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Short Communication

Mixed influenza A and B infections complicate the detection of influenza viruses with altered sensitivities to neuraminidase inhibitors

Peter G. Mohr [a], Henriette Geyer [b,1], Jennifer L. McKimm-Breschkin [b,*]

[a] CSIRO Livestock Industries, Australian Animal Health Laboratory (AAHL), Private Bag 24, Geelong VIC 3220, Australia
[b] CSIRO Materials Science and Engineering, 343 Royal Parade, Parkville, VIC 3052, Australia

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ABSTRACT

Previously, three influenza A(H3N2) isolates with a reduced susceptibility to the neuraminidase inhibitors (NAIs) zanamivir and oseltamivir were identified during screening by the Neuraminidase Inhibitor Susceptibility Network (NISN). The isolates were from untreated patients from the first three years post licensure of the NAIs. We plaque-purified progeny from each of these isolates and determined the NAI sensitivity of each plaqued population. Sequencing and serology for each population revealed that the isolates contained a mix of wild type influenza A(H3N2) and influenza B. The NAI susceptibility reductions that had originally been reported were a consequence of influenza B neuraminidases that have lower relative NAI sensitivities, rather than being due to resistant influenza A(H3N2) viruses. Our study highlights the need to check for mixed influenza infections when isolates with potentially lower sensitivities to NAIs are identified.

Global screening of the susceptibility of 882 influenza A(H3N2) isolates to the neuraminidase inhibitors (NAIs) zanamivir and oseltamivir by the Neuraminidase Inhibitor Susceptibility Network (NISN) previously identified three viruses with decreased susceptibility to oseltamivir in a neuraminidase (NA) enzyme inhibition assay (Monto et al., 2006). One virus, A/Belgium/969/2002, was described as having an NA Q226H mutation, a second, A/Denmark/25/2002, had no apparent NA mutations and a third, A/Greece/110/2000 had an NA E41G mutation. Further to this, we also selected a fourth virus from the same screening, A/Lipetsk/14/2002, as its mean 50% inhibitory concentration (IC_{50}) was higher than the influenza A(H3N2) mean for the same year but it had not fitted the statistical criteria used in the original study. In the original study each virus was initially isolated, amplified and serotyped by hemagglutination inhibition (HAI) in WHO Collaborating Centers, and an aliquot was supplied to Viromed (Minneapolis, MN) where the viruses were amplified and tested in NA enzyme inhibition assays.

We obtained the four potentially oseltamivir resistant viruses from Viromed, amplified them in Madin–Darby canine kidney cells (MDCKs) and tested their sensitivity to zanamivir and oseltamivir with a MUNANA based fluorometric (FL) enzyme inhibition assay (McKimm-Breschkin et al., 1996). The viruses were then plaqued in MDCKs or MDCK-SIATs (that over-express the gene for α-2, 6 linked sialic acids kindly provided by Dr H D Klenk, Marburg Germany) and at least 6 plaques were picked, amplified and again tested in the FL assay. The NA sensitivity of each pure virus population was also determined with a chemiluminescent (CL) enzyme inhibition assay (Wetherall et al., 2003) to enable comparison with the IC_{50} values obtained in the original study. The NA gene of each plaque-purified virus was then amplified by RT-PCR, sequenced (GenBank Accessions HQ695925–HQ695929) and compared to NA sequences in the database of viruses circulating during the same period.

