



# Replacement of neuraminidase inhibitor-susceptible influenza A(H1N1) with resistant phenotype in 2008 and circulation of susceptible influenza A and B viruses during 2009-2013, South Africa

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**Background:** Data on the susceptibility of influenza viruses from South Africa to neuraminidase inhibitors (NAIs) are scarce, and no extensive analysis was done.

**Objectives:** We aimed to determine oseltamivir and zanamivir susceptibility of influenza A and B virus neuraminidases (NAs), 2007-2013, South Africa.

**Patients/Methods:** We enrolled participants through national influenza-like illness surveillance, 2007-2013. Influenza diagnosis was by virus isolation and quantitative polymerase chain reaction (qPCR). Drug susceptibility was determined by chemiluminescence-based NA-STAR/NA-XTD assay. Sanger sequencing was used to determine molecular markers of NAI resistance.

**Results:** Forty percent (6341/15 985) of participants were positive for influenza viruses using virus isolation (2007-2009) and qPCR (2009-2013) methods. A total of 1236/6341 (19.5%) virus isolates were generated of which 307/1236 (25%) were tested for drug susceptibility. During 2007-2008, the median 50% inhibitory concentration (IC<sub>50</sub>) of oseltamivir for seasonal influenza A(H1N1) increased from of 0.08 nmol/L (range 0.01-3.60) in 2007 to 73 nmol/L (range 1.56-305 nmol/L) in

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2008. Influenza A isolates from 2009 to 2013 were susceptible to oseltamivir [A(H3N2) median  $IC_{50}$  = 0.05 nmol/L (range 0.01-0.08); A(H1N1)pdm09 = 0.11 nmol/L (range 0.01-0.78)] and zanamivir [A(H3N2) median  $IC_{50}$  = 0.56 nmol/L (range 0.47-0.66); A(H1N1)pdm09 = 0.35 nmol/L (range 0.27-0.533)]. Influenza B viruses were susceptible to both NAIs. NAI resistance-associated substitutions H275Y, E119V, and R150K (N1 numbering) were not detected in influenza A viruses that circulated in 2009-2013.

**Conclusions:** We confirm replacement of NAI susceptible by resistant phenotype influenza A(H1N1) in 2008. Influenza A and B viruses (2009-2013) remained susceptible to NAIs; therefore, these drugs are useful for treating influenza-infected patients.

#### KEYWORDS

influenza, oseltamivir, South Africa, susceptibility

## 1 | INTRODUCTION

Annually, influenza virus infections account for an estimated 3-5 million cases globally, with 250 000-500 000 deaths.<sup>1</sup> During the 2009 pandemic, an estimated 200 million infections occurred globally and resulted in approximately 138 000 deaths (range 123 000-155 000).<sup>2,3</sup> Following the emergence of adamantane-resistant influenza A viruses, clinical treatment of influenza virus disease is mainly with neuraminidase inhibitors (NAIs): Both zanamivir and oseltamivir was approved in 1999 by the US Food and Drug Administration to treat seasonal influenza.<sup>4,5</sup> Oseltamivir is the most widely used due to ease of oral administration. Data from Australasia and South-East Asia showed that influenza A viruses from 1998 through 2002 were overall more susceptible to the NAIs, oseltamivir, and zanamivir, than influenza B viruses.<sup>6</sup> However, in 2008, oseltamivir resistance was reported at a frequency of 90% globally for seasonal influenza A(H1N1) viruses and was associated with histidine to tyrosine mutation at position 275 (H275Y, N1 numbering) of the NA.<sup>7,8</sup>

During the 2008 influenza season, oseltamivir-resistant influenza A(H1N1) viruses were also isolated from South African patients with influenza-like illness. These influenza A(H1N1) virus isolates (n = 49) had the H275Y substitution and were confirmed to be phenotypically resistant to inhibition by oseltamivir.<sup>9</sup>

Neuraminidase inhibitor resistance associated with the H275Y mutation was reported at a frequency of 3% (169/5152) in influenza A(H1N1)pdm09 virus isolates received at the World Health Organization (WHO) collaborating centers (CCs) from various geographic regions, 2013-2014.<sup>7,10</sup> Global NAI susceptibility surveillance data from WHO-CCs for 2013-2014 include less than 3% African data.<sup>10</sup>

Both oseltamivir and zanamivir are licenced in South Africa. Zanamivir is available since the early 2000s and oseltamivir since 2006.<sup>11,12</sup> Zanamivir is approved for treatment of children aged

