Rapid Communications

Monitoring influenza virus susceptibility to oseltamivir using a new rapid assay, iART

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A new rapid assay for detecting oseltamivir resistance in influenza virus, iART, was used to test 149 clinical specimens. Results were obtained for 132, with iART indicating 41 as ‘resistant’. For these, sequence analysis found known and suspected markers of oseltamivir resistance, while no such markers were detected for the remaining 91 samples. Viruses isolated from the 41 specimens showed reduced or highly reduced inhibition by neuraminidase inhibition assay. iART may facilitate broader antiviral resistance testing.

Early detection of drug-resistant influenza viruses is needed for timely modification of policies and recommendations on the use of antivirals [1]. In many countries, neuraminidase (NA) inhibitor(s) are the medications of choice for treatment and prophylaxis of influenza infections, with oseltamivir being most commonly prescribed. The rapid, global spread of oseltamivir-resistant A(H1N1) viruses that emerged in Norway in 2008, necessitated close monitoring of oseltamivir resistance among circulating influenza viruses [2]. The emergence and subsequent seasonal circulation of the 2009 A(H1N1)pdm09 pandemic virus have further reinforced the need for enhanced surveillance. Moreover, there have been reports of locally transmitted oseltamivir-resistant A(H1N1)pdm09 viruses harbouring the NA amino acid (AA) substitution H275Y [3-5], the marker of clinically relevant resistance to oseltamivir [6,7]. Several genotypic methods (e.g. pyrosequencing) have been implemented by surveillance laboratories to screen clinical specimens for the presence of H275Y [8].

Assays to detect oseltamivir-resistant influenza viruses

Neuraminidase inhibition

Unlike sequence-based assays, the NA inhibition (NAI) assay enables the detection of potentially drug-resistant viruses regardless of underlying genetic change(s). It is the gold standard method for assessing susceptibility to NA inhibitors [9,10]. Interpretation of the NAI assay is based on the determined IC50, a drug concentration needed to inhibit 50% of the NA enzyme activity. Depending on the fold increase of IC50 compared with a control, results are reported as normal (NI), reduced (RI) or highly reduced (HRI) inhibition. In the absence of established laboratory correlates of clinically-relevant oseltamivir resistance, all viruses displaying RI/HRI are considered to be potentially drug resistant and as such are monitored [9,10]. Although useful, this method is labour intensive, complex, and requires propagation of the viruses in cell culture. Additionally, the viral NA sequence from both the isolate and matching clinical specimen should be compared to rule out culturing artefacts [9-11]. Due to the complexity of the assay and data interpretation, testing is mainly performed by specialised surveillance laboratories [10,12,13].

New rapid prototype assay

In this study, we investigated whether the influenza Antiviral Resistance Test (iART), a rapid prototype assay developed by Becton Dickinson R and D for research use only, could be used to improve oseltamivir resistance surveillance by providing a simpler and faster testing method. iART utilises an advanced enzyme substrate that enables measurement of NA activity in virus isolates and in clinical specimens. Unlike the substrate used in the bioluminescence-based assay [14], the substrate used in iART is specific to influenza NA, making it more suitable for testing clinical specimens that may contain other pathogens. In this assay, the sample is divided between two wells of a disposable (Figure), one well containing substrate and the other well containing substrate and oseltamivir carboxylate. A simple device is used to measure the chemiluminescent signal generated from each well of the disposable. The
Built-in software calculates the ratio of signal intensity between the wells (R-factor), which appears on the device's display along with the final result: 'resistant' or 'nonresistant'. The threshold between nonresistant and resistant is different for type A and type B viruses, with R-factors of 0.7 and 2.2, respectively. If the NA activity is too low or absent, the message 'insufficient signal' appears on the display.

Clinical specimens (n=149) were either applied to the gravity-fed column as is, or were diluted fivefold using viral transport medium (VTM). Virus isolates (n=76) were diluted 100- or 1,000-fold using VTM to meet the assay requirement (40,000 < signal < 6,000,000 luminescent units).

