by oseltamivir (Table 2; Table S2). Both I221T with H273Y variants were isolated from Indiana, United States (Table S2; Table S4). B/Yamagata-lineage viruses with H273Y substitution were detected in our global surveillance and showed HRI by peramivir and RI by oseltamivir (Gubareva et al., 2017). The R374K substitution has been detected previously and showed HRI by oseltamivir, zanamivir and peramivir while laninamivir susceptibility was not reported (Burnham et al., 2014).

3. Cap-dependent endonuclease inhibitor

3.1. Analysis of phenotypic baloxavir susceptibility data from WHO CCs

Three WHO CCs (Atlanta, United States; Melbourne, Australia; Tokyo, Japan) assessed phenotypic baloxavir susceptibility of influenza viruses, but different methodologies were used by the laboratories (Gubareva et al., 2019; Koszalka et al., 2019; Takashita et al., 2018). Melbourne and Tokyo CCs determined the 50% effective concentration (EC_{50}) or IC_{50} by use of a Focus Reduction Assay (FRA) (Koszalka et al., 2019; Takashita et al., 2018) and Atlanta CC determined IC_{50} by use of a High-content Imaging Neutralization Test (HINT) (Gubareva et al., 2019). Although comparison of the mean IC_{50} values determined by individual WHO CCs for a panel of reference viruses revealed variation between laboratories, the fold-change in IC_{50} for different reference variant viruses was similar (Table 3). Normalization of distributions using fold-change values was previously implemented for NAI IC_{50} data (Meijer et al., 2014) and subsequently used for all global reports. The WHO-AVWG has established a set of criteria to define the NAI susceptibility of influenza viruses based on the fold-change in IC_{50} value compared with the median value for wild-type viruses from the same type/subtype/lineage (WHO, 2012). More data are needed to establish a similar set of criteria for definition of baloxavir susceptibility.

Table 1
Virus and patient characteristics of 79 influenza type A viruses showing RI or HRI by NAIs: testing performed by WHO CCs.[a]

Virus	n	IC_{50} fold-change compared to reference median IC_{50} values [b]				NA substitution [c]		Patient setting	Antiviral treatment	Immuno-compromised
		Oseltamivir	Zanamivir	Peramivir	Laninamivir	Virus isolate	Clinical specimen			
A(H1N1)pdm09; n = 3937	48	**24–2078**	0.8–2.6	8.6–**495** (43)	1.2–3.4 (43)	H275Y (Y/H mix; 7)	H275Y (31); Y/H mix (6) Not available [d] (11)	Community (23) Hospital (7) Unknown (18)	Yes, oseltamivir (7) Yes, oseltamivir, peramivir (1) Yes, peramivir (1) Yes, peramivir, laninamivir (1) Yes, laninamivir (1) No (19) Unknown (18)	Yes (1) No (23) Unknown (24)
	2	**14–26**	6.2–**11**	n/t [e]	n/t	I223R	Not available (2)	Community (1) Hospital (1)	No (1) Unknown (1)	Unknown (2)
	2	0.8–0.9	**48–142**	**41–162**	7.7–**15**	Q136R (R/Q mix; 1)	None [f] (2)	Community (2)	No (1) Unknown (1)	No (1) Unknown (1)
	1	0.6	**35**	**48**	4.6	V106K/V mix, Q136K/Q mix	Not available	Unknown	Unknown	Unknown
	1	3.4	**10.9**	0.1	1.0	T148A	Not available	Unknown	Unknown	Unknown
	1	**37**	**28**	3.8	7.1	D199Y	D199Y	Unknown	Unknown	Unknown
	1	**23**	8.8	n/t	n/t	I223T	Not available	Hospital	Unknown	No
	1	**37**	**41**	6.2	**10.7**	I427T	Not available	Unknown	Unknown	Unknown
	1	1.5	1.3	**13**	5.7	None	Not available	Unknown	Unknown	Unknown
A(H3N2); n = 5591	2	7.8–9.9	4.3–5.0	**10.5–11**	2.3	V313A	Not available (2)	Unknown (2)	Unknown (2)	Unknown (2)
	2	**20–24**	**14–15**	3.9–5.1	3.0–3.3	S333G	S333G (2)	Community (2)	Unknown (2)	No (1) Unknown (1)
	1	**14**	4.9	n/t	n/t	S334R	Not available	Unknown	Unknown	Unknown
	1	**212**	2.3	1.7	1.9	E119V	Not available	Unknown	Unknown	Unknown
	1	**101**	**13**	n/t	n/t	E119V/E mix, R292K/R mix, △245-248	E119V/E mix, R292K/R mix, △245-248	Hospital	Yes, oseltamivir	Yes
	1	2.6	**13**	n/t	n/t	Q136K/Q mix	Not available	Hospital	Unknown	No
	1	1.3	1.8	**13**	3.0	V143M, S315R	Not available	Unknown	Unknown	Unknown
	1	9.1	**10.5**	0.04	0.4	D161N	Not available	Unknown	Unknown	Unknown
	1	**7735**	6.6	**60**	1.8	R292K	Not available	Hospital	Unknown	Unknown
	1	**13**	**19**	1.8	5.9	V303I, N342K	Not available	Unknown	Unknown	Unknown
	1	**16**	**16**	1.9	4.1	G320R	G320R	Community	No	No
	1	**29**	**19**	3.0	2.7	N329R	N329R	Unknown	No	Unknown
	1	**10.1**	9.3	5.3	2.9	N342K	N342K	Community	No	No
	1	**16**	8.8	**13**	2.9	D463N	Not available	Unknown	Unknown	Unknown
	5	8.7–**19**	4.2–**22**	**10.2–16** (4)	2.3–**17** (4)	None	None (2) Not available (3)	Community (1) Hospital (1) Unknown (3)	Unknown (5)	Unknown (5)

