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Identification of Indonesian clade 2.1 highly pathogenic influenza A(H5N1) viruses with N294S and S246N neuraminidase substitutions which further reduce oseltamivir susceptibility

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ABSTRACT

We have tested the *in vitro* susceptibility to the neuraminidase (NA) inhibitors of 96 highly pathogenic clade 2.1 A(H5N1) viruses from Indonesia, isolated between 2008 and 2011. HPAI virus samples obtained through the Influenza Virus Monitoring (IVM) surveillance program in Indonesia were tested for susceptibility to oseltamivir and zanamivir. The NAs of four viruses were identified as extreme outliers to oseltamivir, based on statistical analysis by box plots, with IC_{50} values ranging from 46 to 62 nM. The NAs of two of these viruses from Sumatra and Aceh, had an N294S substitution, while one virus from Sulawesi had an S246N NA substitution. The NAs of all four viruses showed a specific loss of slow binding to oseltamivir in an IC_{50} kinetics assay. As observed in our previous surveillance, there was only a minimal effect on the sensitivity to zanamivir or peramivir for these mutants or any of the other isolates tested. The continued circulation of subtype H5N1 viruses in avian species poses an on-going zoonotic threat. The fact that we continue to identify avian isolates with naturally occurring mutations conferring reduced oseltamivir susceptibility remains a concern, given oseltamivir will be a key antiviral in the event of a new pandemic emerging.

1. Introduction

Avian influenza A(H5N1) viruses continue to circulate among commercial, domestic and wild birds in Asia, the Middle East and Africa (OIE, 2017; WHO, 2017). Up until 2009 Indonesia had the highest numbers of human cases and a high mortality rate. However, there has been a significant decrease in the numbers of human infections in Indonesia, especially since 2013, with only 2 cases and 2 deaths reported in 2015, none in 2016 and one in 2017 (WHO, 2017). In contrast, Egypt saw a dramatic upsurge in human infections and deaths since 2013, with 136 cases and 39 deaths in 2015, (WHO, 2015), although only 10 cases and 3 deaths were reported for 2016 and 3 cases and one death in 2017 (WHO, 2017).

The neuraminidase inhibitor (NAI), oseltamivir (Tamiflu) remains the drug of choice for the treatment of infected humans. However, resistance is known to arise not only because of oseltamivir treatment but also through random mutations. The latter has been seen in human seasonal influenza viruses (Hurt et al., 2009a; Meijer et al., 2009; Takashita et al., 2015) and in highly pathogenic avian influenza (HPAI) A(H5N1) viruses isolated from infected poultry (McKimm-Breschkin et al., 2007, 2013a; Nguyen et al., 2013). Due to differences in the chemical structures of the NAIs, resistance to oseltamivir may not necessarily confer resistance to the other two licensed NAIs, zanamivir and peramivir (McKimm-Breschkin, 2013). Hence these may be suitable as an alternative therapy. As HPAI (H5N1) viruses remain endemic in many parts of the world, they will not be easily eradicated. Since human (H5N1) virus infections arise from contact with infected poultry the virus is fortunately not yet transmissible between humans, however it remains a potential pandemic threat. Hence, it is important to monitor the emergence and circulation of resistance in avian influenza

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isolates from poultry and other avian species, in order for the appropriate therapy to be used if the viruses become transmissible between humans.

We have previously shown that the NAs from clade 2.1 viruses from Indonesia have a reduced sensitivity to oseltamivir compared to clade 1 viruses from Vietnam (McKimm-Breschkin et al., 2007). This is primarily due to an H252Y substitution in the NA. We also subsequently identified other substitutions which further reduce sensitivity including I222M/V/T and I117V (N2 numbering) (McKimm-Breschkin et al., 2013a). Clade 1 viruses with an S246G also demonstrate reduced oseltamivir sensitivity (McKimm-Breschkin et al., 2007, 2013a). Through the Influenza Virus Monitoring (IVM) surveillance program in Indonesia which primarily aims to monitor antigenic drift of the (H5N1) virus hemagglutinin (Hartaningsih et al., 2015), we further tested samples of (H5N1) viruses obtained from 2008 to 2011 from various regions of Indonesia for sensitivity to the NAIs. We have screened samples for sensitivity to oseltamivir and zanamivir in a fluorescence based enzyme inhibition assay as previously described (McKimm-Breschkin et al., 2013a). We also determined the kinetics of drug binding for those isolates showing altered sensitivity against both these drugs and peramivir (Barrett et al., 2011). Of 96 samples screened, based on box plot statistical analysis, four viruses were considered to be extreme outliers to oseltamivir, with IC_{50} values in the range of 46–62 nM (IC_{50} = concentration of drug to inhibit enzyme activity by 50%). Substitutions were different to those reported in our previous surveillance. Although the oseltamivir resistant viruses demonstrated altered kinetics of drug binding for zanamivir and peramivir, none of the viruses were extreme outliers to these drugs, with all IC_{50} values less than 10 nM.

2. Materials and methods

2.1. Viruses

HPAI (H5N1) viruses isolated primarily from chickens were supplied to the CSIRO Australian Animal Health Laboratory (AAHL) as part of the Indonesian IVM program (Hartaningsih et al., 2015). Viruses were amplified in specific pathogen free eggs under BSL3 conditions at AAHL. Virus cultures were inactivated by gamma irradiation prior to use in the fluorescence based NA inhibition assays.