**Testing viral isolates using the influenza Antiviral Resistance Test**

**International reference panel for neuraminidase inhibition assay**
In the first experiment, the international reference panel for NAI assay was tested using iART and the United States Centers for Disease Control and Prevention (US CDC) standardised fluorescence-based NAI assay [13] (Table 1). Viruses identified as resistant by iART, displayed RI or HRI by NAI assay; viruses with NI were identified as nonresistant, indicating good agreement between the two assays (Table 1).

**A(H1N1)pdm09 virus isolates carrying H275Y mutations**
Monitoring the spread of A(H1N1)pdm09 viruses exhibiting HRI by oseltamivir and carrying H275Y is a priority for surveillance. To evaluate the ability of iART to detect oseltamivir-resistance conferred by H275Y, 13 virus isolates with this mutation, which had been collected between 2009 and 2016 were tested. All these H275Y viruses exhibited HRI by NAI assay and were also identified as resistant by iART with R-factor of 5.3 ± 0.76 (Table 2).

**Virus isolates containing a mix of influenza viruses with and without H275Y mutations**
In some instances, a sample may contain the drug-resistant and wild-type viruses (mix), but still be...
Results of testing viruses from the international reference panel, for resistance to oseltamivir, using the neuraminidase inhibition (NAI) and influenza Antiviral Resistance Test (iART) assays* (n=8)

| Virus Subtype or Lineage | NA amino acid substitution | NAI assay | iART |
|--------------------------|---------------------------|-----------|------|
| A/Mississippi/03/2001 H3N1 | None None | 0.39±0.05 | NI 0.13±0.04 Nonresistant |
| A/Mississippi/03/2001 H3N1 | H275Y H274Y | 337.0±28.93 (876) | HRI 6.06±0.16 Resistant |
| A/Perth/265/2009 H1N1pdm09 | None None | 0.25±0.03 | NI 0.12±0.01 Nonresistant |
| A/Perth/261/2009 H1N1pdm09 | H275Y H274Y | 171.81±20.66 (1,010) | HRI 4.83±0.35 Resistant |
| A/Fukui/20/2004 H3N2 | None None | 0.12±0.02 | NI 0.16±0.05 Nonresistant |
| A/Fukui/45/2004 H3N2 | E119V E119V | 49.53±3.89 (450) | HRI 1.01±0.04 Resistant |
| B/Perth/211/2001 Yamagata | None None | 15.38±0.98 (1) | NI 1.63±0.14 Nonresistant |
| B/Perth/211/2001 Yamagata | D197E D198E | 98.08±20.21 (6) | RI 3.13±0.10 Resistant |

IC50: inhibitory concentration 50%; NA: neuraminidase; R-factor: ratio of chemiluminescent signal intensity generated by viral NA activity on the substrate with and without inhibitor (i.e. oseltamivir carboxylate).

| Item | Description |
|------|-------------|
| a | International Society for Influenza and other Respiratory Virus Diseases Antiviral Group (ISIRV AVG) NAI susceptibility reference panel, a panel of sensitive, resistant and potentially resistant reference viruses to be used as controls for the harmonisation of NAI assays (https://isirv.org/site/index.php/reference-panel). |
| b | NA amino acid substitution position is shown both by straight numbering (type/subtype specific) and N2 subtype numbering. |
| c | Tested using the United States Centers for Disease Control and Prevention (CDC) standardised fluorescence-based NAI Assay [13]. |
| d | IC50, drug concentration required to inhibit 50% of NA activity; mean and standard deviation of at least three independent experiments; Fold, a fold increase in IC50 value compared with the control (IC50 value for the virus lacking the amino acid substitution). |
| e | Criteria for reporting NAI assay results based on IC50 fold increase compared with the reference IC50 value (control virus); for influenza A, normal (<5-fold), reduced (5–50-fold) and highly reduced (>50-fold) inhibition, and for influenza B the same criteria, but using 5-fold, 5–50-fold and >50-fold increases [9]; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition. |
| f | Mean and standard deviation of R-factors; results of at least three independent experiments. |
| g | Output result as shown on the device’s display; result is based on the pre-set cutoffs for influenza A (≥0.7) and B (≥2.2) viruses. |

detected as normally inhibited in the NAI assay. To assess the ability of iART to detect oseltamivir resistance in such samples, we next tested samples with increasing proportions of H275Y (as determined by pyrosequencing [15]). Notably, isolates containing ≥24% of the H275Y variant were identified as resistant by iART, whereas NAI assay required ≥52% of the H275Y variant to detect RI, suggesting that iART was more efficient at this task (Table 3).