RI: reduced inhibition; HRI: highly reduced inhibition; NAI: neuraminidase inhibitor; IC_{50}: 50% inhibitory concentration.
[a] Between brackets the number of viruses for which data was reported if less than the number reported in column 'n'.
[b] RI and HRI fold-change values are displayed underlined and in bold typeface.
[c] Amino acid position numbering is A subtype specific.
[d] Clinical specimen not available for sequencing.
[e] n/t: not tested.
[f] None: no amino acid substitutions compared to viruses with a normal inhibition (NI) phenotype.

Table 2
Virus and patient characteristics of 43 influenza type B viruses showing RI or HRI by NAIs: testing performed by WHO CCs.[a]

Virus	n	IC_{50} fold-change compared to reference median IC_{50} values [b]				NA substitution [c]		Patient setting	Antiviral treatment	Immuno-compromised
		Oseltamivir	Zanamivir	Peramivir	Laninamivir	Virus isolate	Clinical specimen			
B/Victoria- lineage; n = 1415	6	3.0–**5.0**	3.1–**9.9**	2.5–**5.1**	2.7–4.5	D197N	D197N (2) Not available [d] (4)	Community (1) Unknown (5)	No (1) Unknown (5)	No (1) Unknown (5)
	3	**8.7**–**15**	3.3–**18**	**19**–**37**	1.5	D197E	D197E (3)	Community (3)	No (3)	No (3)
	1	1.2	1.1	**7.8**	0.9	G104R/G mix, D432G/D mix	None [e]	Unknown	Unknown	Unknown
	1	0.8	0.7	**7.6**	0.6	G145E	G145E	Unknown	Unknown	Unknown
	1	**24**	**19**	**67**	2.7	A200T	A200T	Unknown	Unknown	Unknown
	1	**9.8**	**7.1**	**26**	**6.6**	I221T	I221T	Hospital	No	Yes
	1	**8.8**	**205**	**10**	4.8	A245T	Not available	Unknown	Unknown	Unknown
	1	1.0	**17**	3.9	3.4	R270K	None	Unknown	Unknown	Unknown
	1	**5.8**	**5.2**	n/t [f]	n/t	I348T	Not available	Hospital	Unknown	No
B/Yamagata- lineage; n = 4466	6	2.1–**5.2**	1.4–**7.4**	**6.4**–**9.3** (5)	0.8–3.2 (5)	D197N	D197N (4) Not available (2)	Community (1) Hospital (1) Unknown (4)	No (1) Unknown (5)	No (1) Unknown (5)
	4	**8.5**–**10**	1.5–3.2	**10**–**29** (2)	1.3–2.3 (2)	I221T	I221T (2) Not available (2)	Community (1) Hospital (2) Unknown (1)	No (1) Unknown (3)	No (3) Unknown (1)
	1	1.9	1.2	**6.9**	1.3	I115T	I115T	Unknown	Unknown	Unknown
	1	**12**	**1250**	**2778**	**971**	E117G	Not available	Unknown	Unknown	Unknown
	1	0.9	**14**	0.9	1.1	V131A	Not available	Unknown	Unknown	Unknown
	1	1.4	0.5	**33**	0.4	H134Y/H mix	None	Community	No	No