2.2. Chemicals and inhibitors

Zanamivir and peramivir were kindly provided by GlaxoSmithKline (Stevenage, UK). Oseltamivir carboxylate was kindly provided by Dr Keith Watson (Walter and Eliza Hall Institute, Australia). Serial $\log_{10}$ dilutions of the inhibitors were prepared in sterile water, ranging from 0.001 nM to 10,000 nM. The fluorogenic substrate 4-methylumbelliferyl N-acetyl-α-D-neuraminic acid (MUNANA) was obtained from Carbosynth (Berkshire, UK).

2.3. Enzyme inhibition assay

Sensitivity to the inhibitors was evaluated in the MUNANA based fluorescence assay (Barrett et al., 2011; Potier et al., 1979). The reaction mix final concentrations were 50 mM sodium acetate pH 5.5, 5 mM $CaCl_2$ and 100 μM MUNANA. Fluorescence was read in a BMG FLUOstar Optima reader, with excitation and emission wavelengths of 355 and 460 nm respectively. Samples were screened in duplicate, with a 30 min preincubation step with inhibitor at room temperature followed by a 60 min reaction at 37 °C after the addition of MUNANA. Reactions were stopped with Na_2CO_3 and total fluorescent units (FU) were then measured. The IC_{50} values were calculated as the concentration of inhibitor resulting in a 50% inhibition of enzyme activity, compared to the uninhibited control. Box and whisker plots were used to identify outliers as previously described (McKimm-Breschkin et al., 2003; Monto et al., 2006). Means of the duplicate $\log_{10}$ IC_{50} values for each sample for each drug were plotted, with the boxes representing the 25%–75% quartiles, with the interquartile range between these (IQR). Mild outliers were between 1.5 and 3.0 times the IQR from the 75th percentile. Extreme outliers were more than 3.0 IQR from the 75th percentile. 95% confidence limits are shown by the whiskers.

Samples showing elevated IC_{50} values were then tested in an IC_{50} kinetics assay to determine whether there was a change in binding or dissociation kinetics as previously observed for many mutants with altered drug sensitivity (Barrett et al., 2011; McKimm-Breschkin et al., 2013a, 2013b). IC_{50} kinetics assays use two reactions to monitor the binding and dissociation steps. In the first reaction virus and inhibitor are incubated for 30 min as in the standard endpoint assay. In the second reaction, virus, inhibitor and MUNANA are all added simultaneously. Fluorescence for both reactions is monitored at 1 min intervals, to confirm stability of the reaction. IC_{50} values are calculated for each 10 min period, and then plotted as bar graphs to demonstrate the rate of change of the IC_{50} values with time. For slow binding inhibitors with wild type NAs the IC_{50} values after preincubation are lower than without preincubation. The IC_{50} values then increase as the drug dissociates. In contrast, the IC_{50} values decrease without preincubation as the inhibitor binds.

2.4. Sequencing

Viral RNA was extracted using the QIAmp RNA Mini Kit (Qiagen, Germany). The Superscript™ III One-Step RT-PCR system (Invitrogen, USA) was used to amplify the viral RNA, using NA specific primers (Supplementary Table 1). After gel purification the amplicons were gel-purified (QIAquick, Qiagen) and directly sequenced according to the manufacturer's instructions using the BigDye Terminator v3.1 Cyle Sequencing Kit (Applied Biosystems). Full length NA gene sequences were submitted to GenBank with the accession numbers MG664572, MG664575–MG664578, MG664581–MG664655, MG664657, and MG664659–MG664668 (Supplementary Table 2). The Bioedit program (Hall, 1999) was used for alignment and translation, to identify possible substitutions associated with altered drug sensitivity.

3. Results

3.1. NAI susceptibility

A total of 96 clade 2.1 (H5N1) virus samples were tested in this study (Table 1 and Supplementary Table 2). Thirty-nine viruses were from the western Indonesian provinces of Sumatra and Aceh that were associated with HPAI outbreaks in 2008–2009. Thirty-two virus samples from 2009 were from the eastern province of South Sulawesi, and two 2008 viruses were from West Sulawesi. Finally, 23 viruses were from mostly central Indonesian provinces in Java, Bali and Kalimantan collected from 2010 to 2011. We used (H5N1) clade 1.1 (A/chicken/Vietnam/08/2004) and clade 2.1 (A/chicken/Wates/126/2005) virus samples from our initial screening study as reference control strains for the enzyme inhibition assays (McKimm-Breschkin et al., 2007, 2013a). The clade 1.1 virus was sensitive to both zanamivir and oseltamivir. The clade 2.1 virus was also sensitive to zanamivir, but had previously demonstrated more than15-fold reduction in sensitivity to oseltamivir compared to the clade 1.1 virus (McKimm-Breschkin et al., 2007, 2013a).

Box plots (Fig. 1) showed that as previously observed the IC_{50} values for oseltamivir were much higher than those for zanamivir (McKimm-Breschkin et al., 2007, 2013a). There were also some obvious outliers, with IC_{50} values greater than the 95th percentile. Statistical analysis (Table 1) of the IC_{50} values showed that the mean IC_{50} values for oseltamivir for viruses from Sumatra and Aceh (24.7 nM) were higher than for viruses from Sulawesi (16.1 nM) or from the central Indonesian regions (15.3 nM). There was statistically one mild outlier to zanamivir,

Table 1
Susceptibility of Indonesian HPAI H5N1 isolates to zanamivir and oseltamivir in the MUNANA enzyme inhibition assay.[a].

