INTRODUCTION

Zoonotic and animal influenza A viruses pose a significant threat to public health; they can cause severe disease in humans with little protection afforded by seasonal vaccination due to antigenic differences.\(^1\) NAIs are routinely used to treat individuals infected with influenza viruses, regardless of subtype, and the oseltamivir is the most commonly prescribed anti-influenza therapeutic. Antiviral resistance can emerge in nature or following treatment with NAIs through changes to the surface antigen NA that affect neuraminidase inhibitor (NAI) binding. Such changes may cause resistance to one or more NAIs.\(^2\)

While NA gene sequence analysis is often used to screen viruses for established markers of resistance, genetic analysis cannot identify viruses carrying new molecular markers, or assess the degree of reduced susceptibility. Thus, phenotypic NAI assays are commonly used to assess viral susceptibility to NAIs.\(^3\) In these assays, virus is diluted to a targeted level of NA activity and tested against serially diluted NAI to determine an IC\(_{50}\), the drug concentration needed to inhibit 50% of NA activity. To report the results for seasonal influenza A viruses, the fold change of the test virus is calculated by comparison to a reference IC\(_{50}\) value, either a subtype-specific median or the IC\(_{50}\) of a control virus lacking the NA change.\(^4\) However, this approach cannot be readily applied to testing and reporting of
non-seasonal influenza virus susceptibility to NAIs because of difficulty of acquiring and testing large numbers of each distinct subtype and wide range of genetic lineages within each subtype. Moreover, NAI results require careful interpretation, as laboratory correlates of clinically relevant resistance have not been established, except for viruses carrying an N1 NA with the H275Y substitution.\(^5\) Infections caused by viruses displaying reduced inhibition (RI) or highly reduced inhibition (HRI) phenotypes may be more difficult to control by therapeutic intervention, which can lead to prolonged illness and virus shedding.\(^6\)

Simple and rapid assays that can be used by surveillance laboratories and in clinical settings are needed to detect viruses with reduced susceptibility to NAIs. As previously reported, the prototype influenza antiviral resistance test (iART), developed by BD Technologies (BARDA Contract HHSO100201300008C), is able to phenotypically detect seasonal influenza viruses that display RI/HRI by oseltamivir.\(^7\) This assay compares influenza-specific sialidase (NA) activity with and without a single drug concentration, requires only 1 hour, and does not need extensive training to carry out. Here, we present similar findings for zoonotic and animal influenza viruses.

## 2 | COMPARISON OF IART TO NAI ASSAY

To verify the ability of iART to efficiently detect NA enzymatic activity and inhibition by oseltamivir of various subtypes (N1 through N9), a variety of zoonotic and animal influenza viruses were tested. This included viruses (n = 45) isolated from wild birds, poultry, a domestic cat, and zoonotic human infections propagated in MDCK cells or fertilized chicken eggs (Table 1). NA sequence analysis did not identify known or suspected markers of resistance to oseltamivir (Table S1). Viruses were tested using both the fluorescence-based NAI and iART assays, as previously described.\(^4\) All virus isolates were found to be susceptible to inhibition by oseltamivir in the iART assay (R-factor ≤0.70). In the NAI assay, all calculated IC\(_{50}\) values were in the nanomolar/sub-nanomolar range; some differences among subtypes were observed, as expected, with the highest IC\(_{50}\) values being for N8 viruses and the lowest for N2 viruses (Table 1). The median IC\(_{50}\) for all subtypes (calculated using an average IC\(_{50}\) for each subtype) was determined to be 0.48 nmol/L (Table S2). Using the median IC\(_{50}\), the fold change was calculated for each isolate. As expected, all tested viruses were determined to be normally inhibited (NI) by oseltamivir, and, therefore, susceptible to this drug, according to the criteria implemented by the Expert Working Group on Antiviral Susceptibility for the WHO Global Influenza Surveillance and Response System\(^2\) (<10-fold increase compared to the median IC\(_{50}\)). The data from the gold standard NAI assay showed good correlation with the results obtained using iART, verifying the test’s ability to detect NA enzymatic activity and inhibition by oseltamivir for non-seasonal influenza viruses.