**Influenza virus isolates with mutations other than H275Y**

Next, we assessed iART ability to detect influenza viruses harbouring NA mutations other than H275Y and displaying RI/HRI against oseltamivir (Table 2): A(H3N1) pdm09 viruses that displayed RI by oseltamivir carrying the S247R (n=3) or I223R (n=1) were identified as resistant with high R-factors for S247R and an R-factor of 1.99±0.30 for I223R. One virus carrying I223K was detected as nonresistant with an R-factor substantially below the resistance threshold (0.42±0.03). The virus carrying D199G displayed NI (eightfold) by NAI assay and was identified as resistant by iART (Table 2). A(H3N2) viruses that display HRI by NAI assay were all identified as resistant by iART. The R-factor of the R292K virus was much greater than those harbouring either E119V or a four-amino acid deletion (del245–248). Three B/Victoria/2/87-lineage viruses – harbouring E117G, N294S or A200T – that displayed RI against oseltamivir were all identified as resistant by iART (Table 2). B/Yamagata/16/98-lineage viruses harbouring E117A, R150K or R374K, that displayed HRI by NAI assay were identified as resistant; and two viruses carrying H273Y and one carrying G407 presenting borderline NI/RI were identified as nonresistant by iART (Table 2). Finally, a group of viruses from both B/lineages – carrying D197N, K152N and I221V – showed borderline NI/RI by NAI assay (4–8-fold), and these viruses were identified as resistant by iART. These results demonstrate that iART may detect some influenza viruses harbouring NA changes in the enzyme active site (e.g. D199G in A(H3N1)pdm09 and I221V in type B) that otherwise would be classified as NI by oseltamivir using NAI assay. Notably, the criteria to separate viruses exhibiting NI from those with RI is arbitrary [9], and can be refined as more data become available. The interpretation of results obtained for viruses displaying borderline IC50 should be made cautiously.

**Testing of clinical specimens**

Because iART was designed to detect oseltamivir-resistant viruses in human respiratory specimens, we next tested a set of 64 well-characterised specimens collected during a clinical study conducted in 2008–2010.
Table 2a

Results from neuraminidase inhibition (NAI) and iART assays for virus isolates carrying NA amino acid mutations conferring various degrees of oseltamivir resistance (n = 42) or no such mutations (controls; n= 4)