	Zanamivir			Oseltamivir		
	Sulawesi (Eastern)	Sumatra/Aceh (Western)	Central Indonesian	Sulawesi (Eastern)	Sumatra/Aceh (Western)	Central Indonesian
Mean[b]	1.2	1.7	1.9	16.1	24.7	15.3
LQ	1.0	1.4	1.5	13.1	22.3	10.5
UQ	1.7	2.1	2.2	17.6	27.1	23.2
IQR[c]	1.7	1.5	1.4	1.3	1.2	2.2
Median	1.1	1.6	1.8	15.1	25.1	18.6
Mild = UQ + 1.5 IQR	3.6	3.7	3.8	27.3	36.3	76.0
Extreme = UQ + 3x IQR	8.0	6.5	6.6	42.4	48.6	249.1
Mild outliers high[d]	0	0	1	0	0	
Extreme outliers high[e]	0	0		2	2	
Min	0.7	1.0	1.0	8.9	10.8	4.1
Max	3.2	2.9	6.4	48.3	61.7	29.7
N = number	34	39	23	34	39	23
Controls						
Vietnam clade 1[f]	2.2 (0.62)		1.9 (0.57)	0.61 (0.14)		0.52 (0.04)
Indonesia clade 2[g]	1.68 (0.2)		2.3 (0.2)	20.3 (4.4)		18.6 (1.8)

[a] Samples were all assayed with a 30 min preincubation with inhibitor, and then MUNANA was added and reactions were stopped after 60 min and read.
[b] Means for each batch were calculated on $\log_{10}$ transformed data, and then back transformed. Samples were tested in duplicate and means and SDs for reference controls are from all assays in each batch.
[c] IQR Interquartile range representing the range in which 50% of values fall (25^{th}-75th percentile).
[d] Mild outliers defined as 1.5xIQR from the 75th percentile.
[e] Extreme outliers defined as 3xIQR from the 75th percentile.
[f] Clade 1 strain sensitive to both zanamivir and oseltamivir.
[g] Clade 2 Indonesian strain with higher reduction in oseltamivir sensitivity.

with an IC_{50} of 6.4 nM, but this was still less than 4-fold higher than the mean. The World Health Organization considers a virus only has reduced sensitivity with a greater than 10-fold increase (WHO, 2012).

There were 4 outliers to oseltamivir in the (H5N1) viruses tested. There were two extreme outliers with IC_{50} values of 59 and 62 nM from Sumatra and Aceh and two extreme outliers with IC_{50} values of 46 and 48 nM from Sulawesi. IC_{50} values for zanamivir for each of these outliers were between 2 and 3 nM, which were all slightly higher than the mean (1.7 nM), but still low. There was a much wider variation in IC_{50} values for oseltamivir in the mix of viruses from the central Indonesian provinces, with several IC_{50} values less than 10 nM, lower than previously observed for the clade 2.1. This makes the interquartile range larger, and hence the definition of an extreme outlier much higher (249 nM). However, even using the IC_{50} values for mild and extreme outliers from western and eastern Indonesia as a reference, there were no mild or extreme outliers in the viruses from Java, Bali and Kalimantan.

There were also a number of additional viruses from central Indonesian provinces for which we were unable to obtain an endpoint in the enzyme inhibition assay. Several of these had Newcastle Disease virus (NDV) co-infections, as we have found previously (McKimm-Breschkin et al., 2013a). NDV also has NA activity, but this is not sensitive to oseltamivir. Hence one has to be aware of the potential complication of an NDV co-infection on IC_{50} results.

3.2. Sequence analysis

Complete NA sequence was obtained for 31 out of 34 HPAI virus samples from the eastern Indonesian provinces of Sulawesi, all 39 of the Sumatran/Aceh samples and 18 of the 23 from Central Indonesia. Three eastern samples only yielded incomplete sequences, missing approximately 60 amino acids, and the other 5 samples from central Indonesia

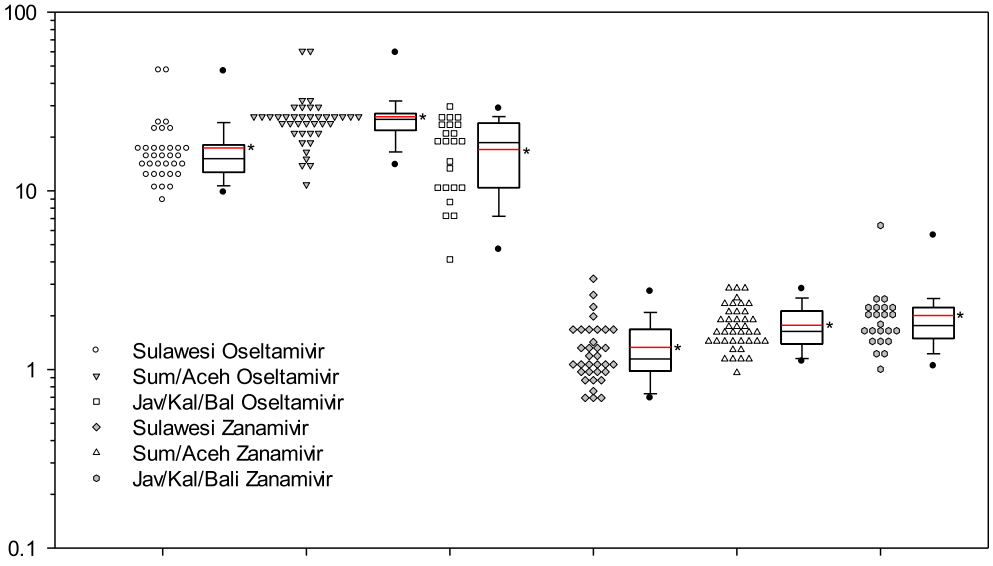

Fig. 1. Box and scatter plots of means of duplicate $\log_{10}$ IC_{50} values Means of the duplicate $\log_{10}$ IC_{50} values for each sample for each drug were plotted, with the boxes representing the 25%–75% quartiles, with the interquartile range between these (IQR). * indicates the median. Outliers are shown by filled circles. Mild outliers were between 1.5 and 3.0 times the IQR from the 75th percentile. Extreme outliers were more than 3.0 IQR from the 75th percentile. 95% confidence limits are shown by the whiskers. Sum/Aceh is Sumatra and Aceh, Jav/Kal/Bal is Java, Kalimantan, Bali.