To verify that iART was able to detect reduced susceptibility to oseltamivir of avian and zoonotic viruses, nine virus isolates with NA amino acid substitutions known to affect oseltamivir susceptibility were tested by both the NAI and iART assays (Table 2). Calculated IC\(_{50}\) values were compared to control viruses that lacked the NA substitution, as well as to the median IC\(_{50}\) value calculated above. The median IC\(_{50}\) fold change calculation is necessary when a matching wild-type virus is not available or a virus with an unknown NA sequence is tested. The method of fold change did not change the interpretation for eight of nine viruses (Table 2). One isolate (Table 2, A/Vietnam/HN30408/2005 clone 1) was interpreted as having RI using the fold change determined with the control virus IC\(_{50}\) normal inhibition (NI) using the fold change determined with the median IC\(_{50}\), and an R-factor that was below the pre-set threshold of 0.70 (0.57). Two viruses (Table 2, A/Ohio/88/2012 and A/Taiwan/1/2013 clone 3) tested as RI by NAI with an R-factor in iART near the threshold (0.62, 0.66). The other six viruses that had RI or HRI phenotypes by the NAI assay showed R-factors above the ≥0.70 threshold in the iART assay.

A wide range of R-factors were observed, which correlated with the range of fold differences determined by NAI assay (Figure S1). Viruses with the highest R-factors (ie, >4.0) were also identified as having HRI by the NAI assay. Viruses with RI or fold change values near the 10-fold cutoff had R-factors near the 0.70 threshold. These results demonstrated that any virus reported as resistant by iART would have RI/HRI by NAI. Non-resistant viruses, particularly those with elevated R-factors, also showed some reduced inhibition by oseltamivir. With further testing and refinement of the R-factor threshold, iART may be able to differentiate between RI and HRI viruses in the future. Alternatively, any specimen with an R-factor above 0.50 could be flagged for sequence analysis and additional testing in the NAI assay. None of the wild-type type viruses shown in Table 1 or seasonal viruses reported previously would be flagged as having potentially reduced susceptibility using a lower threshold for type A viruses.\(^7\)