| Virus                | NA mutations Straight numbering | NA mutations N2 numbering | IC$_{50}$, nM Mean ± SDa | Fold | Interpretationb | R-factor, Mean ± SD | Resultc |
|----------------------|--------------------------------|--------------------------|---------------------------|------|-----------------|---------------------|---------|
| A(H1N1)pdm09         |                                |                          |                           |      |                 |                     |         |
| A/Washington/29/2009  | H275Y                          | H274Y                    | 208.76 ± 27.05            | 1,228| HRI             | 4.1 ± 0.12          | Resistant |
| A/North Carolina/39/2009 | H275Y                          | H274Y                    | 199.43 ± 4.38             | 1,173| HRI             | 5.17 ± 0.12         | Resistant |
| A/India/1027/2013    | H275Y                          | H274Y                    | 185.44 ± 15.95            | 1,091| HRI             | 5.30 ± 0.38         | Resistant |
| A/Delaware/08/2011   | H275Y                          | H274Y                    | 174.53 ± 21.24            | 1,027| HRI             | 4.16 ± 0.73         | Resistant |
| A/Hawaii/67/2014     | H275Y                          | H274Y                    | 171.48 ± 31.01            | 1,009| HRI             | 4.21 ± 0.58         | Resistant |
| A/Michigan/65/2015   | H275Y                          | H274Y                    | 158.76 ± 28.68            | 934  | HRI             | 5.77 ± 0.22         | Resistant |
| A/Denmark/528/2009   | H275Y                          | H274Y                    | 153.26 ± 14.47            | 902  | HRI             | 5.30 ± 0.26         | Resistant |
| A/Georgia/31/2016    | H275Y                          | H274Y                    | 150.48 ± 24.48            | 885  | HRI             | 5.60 ± 0.19         | Resistant |
| A/Maryland/04/2011   | H275Y                          | H274Y                    | 145.64 ± 4.41             | 857  | HRI             | 5.30 ± 0.53         | Resistant |
| A/Washington/31/2016 | H275Y                          | H274Y                    | 141.00 ± 9.58             | 829  | HRI             | 5.76 ± 0.60         | Resistant |
| A/Texas/23/2012      | H275Y                          | H274Y                    | 145.07 ± 30.07            | 805  | HRI             | 5.48 ± 0.67         | Resistant |
| A/Colorado/30/2015   | H275Y                          | H274Y                    | 132.40 ± 32.65            | 779  | HRI             | 5.84 ± 0.30         | Resistant |
| A/Texas/4/2009       | H275Y                          | H274Y                    | 120.22 ± 18.65            | 707  | HRI             | 3.85 ± 0.19         | Resistant |
| A/Bolivia/12/2014    | I223R                          | I222R                    | 11.68 ± 0.15              | 65   | RI              | 1.99 ± 0.30         | Resistant |
| A/Tennessee/24/2016  | S247R                          | S246R                    | 6.61 ± 0.45               | 57   | RI              | 5.67 ± 0.37         | Resistant |
| A/India/18/2016      | S247R                          | S246R                    | 6.31 ± 0.09               | 54   | RI              | 5.78 ± 0.47         | Resistant |
| A/Dnipro/133/2014    | S247R                          | S246R                    | 5.66 ± 0.10               | 31   | RI              | 3.79 ± 0.35         | Resistant |
| A/Chile/17/2009      | I223K                          | I222K                    | 2.84 ± 0.65               | 16   | RI              | 0.42 ± 0.03         | Nonresistant |
| A/Pennsylvania/05/2016 | D199G                          | D198G                    | 1.47 ± 0.03               | 8    | NI              | 1.03 ± 0.26         | Resistant |
| A/California/12/2012 | Control                        |                          | 0.18 ± 0.06               | 1    | NI              | 0.24 ± 0.16         | Nonresistant |
| A(H3N2)              |                                |                          |                           |      |                 |                     |         |
| A/Bethesda/956/2006  | R292K                          | R292K                    | 11,000 ± 114,285          | 7.22 ± 0.24 | HRI              | 7.22 ± 0.24 | Resistant |
| A/Texas/12/2007      | E119V                          | E119V                    | 37.92 ± 5.56              | 542  | HRI             | 1.06 ± 0.11         | Resistant |
| A/Massachusetts/07/2013 | E119V                          | E119V                    | 37.33 ± 10.40             | 533  | HRI             | 1.04 ± 0.04         | Resistant |
| A/Arkansas/13/2013   | E119V                          | E119V                    | 34.88 ± 2.69              | 498  | HRI             | 1.22 ± 0.11         | Resistant |
| A/Illinois/03/2015   | E119V                          | E119V                    | 31.98 ± 3.70              | 458  | HRI             | 1.32 ± 0.14         | Resistant |
| A/Washington/33/2014 | E119V                          | E119V                    | 29.83 ± 6.56              | 426  | HRI             | 1.19 ± 0.08         | Resistant |
| A/Massachusetts/07/2013 | Del245–248                    | Del245–248               | 21.70 ± 3.59              | 310  | HRI             | 1.74 ± 0.06         | Resistant |
| A/Washington/01/2007 | Control                        |                          | 0.07 ± 0.02               | 1    | NI              | 0.16 ± 0.07         | Nonresistant |
| B/Victoria lineage   |                                |                          |                           |      |                 |                     |         |
| B/Florida/103/2016   | A200T                          | A201T                    | 318.19 ± 37.76            | 23   | RI              | 7.30 ± 0.09         | Resistant |
| B/Arkansas/13/2013   | E117G                          | E119G                    | 115.54 ± 10.19            | 8    | RI              | 4.34 ± 0.45         | Resistant |
| B/Washington/14/2016 | N294S                          | N294S                    | 108.37 ± 12.31            | 8    | RI              | 2.29 ± 0.55         | Resistant |
| B/Mexico/4/2016      | I221V                          | I222V                    | 58.57 ± 9.38              | 4    | NI              | 2.42 ± 0.03         | Resistant |
| B/Washington/14/2016 | Control                        |                          | 13.99 ± 0.61              | 1    | NI              | 0.95 ± 0.18         | Nonresistant |