	1	2.1	0.6	**134**	0.4	T146I	None	Unknown	Unknown	Unknown
	1	**47**	29	n/t	n/t	R150K	Not available	Unknown	Unknown	Unknown
	1	0.5	**7.3**	n/t	n/t	R154K	Not available	Hospital	No	Unknown
	1	**18**	1.1	**1360**	0.4	I221T, H273Y	I221T, H273Y	Unknown	Yes, oseltamivir	Unknown
	1	2.6	1.3	**5.2**	1.7	I221V	I221V	Unknown	No	Unknown
	1	2.8	**11**	n/t	n/t	L396H	Not available	Hospital	Unknown	No
	1	1.0	0.7	**8.1**	0.7	D432N	D432N	Unknown	Unknown	Unknown
	1	1.3	0.9	**9.8**	0.7	T436P	None	Unknown	Unknown	Unknown
	1	1.0	1.0	**7.7**	0.5	M464T	M464T	Hospital	No	Unknown
	4	1.1–**7.1**	0.9–**6.2**	2.4–**6.0** (3)	0.9–2.0 (3)	None	None (2) Not available (2)	Community (3) Unknown (1)	No (1) Unknown (3)	No (1) Unknown (3)

RI: reduced inhibition; HRI: highly reduced inhibition; NAI: neuraminidase inhibitor; IC_{50}: 50% inhibitory concentration.

[a] Between brackets the number of viruses for which data was reported if the number reported in column 'n'.

[b] RI and HRI fold-change values are displayed underlined and in bold typeface.

[c] Amino acid position numbering is B lineage specific.

[d] Clinical specimen not available for testing.

[e] None: no amino acid substitutions compared to viruses with a normal inhibition (NI) phenotype.

[f] n/t: not tested.

Table 3
Baloxavir susceptibility results for a panel of reference viruses [a]: testing performed by two WHO CCs.

Virus	Strain designation	PA substitution [b]	Atlanta CC, USA IC_{50}, nM [c]	Tokyo CC, Japan IC_{50}, nM [d]
			(fold-change)	(fold-change)
A(H1N1)pdm09	A/Illinois/08/2018	38I	1.61 ± 0.22	0.58 ± 0.34
A(H1N1)pdm09	A/Illinois/37/2018	I38L	13.50 ± 3.04 (8.4)	4.28 ± 2.04 (7.4)
A(H3N2)	A/Louisiana/50/2017	38I	1.33 ± 0.22	3.91 ± 0.81
A(H3N2)	A/Louisiana/49/2017	I38M	13.88 ± 2.25 (10.4)	38.05 ± 9.74 (9.7)

IC_{50}: 50% inhibitory concentration.
[a] CDC Baloxavir Susceptibility Reference Virus Panel (version 1.0) was obtained from the International Reagent Resource (www.internationalreagentresource.org).
[b] Amino acid position numbering is type A specific.
[c] IC_{50} were determined by use of a High-content Imaging Neutralization Test (HINT). The values are presented as the mean ± SD of ≥ three independent tests.
[d] IC_{50} were determined by use of a Focus Reduction Assay (FRA). The values are presented as the mean ± SD of ≥ three independent tests.