## 3 | RECOMBINANT N9 PROTEINS WITH KNOWN MARKERS OF RI/HRI BY OSELTAMIVIR

Amino acid substitutions known to reduce susceptibility to oseltamivir E119V, I222K/R, H274Y, R292K, and R371K (N2 numbering) have been detected in the NA of A(H7N9) viruses isolated from humans.\(^8\) In addition, I222T was detected in an A(H7N9) virus isolated from a non-human primate after oseltamivir treatment.\(^9\) To determine whether iART is able to identify NA with these changes as resistant to oseltamivir, the respective recombinant N9 (rN9) proteins were generated using the A/Shanghai/2/2013 NA as a backbone, as previously described.\(^10\) The use of recombinant protein allows testing of amino acid changes that reduce enzymatic activity in addition to reducing susceptibility to NAIs, including R292K (R289K in N9 straight numbering), the most commonly identified NA change detected in H7N9 human cases. The R-factors of rN9 proteins carrying substitutions E119V, I222K/R, H274Y, R292K, or R371K categorized them as resistant to oseltamivir and correlated with NAI assay outcomes (Table 3). The range of R-factors also correlated with the range of
| Virus name | HA subtype | NA subtype | NAI assay<sup>a</sup> | Fold change<sup>b</sup> | R-factor<sup>c</sup> |
|------------|------------|------------|------------------------|-------------------------|---------------------|
| A/Iowa/33/2017 | H1v | N1 | 0.12 | 0.25 | 0.08 |
| A/Ohio/09/2015 | H1v | N1 | 0.39 | 0.80 | 0.06 |
| A/Vietnam/1203/2004 | H5 | N1 | 0.76 | 0.58 | 0.11 |
| A/Alberta/01/2014 | H5 | N1 | 1.24 | 0.96 | 0.03 |
| A/duck/Vietnam/NCVD-680/2011 | H5 | N1 | 1.51 | 1.17 | 0.08 |
| A/guinea fowl/Italy/407/2008 | H7 | N1 | 2.94 | 2.26 | 0.07 |
| A/Michigan/09/2007 | H3v | N2 | 0.27 | 0.21 | 0.22 |
| A/Ohio/83/2012 | H3v | N2 | 0.41 | 0.31 | 0.22 |
| A/Iowa/04/2013 | H3v | N2 | 0.54 | 0.42 | 0.25 |
| A/Ohio/02/2014 | H3v | N2 | 0.49 | 0.38 | 0.31 |
| A/Ohio/4319/2014 | H3v | N2 | 0.54 | 0.42 | 0.19 |
| A/Wisconsin/24/2014 | H3v | N2 | 0.57 | 0.44 | 0.24 |
| A/Michigan/83/2016 | H3v | N2 | 0.32 | 0.25 | 0.23 |
| A/Michigan/84/2016 | H3v | N2 | 0.30 | 0.23 | 0.31 |
| A/Ohio/27/2016 | H3v | N2 | 0.24 | 0.19 | 0.23 |
| A/Ohio/28/2016 | H3v | N2 | 0.27 | 0.21 | 0.17 |
| A/northern pintail/Washington/40964/2014 | H5 | N2 | 0.30 | 0.23 | 0.03 |
| A/New York/108/2016 | H7 | N2 | 0.32 | 0.25 | 0.14 |
| A/feline/New York/15-040082-1/2016 | H7 | N2 | 1.11 | 0.85 | 0.41 |
| A/chicken/Bangladesh/OP-4/2013 | H9 | N2 | 0.38 | 0.29 | 0.11 |
| A/chicken/Bangladesh/3C-44/2014 | H9 | N2 | 0.52 | 0.40 | 0.02 |
| A/chicken/Vietnam/NCVD-L552/2016 | H9 | N2 | 2.65 | 2.04 | 0.02 |
| A/duck/Bangladesh/19D691/2016 | H11 | N2 | 0.67 | 0.51 | 0.12 |
| A/chicken/Mexico/8201/12 | H7 | N3 | 0.78 | 0.60 | 0.08 |
| A/duck/Bangladesh/18D59/2016 | H1 | N4 | 1.66 | 1.28 | 0.14 |
| A/nomadic duck/Bangladesh/740/2011 | H2 | N4 | 3.03 | 2.33 | 0.18 |
| A/duck/Bangladesh/17D747/2016 | H3 | N5 | 2.16 | 1.67 | 0.07 |
| A/duck/Peru/MM17/08 | H4 | N5 | 2.35 | 1.81 | 0.2 |
| A/goose/Bangladesh/19D820/2017 | H5 | N6 | 0.78 | 0.60 | 0.37 |
| A/duck/Bangladesh/19D649/2017 | H5 | N6 | 1.01 | 0.78 | 0.37 |
| A/duck/Bangladesh/19D857/2017 | H5 | N6 | 1.01 | 0.78 | 0.26 |
| A/chicken/Vietnam/NCVD-16A26/2016 | H5 | N6 | 3.65 | 2.81 | 0.09 |
| A/duck/Vietnam/NCVD-90911/2013 | H5 | N6 | 1.57 | 1.21 | 0.1 |
| A/waterfowl/Bangladesh/12301/2013 | H6 | N7 | 0.76 | 0.58 | 0.36 |
| A/duck/Bangladesh/18D769/2017 | H6 | N7 | 1.10 | 0.85 | 0.16 |
| A/duck/Bangladesh/20D677/2016 | H3 | N8 | 6.83 | 5.26 | 0.12 |
| A/duck/Vietnam/NCVD-ND4V3P/2016 | H3 | N8 | 2.41 | 1.85 | 0.08 |
| A/gyrfalcon/Washington/41088-6/2014 | H5 | N8 | 1.68 | 1.29 | 0.09 |
| A/turkey/Indiana/1403/2016 | H7 | N8 | 3.95 | 3.04 | 0.14 |
| A/Jiangxi/09037/2014 | H10 | N8 | 2.56 | 1.97 | 0.35 |
| A/Shanghai/1/2013 | H7 | N9 | 0.78 | 0.60 | 0.36 |
| A/Taiwan/1/2013 | H7 | N9 | 0.95 | 0.73 | 0.08 |
| A/Hong Kong/4553/2016 | H7 | N9 | 1.43 | 1.10 | 0.08 |
| A/Hong Kong/61/2016 | H7 | N9 | 1.14 | 0.88 | 0.08 |
| A/Hong Kong/125/2017 | H7 | N9 | 1.22 | 0.94 | 0.26 |
| Overall range | N1-N9 | 0.09-2.53 | 0.19-5.26 | 0.02-0.41 |

<sup>a</sup>Tested using the US Centers for Disease Control and Prevention standardized fluorescence-based NAI assay.

<sup>b</sup>Fold change shows the fold increase in IC<sub>50</sub> value of the test virus compared with the median IC<sub>50</sub> for all subtypes.