Del: deletion; iART: influenza Antiviral Resistance Test; IC$_{50}$: inhibitory concentration 50%; R-factor: ratio of chemiluminescent signal intensity generated by viral neuraminidase activity on the substrate, with and without inhibitor (i.e. oseltamivir carboxylate); SD: standard deviation.

a Mean and standard deviation based on the results from at least three independent experiments.

b Criteria for reporting NAI assay results based on an IC$_{50}$ fold increase compared with the reference IC$_{50}$ value (control virus): for influenza A, normal (< 10-fold), reduced (10–100-fold) and highly reduced (> 100-fold) inhibition, and for influenza B the same criteria, but using < 5-fold, 5–50-fold and > 50-fold increases [9]; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition.

c Output result as shown on the device’s display; result is based on the pre-set cutoffs for influenza A (≥ 0.7) and B (≥ 2.2) viruses.

d Control, a virus lacking NA changes (amino acid substitutions or deletions) associated with altered inhibition by oseltamivir, was included for each antigenic group (type/subtype/lineage) and used to determine a fold change and a degree of inhibition.
Two results were displayed as

Output result as shown on the device’s display; result is based on the pre-set cutoffs for influenza A (≥ 0.7) and B (≥ 2.2) viruses.

Criteria for reporting NAI assay results based on an IC₅₀ fold increase compared with the reference IC₅₀ value (control virus): for influenza A, mean and standard deviation based on the results from at least three independent experiments.

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R-factor of 6.86 ± 1.31. All other specimens were identi-

resistant with a mean

pandemic A(H1N1) viruses harbouring H275Y (n = 32)

[16] (Table 4). All the clinical specimens containing pre-

paradigm A(H1N1) viruses harbouring H275Y (n = 32)

were consistently identified as resistant with a mean

R-factor of 6.86 ± 1.31. All other specimens were identi-

fied as nonresistant (Table 4). As expected, specimens negative for influenza (n = 10) displayed a signal below the level of detection (data not shown). These results

serve as a proof-of-principle that iART can successfully
detect oseltamivir-resistant H275Y viruses directly in

clinical specimens.

Of note, the recommended volume for the iART test in

its current configuration is 0.5 mL of sample, which is

often unavailable at surveillance laboratories. Moreover, clinical specimens submitted to surveillance laboratories commonly undergo freeze-thaw cycles before testing, which adversely affect the integrity of virus particles. To address these concerns, we next tested a set of residual clinical specimens from the 2015/16 US national surveillance that were previously confirmed influenza virus positive; only 0.1 mL of each specimen was used for testing using iART. Of 85 tested, 17 samples (20%) had a signal below the limit of detection; 59 samples (69%) were identified as nonresistant; and nine samples (11%) as resistant (Table 5). These nine harboured H275Y, E119Y or K152N. The matching isolates of these nine clinical specimens displayed RI/HRI in the NAI assay, while the other virus isolates showed NI.

Conclusion

A limitation of this study is that the effect of viral loads in relation to the performance of iART was not investigated. As the iART detects NA activity, one challenge is the difference in NA specific activities of seasonal wild-type viruses, whereby the minimal viral load needed for the iART assay may depend on the virus type/subtype and might not be generalisable. More studies are needed to establish the type/subtype specific limit of detection. Moreover, NA mutations that confer oseltamivir resistance may or may not affect the NA specific activity, so the influence of this on viral load appropriate for the assay would also have to be investigated independently for such viruses.