7 years and older, whereas oseltamivir can be given to individuals of all ages.<sup>13,14</sup> Although generally thought not to be widely prescribed, limited reports are available on the use of NAIs in South Africa. Benefit of oseltamivir for both treatment of and prophylaxis against influenza-associated respiratory illness in South African infants with low birthweight was reported.<sup>15</sup> Influenza A(H1N1) pdm09 H275Y-resistant phenotype viruses were reported in 1 of 54 (2%) of patients following 5-day standard dose oseltamivir treatment.<sup>16</sup>

We aimed to determine oseltamivir and zanamivir susceptibility of influenza A and B virus NAs, 2007-2013, South Africa, and to investigate amino acid polymorphisms in NA.

## 2 | METHODS

### 2.1 | Surveillance programs

A sentinel surveillance program for influenza-like illness (ILI) (Viral Watch [VW] program) recruited outpatients with a measured fever  $\geq 38^{\circ}\text{C}$  and cough, headache, myalgia, or sore throat (of duration  $\leq 10$  days) during 2007-2013, through medical practitioners in all nine provinces of South Africa.<sup>17</sup>

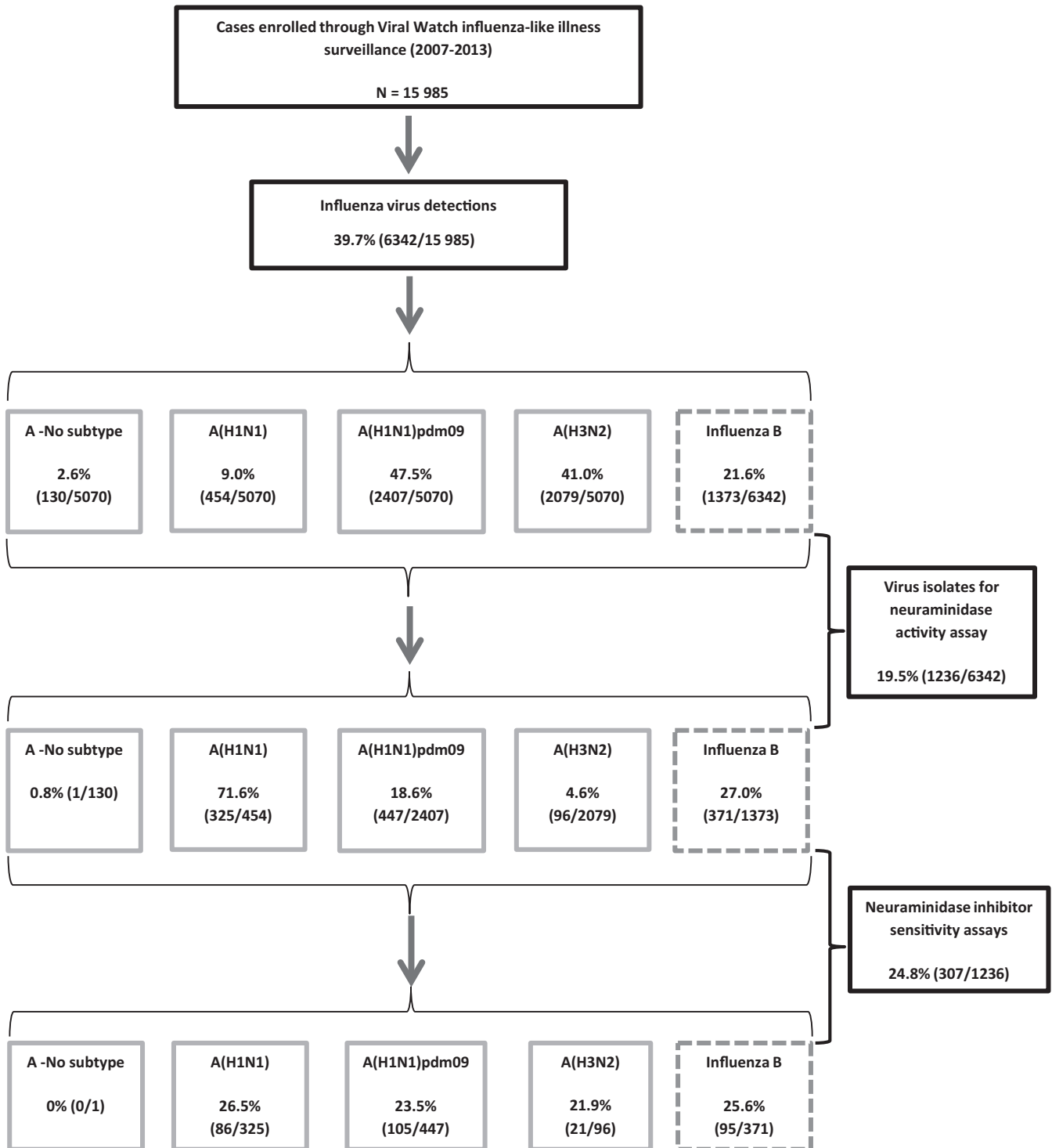
### 2.2 | Study specimens

Respiratory specimens collected included primarily nose and throat swabs collected at the time of diagnosis of the acute respiratory illness episode prior to the initiation of treatment. All upper respiratory tract specimens from patients enrolled from 2007 to 2013 (Figure 1) were collected in viral transport medium (Highveld Biological, Johannesburg, South Africa) or universal transport medium (Copan, Murrieta, CA, USA) as previously described.<sup>18,19</sup> Isolation of respiratory viruses including influenza A and B in Madin-Darby canine

kidney (MDCK) cell cultures was done for specimens submitted during 2007-2009. From 2009, real-time or quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were introduced to test respiratory specimens by single or multiplex respiratory virus qPCR assays, which included diagnosis of influenza A and B viruses.<sup>18,20,21</sup>

### 2.3 | Influenza virus detection and isolation

Before 2009, influenza virus isolation was performed using MDCK cells and immunofluorescence assays (IFAs) with monoclonal antibodies from Light Diagnostics™ (EMD Millipore Corp, Temecula, CA, USA) were used to confirm influenza A and B positive cultures.