3.2. Frequency of PA amino acid substitutions associated with reduced susceptibility to baloxavir in sequence databases

We analyzed PA sequences from viruses collected between weeks 21/2017 and 20/2018 that had been deposited in either GISAID or NCBI-IVR databases. Deduplication by strain name was performed, with preference given to sequences from original clinical specimens, where available. Sequences were screened for amino acid substitutions known to be associated with reduced susceptibility to baloxavir (PA E23G/K/R, PA A36V, PA A37T, PA I38F/M/T/L, PA E119D, PA E199G) (Gubareva et al., 2019).

A total of 13523 sequences (2969 A(H1N1)pdm09, 5781 A(H3N2), 1020 B/Victoria-lineage and 3753 B/Yamagata-lineage) were analyzed, 6498 (48.1%) of which were from viruses from the United States. Eleven (0.08%) viruses possessed PA substitutions associated with reduced susceptibility to baloxavir (Table 4). These PA variants were isolated from five countries; France (1), Japan (1), Singapore (1), United Kingdom (1) and United States (7).

Of the 2969 A(H1N1)pdm09 sequences, 7 (0.2%) contained PA E23G (n = 2), PA A37T/A polymorphism, PA I38L (n = 2), PA I38M/I polymorphism or PA E199G substitutions (Table 4). The E23G and I38L variants were included in phenotypic baloxavir susceptibility analysis by the Atlanta CC and showed 6–9-fold reduced susceptibility to baloxavir (Gubareva et al., 2019). Notably, A/Iowa/33/2017, with an HA of A(H1N1)pdm09 subtype (Table 4), should be classified as a 'variant' virus [A(H1N1)v] as its contains genes from swine influenza viruses (https://www.who.int/influenza/vaccines/virus/201802_zoonotic_vaccinevirusupdate.pdf) with the PA having I38M/I polymorphism. Of the 5781 A(H3N2) sequences, three (0.05%) contained PA A37T, PA A37T/A polymorphism or PA I38M (Table 4). The A37T/A polymorphic and I38M variants were included in phenotypic baloxavir susceptibility analysis by Tokyo and Atlanta CCs and showed 2- and 17-fold reduced susceptibility to baloxavir, respectively (Gubareva et al., 2019). One (0.03%) of the 3753 B/Yamagata-lineage PA sequences had PA I38T substitution but the variant was not tested phenotypically (Table 4).

4. Concluding remarks

This is the 6th global update on influenza NAI susceptibility and the first of these global reports to include data on baloxavir susceptibility. The update was based on analyses of data generated by WHO CCs from samples shared by GISRS laboratories. The proportion of viruses showing RI or HRI by NAIs in this study (0.8%) was similar to those reported for the five previous periods; 0.6% in 2012–13, 1.9% in 2013–14, 0.5% in 2014–15, 0.8% in 2015–16 and 0.2% in 2016–17. The slight increase in 2013–14 was due to several clusters of untreated cases of A(H1N1)pdm09 viruses carrying NA H275Y substitution in China, Japan and the United States (Takashita et al., 2015). In this study, 60% (73/122) of viruses showing RI or HRI by NAIs were isolated from Japan, United States and Australia, while the frequency of these variants in Japan, United States, Australia and the rest of the world was 3.6% (34/945), 0.6% (22/3752), 0.8% (17/2182) and 0.6% (49/8530), respectively. In the 2017–18 season, NAIs were supplied to medical institutions that together served about 15 million people in Japan (i.e., one-ninth of the Japanese population). A(H1N1)pdm09 viruses with the NA H275Y substitution were the most frequently detected in this study. Of the 48 NA H275Y variants detected, 19 (40%) cases from Japan (14) and the United States (5) had not received any NAIs prior to specimen collection, suggesting human-to-human transmission of the H275Y variants. Since we found that 50 A(H3N2) viruses contained NA substitutions associated with HRI or RI but showed phenotypic NI, a combination of phenotypic methods analyzing antiviral susceptibility and genotypic methods detecting amino acid substitutions is valuable for surveillance purposes.