Taken together, however, the data presented here show that the iART assay can become a valuable tool for surveillance laboratories. iART offers a fast mean for detecting viruses displaying RI/HRI against oseltamivir in either isolates or clinical specimens. It is a simple approach where signal measurement, data analysis and interpretation are done by a compact portable device. The assay robustness is evident from its ability to test specimens under less than optimal conditions (i.e. interference from virus transport media (VTM), multiple freeze/thaw cycles, limited volume). Although iART is not a substitute for NAI assay employed by specialised laboratories, it has great potential to enable a

Table 28
Results from neuraminidase inhibition (NAI) and iART assays for virus isolates carrying NA amino acid mutations conferring various degrees of oseltamivir resistance (n = 42) or no such mutations (controls; n= 4)

| Virus                  | NA mutations | NAI assay | iART |
|-----------------------|--------------|-----------|------|
|                       | Straight      | N2        | IC₅₀  | Fold | Interpretation | R-factor | Result |
|                       | numbering    | numbering | Mean ± SD |      |               | Mean ± SD |       |
|                       |              |           |        |      |               |           |       |
| B/Yamagata lineage    |              |           |        |      |               |           |       |
| B/Illinois/03/2008    | E117A        | E119A     | 1,100 | 112  | HRI           | 10.44 ± 0.26 | Resistant |
| B/Hong Kong/36/2005   | R374K        | R371K     | 1,100 | 112  | HRI           | 9.11 ± 0.28 | Resistant |
| B/Memphis/20/1996     | R150K        | R152K     | 591.47 ± 61.79 | 66  | HRI           | 3.99 ± 0.36 | Resistant |
| B/Vermont/15/2015     | D197N        | D198N     | 73.76 ± 18.17 | 8   | RI            | 2.39 ± 0.18 | Resistant |
| B/Santiago/77552/2015 | D197N        | D198N     | 54.81 ± 6.48 | 6   | RI            | 2.59 ± 0.24 | Resistant |
| B/Gorbea/75877/2015   | D197N        | D198N     | 49.51 ± 8.85 | 6   | RI            | 2.49 ± 0.03 | Resistant |
| B/Ontario/110/2011    | H273Y        | H274Y     | 57.48 ± 6.98 | 6   | RI            | 1.66 ± 0.16 | Nonresistant |
| B/California/88/2015  | H273Y        | H274Y     | 50.18 ± 7.58 | 6   | RI            | 1.78 ± 0.34 | Nonresistant |
| B/Florida/05/2016     | K152N        | K154N     | 43.59 ± 4.88 | 5   | RI            | 4.09 ± 0.29 | Resistant |
| B/Utah/15/2016       | D197N        | D198N     | 38.22 ± 3.19 | 4   | NI            | 3.06 ± 0.58 | Resistant |
| B/Rochester/02/2001   | D197N        | D198N     | 37.08 ± 1.96 | 4   | NI            | 2.40 ± 0.33 | Nonresistant |
| B/Wisconsin/42/2016   | G407S        | G402S     | 36.08 ± 3.52 | 4   | NI            | 1.99 ± 0.10 | Nonresistant |
| B/Rochester/02/2001   | Control      |           | 8.93 ± 0.82 | 1   | NI            | 0.97 ± 0.12 | Nonresistant |

Del: deletion; iART: Influenza Antiviral Resistance Test; IC₅₀: inhibitory concentration 50%; R-factor: ratio of chemiluminescent signal intensity generated by viral neuraminidase activity on the substrate, with and without inhibitor (i.e. oseltamivir carboxylate); SD: standard deviation.

a Mean and standard deviation based on the results from at least three independent experiments.
b Criteria for reporting NAI assay results based on an IC₅₀ fold increase compared with the reference IC₅₀ value (control virus): for influenza A, normal (< 10-fold), reduced (10–100-fold) and highly reduced (> 100-fold) inhibition, and for influenza B the same criteria, but using < 5-fold, 5–50-fold and > 50-fold increases [9]; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition.
c Output result as shown on the device’s display; result is based on the pre-set cutoffs for influenza A (0.7) and B (2.2) viruses.
d Two results were displayed as resistant and one as nonresistant.
broader adoption of influenza antiviral resistance testing in various settings.