After our initial amplification of the Viromed samples, we confirmed that A/Belgium/969/2002, A/Denmark/25/2002 and A/Lipetsk/14/2002 had reduced susceptibility to oseltamivir in the FL assay with IC_{50}s of 23, 19 and 18 nM, respectively. In contrast to the original study, however, A/Greece/110/2000 had an oseltamivir IC_{50} of 0.3 nM, that was similar to that of a reference influenza A(H3N2) virus. Plaque-purification of A/Belgium/969/2002 revealed two distinct populations based on their sensitivity in the FL assay (Table 1). Plaque population (pp) 1 represented plaques with IC_{50} values similar to a reference influenza A (H3N2) wild-type strain. The full length N2 gene was sequenced from this population and found to contain the Q226H mutation initially reported by Monto et al. (2006) and postulated to cause the altered drug sensitivity for this isolate. However, plaque-purification in our study clarified that pp1 with the N2 Q226H mutation did not have altered drug sensitivity in the FL assay. The second population, pp2 had

* Corresponding author: Tel.: +61 3 9662 7257; fax: +61 3 9662 7101.
E-mail addresses: peter.mohr@csiro.au (P.G. Mohr), henriettegeyer@googlemail.com (H. Geyer), mck245@csiro.au (J.L. McKimm-Breschkin).
[1] Present address: Robert Koch Institute, Nordufer 20, D-13353 Berlin-Wedding, Germany.

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Table 1
Sensitivities of influenza populations to NAIs determined by fluorescent (FL) and chemiluminescent (CL) enzyme inhibition assays.

Influenza	NA sequence change(s)	Influenza serotype[a]		Oseltamivir IC_{50} (nM)[b]		Zanamivir IC_{50} (nM)[b]	
		A	B	FL	CL	FL	CL
Reference strains							
Influenza A(H3N2)							
A/Fukui/45/2004	N2	+	–	0.46	0.03	0.67	1.45
A/Fukui/45/2004	N2 (E119V)[c]	NA	NA	68.91	2.42	1.06	0.81
Influenza B							
B/Perth/211/2001	B NA	–	+	35.95	2.27	5.10	2.01
B/Perth/211/2001	B NA (D197E)[d]	NA	NA	771.13	13.97	335.28	3.46
Plaque-purified populations							
A/Belgium/969/2002 pp1	N2 (Q226H)[e]	+	–	1.53	0.04	1.80	0.52
A/Belgium/969/2002 pp2	B NA 2002-like	–	+	43.78	2.63	12.40	2.10
A/Denmark/25/2002 pp1	B NA 2002-like	–	+	42.92	2.45	9.93	1.77
A/Lipetsk/14/2002 pp1	N2 (I17L, D151G)[e]	+	–	0.12	0.03	0.86	1.53
A/Lipetsk/14/2002 pp2	B NA 2002-like	–	+	42.70	3.81	10.93	2.06

[a] α-VV NP influenza A (CSIRO Livestock Industries, Geelong, Australia) and α-B/Sichuan/379/99 (WHO, Melbourne, Australia) sera were used in a slot blot to detect influenza A or B, respectively (+ positive, – negative, NA not assessed).
[b] IC_{50} values are means of at least two independent experiments.
[c] Tashiro et al. (2009) E119V strain is less susceptible to oseltamivir than the wild type (wt) strain.
[d] Hurt et al. (2006) D197E strain is less susceptible than the wild-type (wt) strain to both oseltamivir and zanamivir.
[e] Influenza A N2 sequence change (N2 numbering) determined by alignment with consensus sequence obtained from Influenza Virus Resource (Bao et al., 2008).

reduced sensitivity to both NAIs in the FL assay but only to oseltamivir in the CL assay. Intriguingly, primers specific for the N2 NA gene were unable to yield a RT-PCR product for pp2; however, primers specific for an influenza B NA were successful. Sequencing confirmed that pp2 was influenza B. Furthermore, the IC_{50} values for pp2 were also similar to our reference influenza B wild-type strain. Thus, the reported decrease in the susceptibility of the isolate was due to the presence of an influenza B wild type virus that had reduced sensitivity to the NAIs when compared with influenza A (H3N2) viruses. Supporting this, influenza B viruses have previously been shown to have lower levels of sensitivity to oseltamivir than influenza A (H3N2) viruses in FL and CL assays (McKimm-Breschkin et al., 2003).