The cap-dependent endonuclease inhibitor baloxavir was approved on 23 February 2018 in Japan and on 24 October 2018 in the United States for the treatment of influenza A and B virus infections. The frequency of viruses with PA substitutions associated with reduced susceptibility to baloxavir in this period (0.08%) was low. The most PA variants were isolated from the United States, while the frequency of these variants in the United States and the rest of the world was 0.1% (7/6497) and 0.06% (4/7026), respectively. Usage of baloxavir has increased during the 2018–19 influenza season. In Japan, baloxavir was supplied to medical institutions that together served about 0.4 and 5.3 million people in the 2017–18 and 2018–19 season, respectively. Furthermore, human-to-human transmission of A(H3N2) viruses with PA I38T substitution has been detected in Japan (Takashita et al., 2019a, Takashita et al., 2019). Therefore, continued monitoring of circulating viruses for NAI and baloxavir susceptibility is important to ensure antiviral treatment guidelines remain appropriate.

Contributions

All WHO-AVWG Members and WHO headquarters and Regional Office Staff named were involved in the development of this global update. ET/SF, RSD/VG, LVG/HTN, ACH/MR and DW/WH generated and provided the NAI susceptibility data and molecular analysis data. ET and LVG generated and provided the baloxavir susceptibility data and molecular analysis data. AM performed analysis of the phenotypic data from the WHO CCs and drafted tables and figures. ET and SF performed analysis of the sequence data from GISAID and NCBI-IVR databases. ET drafted the manuscript and all authors contributed to editing the final manuscript.

We gratefully acknowledge the work of our late colleague, Vicki Gregory, who contributed significantly to the WHO-AVWG annual reports over the years.

Table 4
Influenza types A and B viruses carrying PA substitutions associated with reduced susceptibility to baloxavir (n = 11).

Virus	Strain designation	Submitting laboratory	Fold change in IC_{50}	PA substitution [a]	Passage details/ history [b]	PA GISAID Acc.No.	Country of specimen collection	Date of collection (y/m/d)
A(H1N1)pdm09	A/Florida/20/2018	Centers for Disease Control and Prevention	6	E23G	Original	EPI1196011	United States	2018-02-02
A(H1N1)pdm09	A/Singapore/NUH0016/2017	Ministry of Health Singapore	n/t [c]	E23G	MDCK1	EPI1076747	Singapore	2017-06-09
A(H1N1)pdm09	A/New/York/11/2018	Centers for Disease Control and Prevention	n/t	A37T/A mix	Original	EPI1195905	United States	2018-02-04
A(H1N1)pdm09	A/Illinois/37/2018	Centers for Disease Control and Prevention	9	I38L	Original	EPI1250007	United States	2018-02-08
A(H1N1)pdm09	A/Illinois/38/2018	Centers for Disease Control and Prevention	8	I38L	Original	EPI1250014	United States	2018-02-08
A(H1N1)pdm09	A/Iowa/33/2017[d]	Centers for Disease Control and Prevention	n/t	I38M/I mix	Original	EPI1311341	United States	2017-10-22
A(H1N1)pdm09	A/Pennsylvania/108/2018	Centers for Disease Control and Prevention	n/t	E199G	Original	EPI1452257	United States	2018-02-18
A(H3N2)	A/England/81140621/2018	Microbiology Services Colindale, Public Health England	n/t	A37T	Original	EPI1242465	United Kingdom	2018-03-06
A(H3N2)	A/IWATE/30/2018	National Institute of Infectious Diseases	2	A37T/A mix	MDCK 1 +SIAT1	EPI1265721	Japan	2018-05-07
A(H3N2)	A/Louisiana/49/2017	Centers for Disease Control and Prevention	17	I38M	Original	EPI1059954	United States	2017-08-21
B Yamagata	B/Picardie/1392/2018	Institut Pasteur	n/t	I38T	PI	EPI1216455	France	2018-03-12

IC_{50}: 50% inhibitory concentration; GISAID: Global Initiative on Sharing All Influenza Data.
[a] Amino acid position numbering is subtype/lineage-specific.
[b] Passage as shown in the sequence databases.
[c] n/t: not tested.
[d] A/Iowa/33/2017, carrying HA and NA genes of the A(H1N1)pdm09 subtype and other genes from swine influenza viruses, is designated as A(H1N1)v (https://www.who.int/influenza/vaccines/virus/201802_zoonotic_vaccinevirusupdate.pdf).

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2020.104718.

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