yielded no sequence. (Supplementary Table 2). IC_{50} values for the NAs unable to be sequenced fell within the normal range, so were not pursued further. Alignment of the NA amino acid sequences showed all NAs still had the Y252 previously associated with reduced oseltamivir sensitivity, but there were several common amino acid differences between the Sulawesi and Sumatra/Aceh (western) virus batches. There were five changes in the NA stalk region, plus V232A, E341N, I384T in the head, which were seen in the NAs of all the western province (Sumatra/Aceh) viruses. These NA substitutions may contribute to the subtle differences in susceptibility to oseltamivir between the western and eastern batches.

Around half of the NAs of viruses from geographically central provinces of Indonesia had IC_{50} values for oseltamivir between 4 and 10 nM, much lower than previously seen for the clade 2.1 viruses. Sequence comparisons of their NAs, showed that all of these viruses had common amino acid differences to those NAs with higher IC_{50} values in this same batch, with the NAs with lower IC_{50} values having Q136H, T239V, R258K and I303V. Substitutions of Q136K and Q136R in human viruses and Q136L in HPAI (H5N1) viruses are known to decrease sensitivity to the NAIs, especially to zanamivir (Hurt et al., 2009b, 2010; Little et al., 2015; Okomo-Adhiambo et al., 2010), so it is possible this Q136H substitution may contribute to the enhanced oseltamivir sensitivity in this subgroup.

There was only a single amino acid substitution in the mild outlier to zanamivir, L25I. As this was in the transmembrane region it is unlikely this affected the NAI sensitivity.

We also identified unique substitutions in the extreme outliers. The A/chicken/Aceh Timur/477-08-2/2008 and A/chicken/Aceh Timur/477-08-3/2008 viruses with IC_{50} values of 59 and 62 nM for oseltamivir, both had an N294S substitution. The third outlier, A/chicken/Maros/315/2009 from South Sulawesi had an S246N substitution, with an IC_{50} of 48 nM for oseltamivir. The fourth virus, A/chicken/Sidrap/40/2009 an outlier also from South Sulawesi had an IC_{50} of 46 nM for oseltamivir, which did not appear to have any unique substitutions.

3.3. IC_{50} kinetics

We have developed a 96-well based assay, which demonstrates differences in the binding and dissociating kinetics of the NAIs (Barrett et al., 2011; McKimm-Breschkin et al., 2013a, 2013b; Oakley et al., 2010). For wild type viruses, the NAIs are slow binding, and generally slow dissociating. In contrast, we have shown that most mutations leading to reduced NAI susceptibility result in both faster binding and dissociation (Barrett and McKimm-Breschkin, 2014; McKimm-Breschkin et al., 2012; McKimm-Breschkin et al., 2013b; Oakley et al., 2010). We have established that dissociation of oseltamivir is faster from clade 2.1 viruses from Indonesia compared to clade 1 viruses from Vietnam (McKimm-Breschkin et al., 2013a). We wanted to determine if the four outliers identified here also demonstrated altered binding kinetics as a way of confirming the higher IC_{50} values. IC_{50} kinetics were initially analyzed for oseltamivir with and without preincubation (Fig. 2).

The Vietnam clade 1 NA control was used to show the difference in the kinetics for oseltamivir compared to the normal Indonesian clade 2.1 NA. Both the Aceh Timur/477/08 N294S NAs and the Sidrap/40/09 NA, showed faster dissociation (increases in IC_{50} after preincubation) compared to the Indonesian control. Despite a higher IC_{50}, dissociation of the Maros/315/09 S246N NA was only marginally faster than the control.

Each of these four outliers was then tested against zanamivir and peramivir in the preincubation reaction which provides information on relative dissociation, as we had seen some small increase in zanamivir IC_{50} values. Although the Aceh Timur/477/08 and Sidrap/40/09 viruses had marginally higher IC_{50} values for zanamivir the change in IC_{50} from 10 to 60 min were comparable to the control. For peramivir there was a faster dissociation for all four viruses, with slightly higher IC_{50} values correlating with larger increases in IC_{50} values from 10 to 20 min especially. Changes in the IC_{50} kinetics confirm that the differences in IC_{50} do reflect a difference in inhibitor binding and are not just experimental variation.

4. Discussion

The WHO laboratories and National Influenza Centers are now more actively involved in surveillance and testing the sensitivity of human influenza isolates to the NAIs (Hurt et al., 2016). However, there remains limited surveillance of the NAI sensitivity of circulating avian influenza isolates, especially the HPAI (H5N1) and A(H7N9) viruses. Yet these remain as potential pandemic threats, and the NAIs remain the only globally approved influenza-specific antiviral.

We have previously demonstrated that clade 2.1 HPAI (H5N1) viruses from Indonesia had reduced sensitivity to oseltamivir, compared to the clade 1 viruses (McKimm-Breschkin et al., 2007). Subsequently we also identified additional substitutions at I222 to M/T/V in the NA of clade 2.1 viruses, which further reduced oseltamivir sensitivity (McKimm-Breschkin et al., 2013a).