All A/Denmark/25/2002 plaques that we purified had reduced sensitivity to both zanamivir and oseltamivir in the FL assay. Monto et al. (2006) found only drift mutations in the N2 sequences for this isolate. However, we were unable to amplify the N2 gene by RT-PCR, but we were able to amplify the NA gene using influenza B primers. The IC_{50} values for this influenza B population were similar to the reference influenza B wild-type strain. It is therefore likely that the influenza B population we plaque-purified from the amplified stock for this isolate had overgrown the influenza A that was also originally present. Therefore it would appear that the decrease in sensitivity to NAIs reported for the original isolate was again due to the presence of a wild-type influenza B virus.

Testing purified plaques from A/Lipetsk/14/2002 revealed that this isolate also contained two populations represented by pp1 and pp2. Pp1 had IC_{50} values in the FL assay similar to an influenza A (H3N2) wild-type reference strain. Although two mutations (I17L and D151G) were identified in its N2 gene, these did not appear to affect the NAI sensitivity of this virus. This is consistent with previous screening by NISN, (McKimm-Breschkin et al., 2003) although others have reported mutations at D151 can affect the susceptibility of influenza A viruses to NAIs (Lin et al., 2010). Pp2 had an IC_{50} value similar to an influenza B wild-type strain and NA sequencing confirmed that the population was indeed an influenza B.

Serological analysis for each plaque purified population from the three isolates confirmed our RT-PCR detection of either influenza A or B populations (Table 1). The original clinical isolates had been destroyed before our study began and therefore we could not confirm the presence of influenza A and B in these samples. However, we were able to detect both influenza A and B by RT-PCR and sequencing in the stocks supplied by Viromed for A/Belgium/969/2002, A/Denmark/25/2002 and A/Lipetsk/14/2002 (data not shown) which had been used in the assays reported by Monto et al. (2006). BLASTP analysis of the influenza B NAs from the three isolates revealed that they were identical to several influenza B isolates circulating internationally in 2002 (e.g. B/Brisbane/32/02, B/New York/1/02 and B/Tehran/80/02), the same time as the isolation of the influenza A viruses. We therefore speculate that the influenza A and B populations we have characterized for each isolate were present in the patients originally sampled. This finding is supported by the recent characterization of mixed influenza A and B infections in clinical samples from individual patients (Falchi et al., 2008; Ghedin et al., 2009). However, we cannot entirely rule out the possibility that the mixed populations may have arisen during laboratory passage.

A/Greece/110/2000 was identified by Monto et al. (2006) with a reduced sensitivity to oseltamivir similar to A/Belgium/969/2002 and A/Denmark/25/2002. In our laboratory, the NAI sensitivities of A/Greece/110/2000 plaque-purified progeny (IC_{50}s oseltamivir 0.8 nM and zanamivir 0.3 nM) were comparable to an influenza A (H3N2) wild-type strain. We were also only able to detect influenza A (H3N2) by RT-PCR in the Viromed stock. Based on these observations we were unable to determine the source of oseltamivir resistance originally detected by Monto et al. (2006).

In summary, we have identified three cases (A/Belgium/969/2002, A/Denmark/25/2002 and A/Lipetsk/14/2002) where an isolate that was initially serotyped as influenza A (H3N2) with an altered sensitivity to NAIs also contained an influenza B virus. Although each of these influenza B viruses had the same sensitivity as a wild-type influenza B reference strain, the higher basal IC_{50} values of influenza B strains (McKimm-Breschkin et al., 2003) appears to have led to the misidentification of an influenza A (H3N2) isolate with altered NAI sensitivity. Our research demonstrates that it may be prudent in future screens for any influenza isolates with reduced NAI sensitivities to be retested for the presence of other influenza strains, especially when sub-typing relies on the HAI assay, rather than PCR. Furthermore, we have also highlighted the need for plaque-purification to confirm the role of potential NA mutations when screening unselected isolates.

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