<sup>c</sup>R-factor: ratio of chemiluminescent signal intensity generated by viral NA activity on the substrate with and without inhibitor (ie, oseltamivir carboxylate).
| Virus name                         | Subtype       | NA amino acid substitutiona | NAI assayb | Fold change vs control virus | Fold change vs median of all subtypes | Interpretationc | iART | Result     |
|-----------------------------------|---------------|-----------------------------|------------|------------------------------|--------------------------------------|-----------------|------|------------|
| A/Vietnam/HN30408/2005 clone 1    | H5N1 Clade 1  | n/a N294S                   | 2.76 ± 0.37| 10                           | 6                                    | RI/NI           | 0.57 | Non-resistant |
| A/Vietnam/HN30408/2005 clone 2    | H5N1 Clade 1  | n/a H274Y                   | 189.94 ± 36.95| 687                          | 396                                  | HRI             | 4.06 | Resistant   |
| A/duck/Vietnam/NCVD-664/2010      | H5N1 Clade 2.3.2.1 | n/a H274Y               | 259.91 ± 46.79| 466                          | 541                                  | HRI             | 6.37 | Resistant   |
| A/Ohio/88/2012                    | H3N2v         | n/a S247P                   | 5.22 ± 0.97| 54                           | 11                                   | RI              | 0.62 | Non-resistant |
| A/Taiwan/1/2013 clone 1           | H7N9          | E115V E119V                 | 27.79 ± 2.86| 79                           | 58                                   | RI              | 2.10 | Resistant   |
| A/Taiwan/1/2013 clone 2           | H7N9          | I219R I222R                 | 14.97 ± 6.73| 43                           | 31                                   | RI              | 1.00 | Resistant   |
| A/Taiwan/1/2013 clone 3           | H7N9          | I219K I222K                 | 8.82 ± 0.27| 24                           | 18                                   | RI              | 0.66 | Non-resistant |
| A/Taiwan/1/2013 clone 4           | H7N9          | R289K R292K                 | >1000       | >3000                         | >2000                                | HRI             | 9.84 | Resistant   |
| A/Shanghai/1/2013 clone 1         | H7N9          | R289K R292K                 | >1000       | >3000                         | >2000                                | HRI             | 8.39 | Resistant   |

aNA amino acid substitution position shown using both straight numbering and N2 subtype numbering.
bTested using the US Centers for Disease Control and Prevention standardized fluorescence-based NAI assay. Mean and standard deviation (SD) of at least three independent experiments shown; fold change shows the fold increase in IC_{50} value of the test virus compared with a control virus IC_{50} value (for the virus lacking the amino acid substitution) and using the median IC_{50} of all subtypes.
cCriteria for interpreting NAI assay results based on IC_{50} fold increase compared with the control virus/median IC_{50} value: normal inhibition (NI) <10-fold, reduced inhibition (RI) 10- to 100-fold, and highly reduced inhibition (HRI) >100-fold.
dR-factor: ratio of chemiluminescent signal intensity generated by viral NA activity with and without inhibitor (ie, oseltamivir carboxylate). R-factor interpretation based on pre-set cutoff for influenza A (resistance is ≥0.70).
IC$_{50}$ values (Figure S1); all rN9 with R-factors above 2.0 were identified as having HRI by the NAI assay. The rN9 protein with I222T was identified as non-resistant by iART. In the NAI assay, the fold change conferred by this substitution was below the threshold of 10, further confirming the correlation between the two assays.

IC$_{50}$ values (Figure S1); all rN9 with R-factors above 2.0 were identified as having HRI by the NAI assay. The rN9 protein with I222T was identified as non-resistant by iART. In the NAI assay, the fold change conferred by this substitution was below the threshold of 10, further confirming the correlation between the two assays.

### 4 | IART VS NAI ASSAY UNDER LOW PH CONDITIONS (PH 5.3 VS 6.8)

As mentioned above, R292K is the most commonly reported NA marker in oseltamivir-treated patients infected with A(H7N9) viruses. In addition, this change is also known to reduce enzymatic activity, making detection of drug resistance difficult using the standard NAI assay due to insufficient activity for testing or wild-type activity masking resistance. It was previously reported that detection of R292K viruses could be improved by NAI testing at an acidic pH. To confirm this finding, testing was performed on a highly pathogenic avian influenza A(H7N9) isolate, A/Taiwan/1/2017, containing the R292K substitution. At a standard pH of 6.8, the NAI assay was unable to test this virus isolate as NA activity was below the threshold needed for testing (Table 4). At a pH of 5.3, however, this virus had sufficient NA activity and displayed an HRI phenotype. Notably, iART was able to detect resistance caused by R292K, without modifying the pH conditions of the assay. We previously showed that clinical specimens can be tested directly by iART, even when NA activity is insufficient for testing by NAI. These results confirm and extend those findings and suggest the greater sensitivity of iART to detect resistance in low-activity NA viruses.