The prototype of the iART system tested in this study was configured by the developers for surveillance applications to detect viruses that could be identified by the gold standard NAI assay. Of note, samples collected by surveillance laboratories may be stored in a variety of storage media (e.g. VTM). To accommodate various types of sample media, the current iART workflow includes a buffer exchange to remove media components that interfere with the assay. If this assay is to be used at clinical care settings, this step is not needed, since a buffer optimised for the iART assay can be used for sample collection.

Larger studies are desirable to provide a better understanding of the performance and utility of the iART assay and to establish laboratory correlates (e.g. R-factor threshold) for clinically-relevant resistance. As iART was designed to test influenza A viruses, regardless of their antigenic subtype, the utility of this rapid test in detecting oseltamivir resistance in zoonotic influenza viruses (e.g. avian A(H7N9)) needs to be evaluated, as this would facilitate pandemic preparedness. Nonetheless, we are confident that the implementation of this assay, which is available for national public health agencies, e.g. the US CDC and application by its network of influenza surveillance laboratories, can facilitate timely detection of oseltamivir resistance emergence and spread.

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### Table 3

Results from neuraminidase inhibition (NAI) and iART assays on mixtures of influenza A(H1N1)pdm09 viruses containing different proportions of mutants with H275Y in the neuraminidase (n = 22)

| Virus                  | Pyrosequencing (%) | NAI assay | iART |
|------------------------|--------------------|-----------|------|
|                        | H275   | H275Y | IC₅₀⁻⁻⁻⁻⁻⁻ | R-factor | Result |
| A/Louisiana/08/2013    | 0  | 100  | 190.84 (1,004) | HRI | 5.97   | Resistant |
| A/Mississippi/11/2013  | 3  | 97   | 177.62 (935)  | HRI | 6.67   | Resistant |
| A/North Carolina/04/2014 | 3  | 97   | 199.91 (1,052) | HRI | 6.17   | Resistant |
| A/Michigan/73/2016     | 3  | 97   | 157.39 (828)  | HRI | 5.89   | Resistant |
| A/Texas/09/2014       | 7  | 93   | 131.02 (690)  | HRI | 5.39   | Resistant |
| A/Texas/100/2013      | 9  | 91   | 150.21 (791)  | HRI | 5.03   | Resistant |
| A/Massachusetts/06/2016| 10 | 90   | 121.85 (641)  | HRI | 6.03   | Resistant |
| A/Pennsylvania/18/2014 | 11 | 89   | 127.1 (669)   | HRI | 6.20   | Resistant |
| A/Florida/10/2014     | 14 | 86   | 111.35 (586)  | HRI | 6.46   | Resistant |
| A/Colorado/07/2014    | 16 | 84   | 110.24 (580)  | HRI | 6.22   | Resistant |
| A/Brazil/0257 S2/2016 | 25 | 75   | 97.73 (514)   | HRI | 4.92   | Resistant |
| A/Brazil/9061/2014    | 32 | 68   | 39.32 (207)   | HRI | 3.47   | Resistant |
| A/Quebec/RV1424/2016  | 48 | 52   | 4.14 (22)     | R-factor | 1.93 | Resistant |
| Mix #1*               | 63 | 37   | 1.37 (8)      | NI | 1.09   | Resistant |
| A/Utah/10/2013       | 68 | 32   | 0.98 (5)      | NI | 1.25   | Resistant |
| A/North Carolina/21/2013 | 72 | 28   | 0.95 (5)      | NI | 1.28   | Resistant |
| Mix #2               | 76 | 24   | 0.73 (4)      | NI | 0.71   | Resistant |
| Mix #3               | 84 | 16   | 0.49 (3)      | NI | 0.46   | Nonresistant |
| A/Michigan/36/2016    | 89 | 11   | 0.57 (3)      | NI | 0.43   | Nonresistant |
| Mix #4               | 92 | 8    | 0.37 (2)      | NI | 0.28   | Nonresistant |
| Mix #5               | 96 | 4    | 0.35 (2)      | NI | 0.12   | Nonresistant |
| A/Maryland/08/2013    | 100| 0    | 0.22 (1)      | NI | 0.18   | Nonresistant |

iART: influenza Antiviral Resistance Test; IC₅₀⁻⁻⁻⁻⁻⁻: inhibitory concentration 50%; R-factor: ratio of chemiluminescent signal intensity generated by viral neuraminidase activity on the substrate, with and without inhibitor (i.e. oseltamivir carboxylate).