In the screening carried out here testing 96 samples from 2008 to 2011, we did not identify any substitutions at I222, however we did identify four samples which had higher IC_{50} values for oseltamivir. These viruses demonstrated altered kinetics of binding of oseltamivir and sequence analysis identified NA substitutions known to cause reduced oseltamivir sensitivity. Two NAs had an N294S substitution, a third had an S246N NA substitution, but we could not identify a unique change in the fourth.

Others have also more recently detected three of 30 clade 2.3.4 viruses from Vietnam with I222T NA substitutions, with reduced sensitivity to oseltamivir (Nguyen et al., 2013). In addition, they also found one of 70 clade 2.3.2.1 viruses had an H275Y substitution, with highly reduced sensitivity to oseltamivir. Its replicative fitness was comparable to wild type. Each of the mutant viruses we identified from Indonesia was clearly the predominant phenotype and genotype in its sample, as the sequence chromatograms did not reveal any contaminating peaks at the site of the mutation. Hence there was no evidence of a mixed population of wild type and mutant viruses. Since two separate samples had viruses with the same N294S substitution, this suggests these viruses were fit and able to transmit within poultry flocks.

The N294S substitution has been reported in viruses isolated from oseltamivir treated patients infected with either seasonal or (H5N1) viruses, (Kiso et al., 2004; Le et al., 2005). Some Egyptian (H5N1) viruses from untreated humans were also reported to have an N294S NA substitution, which conferred a 12–15-fold increase in IC_{50} in an NA inhibition assay (Earhart et al., 2009; Kayali et al., 2011). (H5N1) viruses with this substitution have also emerged spontaneously after passage in ferrets (Govorkova et al., 2010) in both clade 1 and clade 2 viruses. While many substitutions may compromise virus fitness, one study found that although human (H5N1) viruses with the N294S substitution were attenuated in mice and ferrets compared to the parent virus, one of the infected ferrets died from a systemic infection, demonstrating that the potential for lethality remained (Kiso et al., 2011). Additionally, the efficacy of oseltamivir against this mutant virus was substantially impaired in ferrets. Similarly both clade 1 and clade 2 (H5N1) N294S mutant viruses generated by reverse genetics, demonstrated reduced oseltamivir susceptibility but retained their lethality in mice or ferrets respectively (Ilyushina et al., 2010; Yen et al., 2007).

We had previously tested NAI sensitivity of (H5N1) viruses from Cambodia, and found that reduced oseltamivir sensitivity correlated with an S246G substitution (McKimm-Breschkin et al., 2013a). Screening of (H5N1) clade 2.3.2 viruses from Lao People's Democratic Republic in 2006–2008 identified three outliers with reduced oseltamivir susceptibility, including two with an S246N substitution (Boltz et al., 2010). This decreased oseltamivir sensitivity by 24-fold. Serine 246 is reported to mediate hydrogen-bonded ligand contacts with sialic