**Table 4**

| Virus name | Subtype | NAI assay | Modified pH 5.3 | iART |
|------------|---------|-----------|----------------|------|
| A/Taiwan/01/2017 | HPAI H7N9 | IC$_{50}$ (nmol/L) | IC$_{50}$ (nmol/L) | IC$_{50}$ (nmol/L) |
| IC$_{50}$ | Fold | Result | IC$_{50}$ | Result |
| Standard pH 6.8 | Modified pH 5.3 | R-factor | Result |
| A/Taiwan/01/2017 | HPAI H7N9 | N/A | >1000 | >1500 | HRI | 9.90 ± 1.43 | Resistant |

*NA/Not available because NA enzyme activity level was insufficient for testing. Results confirm and extend those findings and suggest the greater sensitivity of iART to detect resistance in low-activity NA viruses.

Influenza antiviral resistance test is a rapid and sensitive phenotypic assay for the detection of influenza viruses with reduced inhibition by oseltamivir. Unlike sequence-based methods, iART provides phenotypic data that are valuable for the identification of viruses carrying both known and unknown molecular markers.

#### Table 3

**Recombinant neuraminidase (NA) proteins of A/Shanghai/2/2013 (H7N9) with substitutions conferring (highly) reduced inhibition by oseltamivir**

| NA amino acid substitution$^a$ | NAI assay$^b$ | iART |
|-----------------------------|-------------|------|
| Recombinant N9 (straight N9 numbering) | IC$_{50}$ (nmol/L) | Fold change | Interpretation$^c$ | R-factor$^d$ | Result |
| Shanghai/2/2013 | None | 0.31 ± 0.02 | 1 | NI | 0.09 ± 0.08 | Non-resistant |
| E115V | E119V | 55.18 ± 1.02 | 176 | HRI | 2.10 ± 0.22 | Resistant |
| I219K | I222K | 14.89 ± 0.39 | 48 | RI | 0.88 ± 0.14 | Resistant |
| I219R | I222R | 27.01 ± 0.62 | 86 | RI | 1.34 ± 0.05 | Resistant |
| I219T | I222T | 2.79 ± 0.04 | 9 | RI | 0.23 ± 0.05 | Non-resistant |
| H271Y | H274Y | 36.71 ± 0.86 | 117 | HRI | 2.04 ± 0.10 | Resistant |
| R289K | R292K | >1000 | >3192 | HRI | 9.48 ± 0.14 | Resistant |
| R367K | R371K | 24.56 ± 1.26 | 78 | RI | 1.71 ± 0.20 | Resistant |

$^a$NA amino acid substitution position is shown using both straight numbering and N2 subtype numbering.

$^b$Tested using the US Centers for Disease Control and Prevention standardized fluorescence-based NAI assay. Mean and standard deviation (SD) of at least three independent experiments shown; fold change shows the fold increase in IC$_{50}$ value of the test recombinant NA protein compared with the A/Shanghai/2/2013 NA protein IC$_{50}$ value.

$^c$Criteria for interpreting NAI assay results based on the fold increase in IC$_{50}$ value of the test NA compared with the wild-type A/Shanghai/2/2013 NA protein IC$_{50}$ value: normal inhibition (NI) <10-fold, reduced inhibition (RI) 10- to 100-fold, and highly reduced inhibition (HRI) >100-fold.

$^d$R-factor: ratio of chemiluminescent signal intensity generated by viral NA activity on the substrate with and without inhibitor (ie, oseltamivir carboxylate). Mean and standard deviation of R-factors from three independent experiments. R-factor interpretation based on pre-set cutoff for influenza A (resistance is ≥0.70).
associated with reduced susceptibility. As new animal and zoonotic subtype viruses emerge, it is critical to determine their drug phenotype rapidly so that public health authorities and clinicians can better assess treatment options. iART is currently not commercially available, though another influenza-specific assay (QFlu Combo Test by Cellex) uses a similar principal of oseltamivir resistance detection. The future availability of iART depends on demand for point of care assays to detect antiviral resistance.

While the gold standard NAI assay continues to be the assay of choice for surveillance laboratories, it is cumbersome and requires highly trained personnel. iART provides an alternative, simple method for detecting oseltamivir-resistant viruses using a small and portable device with built-in software for data interpretation. Viruses detected by iART with elevated R-factors can be flagged for genetic analysis and comprehensive phenotypic evaluation. This design and ease of use may allow oseltamivir susceptibility testing in locations currently unable to carry out the NAI assay.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.