* Proportion of H275 and H275Y virus subpopulations was determined by a single-nt polymorphism (SNP) pyrosequencing analysis in allele quantification mode (AQ) as described in reference [15].

* Fold increase calculated using the median oseltamivir IC₅₀⁻⁻⁻⁻⁻⁻ for influenza A(H1N1)pdm09 viruses circulating during 2015/16 influenza season.

* Interpretation of NAI assay results based on the fold increase in IC₅₀⁻⁻⁻⁻⁻⁻ value: normal (<10-fold), reduced (10–100-fold) and highly reduced (>100-fold) inhibition; NI, normal inhibition; R-factor, highly reduced inhibition.

* Output result as shown on the device’s display; result is based on the pre-set cutoffs for influenza A (≥0.7) and influenza B (≥2.2) viruses.

* H275 and H275Y mixes were prepared by combining the two virus isolates A/Maryland/08/2013 and A/Louisiana/08/2013, at different ratios.
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Conflict of interest

EF, KW and RJ are employees of Becton Dickinson.

### Table 4

| Type and subtype | Number of specimens | Ct \(\pm\) | iART/R-factor | iART/result |
|------------------|---------------------|---------|----------------|-------------|
| A(H1N1)pdm09    | 32                  | 24.40 ± 2.63 | 6.86 ± 1.31 | Resistant  |
| A(H1N1)pdm09    | 12                  | 21.31 ± 3.25 | 0.06± 0.02  | Nonresistant|
| A(H3N2)         | 10                  | 21.75 ± 2.13 | 0.25± 0.11  | Nonresistant|
| B                | 10                  | 24.46 ± 1.81 | 0.99± 0.10  | Nonresistant|

Ct: cycle threshold; iART: influenza Antiviral Resistance Test; R-factor: ratio of chemiluminescent signal intensity generated by viral neuraminidase activity on the substrate, with and without inhibitor (i.e. oseltamivir carboxylate).

CT value as determined using RT-PCR assay according to the CDC protocols.

Table 5

| Type and subtype | Number of specimens tested | Number of indeterminate | Number of nonresistant | Number of resistant | NA mutation in resistant viruses |
|------------------|---------------------------|-------------------------|------------------------|-------------------|-------------------------------|
| A(H1N1)pdm09     | 34                        | 9                       | 19                     | 6                 | H275Y                          |
| A(H3N2)          | 25                        | 5                       | 18                     | 2                 | E119V                          |
| B                | 26                        | 3                       | 22                     | 1                 | K152N                          |
| Total            | 85                        | 17                      | 59                     | 9                 | Not applicable                 |

NA: neuraminidase.

CT value as determined using RT-PCR assay according to the CDC protocols.

| Type and subtype | Number of specimens tested | Number of indeterminate | Number of nonresistant | Number of resistant | NA mutation in resistant viruses |
|------------------|---------------------------|-------------------------|------------------------|-------------------|-------------------------------|
| A(H1N1)pdm09     | 34                        | 9                       | 19                     | 6                 | H275Y                          |
| A(H3N2)          | 25                        | 5                       | 18                     | 2                 | E119V                          |
| B                | 26                        | 3                       | 22                     | 1                 | K152N                          |
| Total            | 85                        | 17                      | 59                     | 9                 | Not applicable                 |

NA: neuraminidase.

CT value as determined using RT-PCR assay according to the CDC protocols.

Authors’ contributions

Designed the study: LVG, KW. Generated and analysed antiviral susceptibility data: VPM, EF and EH. Generated sequencing data: JB. Clinical data analysis and interpretation: AMF and AB. Revised the article: WK, LVG and KW. Provided supervisory oversight: DEW, RJ and RS. All authors further edited the manuscript and approved the final version of the paper.

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