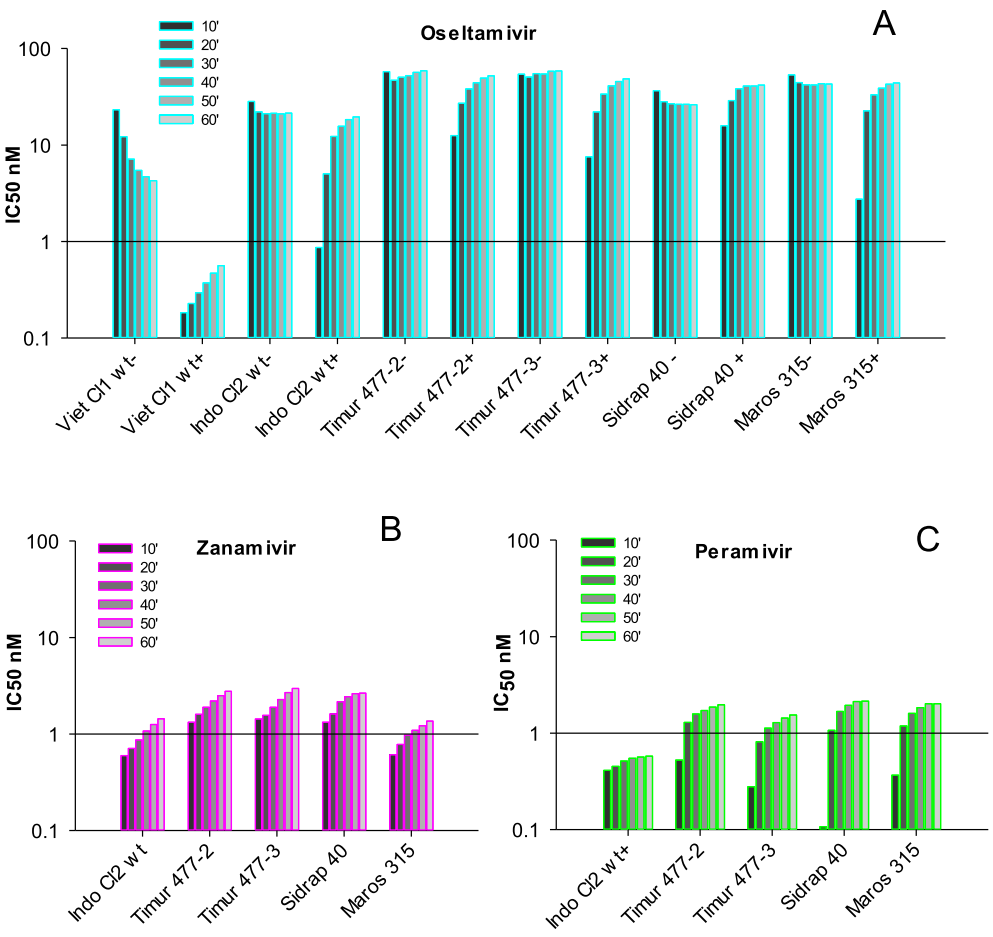

Fig. 2. IC_{50} kinetics of binding of oseltamivir, zanamivir and peramivir Virus abbreviations are as follows: Viet Cl1 = clade 1 A/chicken/Vietnam/08/2004, Indo Cl2 = Indonesian clade 2.1 A/chicken/Wates/126/2005, Timur 477-2 = A/chicken/Aceh Timur/477-08-2/2008, Timur 477-3 = A/chicken/Aceh Timur/477-08-3/2008, Maros-315 = A/chicken/Maros/315/2009, Sidrap-40 = A/chicken/Sidrap/40/2009. + is virus and drug preincubated, - no preincubation. A) The clade 1 Vietnam virus shows slow binding of oseltamivir, with the IC_{50} much lower with preincubation compared to no preincubation. In contrast, rapid dissociation occurs after preincubation with oseltamivir for the clade 2 Wates virus, and the final IC_{50} is similar to without preincubation. IC_{50} values are higher and dissociation for the mutants is even more rapid for oseltamivir. B) Minimal difference is seen for zanamivir. C) Faster dissociation is obvious for all mutants after incubation with peramivir, compared to the wild type.

acid (Landon et al., 2008) and clearly S246G or S246N as we have shown here, can impact on oseltamivir sensitivity.

Thus, we have yet again demonstrated that oseltamivir resistant (H5N1) viruses continue to emerge in the natural reservoirs of avian species. It is also disturbing and puzzling why the incidence of viruses demonstrating reduced oseltamivir sensitivity remains higher, (4/96 or 4% here) in the avian samples than seen in surveillance of human H1N1, H3N2 and B isolates (1–2%) (Hurt et al., 2016), despite the possible selection in humans due to the use of oseltamivir.

Similarly the avian influenza A(H7N9) viruses are also now a source of avian to human infection (Chen et al., 2013; FAO, 2017; Gao et al., 2013; WHO, 2014) with 1622 reported cases and 619 deaths between 2013 and December 2017 (FAO, 2017). While samples from the environment and chickens, pigeons and ducks have tested positive for the virus (FAO, 2017), no surveillance of NAI susceptibility of avian isolates has yet been reported. It has been shown that oseltamivir treatment of humans infected with (H7N9) virus, can lead to the selection of highly resistant viruses with an R292K substitution (Yen et al., 2013).

With the potential for emergence of a pandemic strain from this avian reservoir, it is critical to continue to carry out surveillance in poultry and avian species of not only (H5N1) viruses, but also other avian viruses including (H7N9) viruses, in order to have appropriate strategies in place for antiviral therapy in the event of a new pandemic.

Declarations of interest

None.

Author's contribution

Conceived and designed the experiments: JMB. Performed the experiments: JMB, SB, FW, PS, KRD. Analyzed the data: JMB, SB, FW, KRD. Contributed reagents/materials/analysis tools: P, MA, NH, JM. Wrote the paper: JMB, FW.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2018.03.007.

References

Barrett, S., McKimm-Breschkin, J.L., 2014. Solid phase assay for comparing reactivation rates of neuraminidases of influenza wild type and resistant mutants after inhibitor removal. Antivir. Res. 108, 30–35.

Barrett, S., Mohr, P.G., Schmidt, P.M., McKimm-Breschkin, J.L., 2011. Real time enzyme inhibition assays provide insights into differences in binding of neuraminidase inhibitors to wild type and mutant influenza viruses. PLoS One 6, e23627.

Boltz, D.A., Douangngeun, B., Phommachanh, P., Sinthasak, S., Mondry, R., Obert, C., Seiler, P., Keating, R., Suzuki, Y., Hiramatsu, H., Govorkova, E.A., Webster, R.G., 2010. Emergence of H5N1 avian influenza viruses with reduced sensitivity to neuraminidase inhibitors and novel reassortants in Lao People's Democratic Republic. J. Gen. Virol. 91, 949–959.

Chen, Y., Liang, W., Yang, S., Wu, N., Gao, H., Sheng, J., Yao, H., Wo, J., Fang, Q., Cui, D., Li, Y., Yao, X., Zhang, Y., Wu, H., Zheng, S., Diao, H., Xia, S., Chan, K.H., Tsoi, H.W., Teng, J.L., Song, W., Wang, P., Lau, S.Y., Zheng, M., Chan, J.F., To, K.K., Chen, H., Li, L., Yuen, K.Y., 2013. Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. Lancet. http://dx.doi.org/10.1016/s0140-6736(13) 60903–60904.

Earhart, K.C., Elsayed, N.M., Saad, M.D., Gubareva, L.V., Nayel, A., Deyde, V.M., Abdelsattar, A., Abdelghani, A.S., Boynton, B.R., Mansour, M.M., Essmat, H.M., Klimov, A., Shuck-Lee, D., Monteville, M.R., Tjaden, J.A., 2009. Oseltamivir resistance mutation N294S in human influenza A(H5N1) virus in Egypt. J. Infect. Public Health 2, 74–80.

FAO, 2017. H7N9 Situation Update. http://www.fao.org/ag/againfo/programmes/en/empres/H7N9/situation_update.html, Accessed date: 12 December 2017.

Gao, R., Cao, B., Hu, Y., Feng, Z., Wang, D., Hu, W., Chen, J., Jie, Z., Qiu, H., Xu, K., Xu, X., Lu, H., Zhu, W., Gao, Z., Xiang, N., Shen, Y., He, Z., Gu, Y., Zhang, Z., Yang, Y., Zhao, X., Zhou, L., Li, X., Zou, S., Zhang, Y., Li, X., Yang, L., Guo, J., Dong, J., Li, Q., Dong, L., Zhu, Y., Bai, T., Wang, S., Hao, P., Yang, W., Zhang, Y., Han, J., Yu, H., Li, D., Gao, G.F., Wu, G., Wang, Y., Yuan, Z., Shu, Y., 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. N. Engl. J. Med. 368, 1888–1897.

Govorkova, E.A., Ilyushina, N.A., Marathe, B.M., McClaren, J.L., Webster, R.G., 2010. Competitive fitness of oseltamivir-sensitive and -resistant highly pathogenic H5N1 influenza viruses in a ferret model. J. Virol. 84, 8042–8050.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hartaningsih, N., Wibawa, H., Pudjiatmoko, Rasa, F.S., Irianingsih, S.H., Dharmawan, R., Azhar, M., Siregar, E.S., McGrane, J., Wong, F., Selleck, P., Allen, J., Broz, I., Torchetti, M.K., Dauphin, G., Claes, F., Sastraningrat, W., Durr, P.A., 2015. Surveillance at the molecular level: developing an integrated network for detecting variation in avian influenza viruses in Indonesia. Prev. Vet. Med. 120, 96–105.

Hurt, A.C., Besselaar, T.G., Daniels, R.S., Ermetal, B., Fry, A., Gubareva, L., Huang, W., Lackenby, A., Lee, R.T., Lo, J., Maurer-Stroh, S., Nguyen, H.T., Pereyaslov, D., Rebelo-de-Andrade, H., Siqueira, M.M., Takashita, E., Tashiro, M., Tilmanis, D., Wang, D., Zhang, W., Meijer, A., 2016. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2014-2015. Antivir. Res. 132, 178–185.

Hurt, A.C., Ernest, J., Deng, Y.M., Iannello, P., Besselaar, T.G., Birch, C., Buchy, P., Chittaganpitch, M., Chiu, S.C., Dwyer, D., Guigon, A., Harrower, B., Kei, I.P., Kok, T., Lin, C., McPhie, K., Mohd, A., Olveda, R., Panayotou, T., Rawlinson, W., Scott, L., Smith, D., D'Souza, H., Komadina, N., Shaw, R., Kelso, A., Barr, I.G., 2009a. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in oceania, South East Asia and South Africa. Antivir. Res. 83, 90–93.

Hurt, A.C., Holien, J.K., Parker, M., Kelso, A., Barr, I.G., 2009b. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation. J. Virol. 83, 10366–10373.

Hurt, A.C., Lowther, S., Middleton, D., Barr, I.G., 2010. Assessing the development of oseltamivir and zanamivir resistance in A(H5N1) influenza viruses using a ferret model. Antivir. Res. 87, 361–366.

Ilyushina, N.A., Seiler, J.P., Rehg, J.E., Webster, R.G., Govorkova, E.A., 2010. Effect of neuraminidase inhibitor-resistant mutations on pathogenicity of clade 2.2 A/Turkey/15/06 (H5N1) influenza virus in ferrets. PLoS Pathog. 6 e1000933.

Kayali, G., El-Shesheny, R., Kutkat, M.A., Kandeil, A.M., Mostafa, A., Ducatez, M.F., McKenzie, P.P., Govorkova, E.A., Nasraa, M.H., Webster, R.G., Webby, R.J., Ali, M.A., 2011. Continuing threat of influenza (H5N1) virus circulation in Egypt. Emerg. Infect. Dis. 17, 2306–2308.

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

Kiso, M., Ozawa, M., Le, M.T., Imai, H., Takahashi, K., Kakugawa, S., Noda, T., Horimoto, T., Kawaoka, Y., 2011. Effect of an asparagine-to-serine mutation at position 294 in neuraminidase on the pathogenicity of highly pathogenic H5N1 influenza A virus. J. Virol. 85, 4667–4672.

Landon, M.R., Amaro, R.E., Baron, R., Ngan, C.H., Ozonoff, D., McCammon, J.A., Vajda, S., 2008. Novel druggable hot spots in avian influenza neuraminidase H5N1 revealed by computational solvent mapping of a reduced and representative receptor ensemble. Chem. Biol. Drug Des. 71, 106–116.

Le, Q.M., Kiso, M., Someya, K., Sakai, Y.T., Nguyen, T.H., Nguyen, K.H., Pham, N.D., Ngyen, H.H., Yamada, S., Muramoto, Y., Horimoto, T., Takada, A., Goto, H., Suzuki, T., Suzuki, Y., Kawaoka, Y., 2005. Avian flu: isolation of drug-resistant H5N1 virus. Nature 437, 1108.

Little, K., Leang, S.K., Butler, J., Baas, C., Harrower, B., Mosse, J., Barr, I.G., Hurt, A.C., 2015. Zanamivir-resistant influenza viruses with Q136K or Q136R neuraminidase residue mutations can arise during MDCK cell culture creating challenges for antiviral susceptibility monitoring. Euro Surveill. 20.

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

McKimm-Breschkin, J.L., 2013. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. Influenza Other Respi Viruses 7 (Suppl. 1), 25–36.

McKimm-Breschkin, J.L., Barrett, S., Pudjiatmoko, Azhar, M., Wong, F.Y., Selleck, P., Mohr, P.G., McGrane, J., Kim, M., 2013a. I222 neuraminidase mutations further reduce oseltamivir susceptibility of Indonesian clade 2.1 highly pathogenic avian influenza A(H5N1) viruses. PLoS One 8, e66105.

McKimm-Breschkin, J.L., Rootes, C., Mohr, P.G., Barrett, S., Streltsov, V.A., 2012. In vitro passaging of a pandemic H1N1/09 virus selects for viruses with neuraminidase mutations conferring high-level resistance to oseltamivir and peramivir, but not to zanamivir. J. Antimicrob. Chemother. 67, 1874–1883.

McKimm-Breschkin, J.L., Selleck, P.W., Usman, T.B., Johnson, M.A., 2007. Reduced sensitivity of influenza A (H5N1) to oseltamivir. Emerg. Infect. Dis. 13, 1354–1357.

McKimm-Breschkin, J.L., Williams, J., Barrett, S., Jachno, K., McDonald, M., Mohr, P.G., Saito, T., Tashiro, M., 2013b. Reduced susceptibility to all neuraminidase inhibitors of influenza H1N1 viruses with haemagglutinin mutations and mutations in non-conserved residues of the neuraminidase. J. Antimicrob. Chemother. 68, 2210–2221.

Meijer, A., Lackenby, A., Hungnes, O., Lina, B., van-der-Werf, S., Schweiger, B., Opp, M., Paget, J., van-de-Kassteele, J., Hay, A., Zambon, M., 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season. Emerg. Infect. Dis. 15, 552–560.

Monto, A.S., McKimm-Breschkin, J.L., Macken, C., Hampson, A.W., Hay, A., Klimov, A., Tashiro, M., Webster, R.G., Aymard, M., Hayden, F.G., Zambon, M., 2006. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob. Agents Chemother. 50, 2395–2402.

Nguyen, H.T., Nguyen, T., Mishin, V.P., Sleeman, K., Balish, A., Jones, J., Creanga, A., Marjuki, H., Uyeki, T.M., Nguyen, D.H., Nguyen, D.T., Do, H.T., Klimov, A.I., Davis, C.T., Gubareva, L.V., 2013. Antiviral susceptibility of highly pathogenic avian influenza A(H5N1) viruses isolated from poultry, Vietnam, 2009–2011. Emerg. Infect. Dis. 19, 1963–1971.

Oakley, A.J., Barrett, S., Peat, T.S., Newman, J., Streltsov, V.A., Waddington, L., Saito, T., Tashiro, M., McKimm-Breschkin, J.L., 2010. Structural and functional basis of resistance to neuraminidase inhibitors of influenza B viruses. J. Med. Chem. 53, 6421–6431.

OIE, 2017. Update on Avian Influenza in Animals (Types H5 and H7). http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2017/, Accessed date: 22 May 2017.

Okomo-Adhiambo, M., Nguyen, H.T., Sleeman, K., Sheu, T.G., Deyde, V.M., Garten, R.J., Xu, X., Shaw, M.W., Klimov, A.I., Gubareva, L.V., 2010. Host cell selection of influenza neuraminidase variants: implications for drug resistance monitoring in A(H1N1) viruses. Antivir. Res. 85, 381–388.

Potier, M., Mameli, L., Belisle, M., Dallaire, L., Melancon, S.B., 1979. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl- alpha-D-N-acetylneuraminate) substrate. Anal. Biochem. 94, 287–296.

Takashita, E., Meijer, A., Lackenby, A., Gubareva, L., Rebelo-de-Andrade, H., Besselaar, T., Fry, A., Gregory, V., Leang, S.K., Huang, W., Lo, J., Pereyaslov, D., Siqueira, M.M., Wang, D., Mak, G.C., Zhang, W., Daniels, R.S., Hurt, A.C., Tashiro, M., 2015. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2013-2014. Antivir. Res. 117, 27–38.

WHO, 2012. Meetings of the WHO working group on surveillance of influenza antiviral susceptibility - Geneva, November 2011 and June 2012. Wkly. Epidemiol. Rec. 87, 369–374.

WHO, 2014. Human Infections with Avian Influenza A(H7N9) Virus -Summary of Surveillance and Investigation Findings. http://www.who.int/influenza/human_animal_interface/influenza_h7n9/riskassessment_h7n9_27june14.pdf, Accessed date: 30 September 2014.

WHO, 2015. Cumulative Number of Confirmed Human Cases for Avian Influenza A(H5N1) Reported to WHO, 2003-2015. http://www.who.int/influenza/human_animal_interface/EN_GIP_20150717cumulativeNumberH5N1cases.pdf?ua=1, Accessed date: 29 September 2015.

WHO, 2017. Cumulative Number of Confirmed Human Cases for Avian Influenza A(H5N1) Reported to WHO, 2003-2017. http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/, Accessed date: 12 December 2017.

Yen, H.L., Ilyushina, N.A., Salomon, R., Hoffmann, E., Webster, R.G., Govorkova, E.A., 2007. Neuraminidase inhibitor-resistant recombinant A/Vietnam/1203/04 (H5N1) influenza viruses retain their replication efficiency and pathogenicity in vitro and in vivo. J. Virol. 81, 12418–12426.

Yen, H.L., McKimm-Breschkin, J.L., Choy, K.T., Wong, D.D., Cheung, P.P., Zhou, J., Ng, I.H., Zhu, H., Webby, R.J., Guan, Y., Webster, R.G., Peiris, J.S., 2013. Resistance to neuraminidase inhibitors conferred by an R292K mutation in a human influenza virus H7N9 isolate can be masked by a mixed R/K viral population. MBio 4.