**FIGURE 1** Flow diagram of influenza virus detections, virus isolates, and neuraminidase inhibitor susceptibility testing for cases enrolled through influenza-like illness (Viral Watch) surveillance program, South Africa, 2007-2013

Briefly, MDCK cells were seeded in shell vials and maintained with Dulbecco's modified Eagle's medium (DMEM, Lonza, Allendale, NJ, USA) containing 4.5 g/L glucose, with L-glutamine, 10% fetal bovine serum, 2 mmol/L L-glutamine, and antibiotics. After inoculation with 250 microliter clinical specimen for 2 hours on washed serum-free cell monolayers, the cultures were incubated with DMEM serum media at 37°C and harvested 24-73 hours later. Culture supernatant fluids from influenza virus cultures were stored at -70°C.<sup>22,23</sup> Influenza virus isolates (2007-2009) were subtyped serologically by hemagglutination inhibition (HAI) and subtype-specific antisera.<sup>23-25</sup>

Influenza viruses were detected by qRT-PCR, and influenza A positives were subtyped using a qRT-PCR assay from Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA).<sup>18,20,21,26</sup> From 2009 to 2013, influenza-positive specimens with qPCR with cycle threshold ( $C_t$ ) values <30 were selected for virus isolation.

In addition, we selected specimens for influenza virus isolation at the beginning, middle, and end of the South African influenza season (typically May through September).

## 2.4 | Phenotypic drug resistance assays

### 2.4.1 | Neuraminidase inhibitor-susceptible and neuraminidase inhibitor-resistant reference virus isolates

Reference virus isolates (received from Dr L. Gubareva, CDC, GA, USA) were used as controls: (a) A/Washington/10/2008 [A(H1N1)], wild-type strain, oseltamivir and zanamivir susceptible; (b) A/Florida/21/2008 [A(H1N1)], H275Y NA substitution, oseltamivir resistant and zanamivir susceptible; (c) A/Washington/01/2007 [A(H3N2)], wild-type strain, oseltamivir and zanamivir susceptible; (d) A/Texas/12/2007 [A(H3N2)], E119V NA substitution, oseltamivir resistant and zanamivir susceptible; (e) B/Memphis/20/1996 (influenza B), wild-type strain, oseltamivir and zanamivir susceptible; (f) B/Memphis/20/1996 (influenza B), R152K (N2 numbering) or R150K (N1 numbering); NA substitution, oseltamivir and zanamivir resistant; (g) A/California/07/2009 [A(H1N1)pdm09], wild-type strain, oseltamivir and zanamivir susceptible; and (h) A/Texas/48/2009 [A(H1N1)pdm09], H275Y NA substitution, oseltamivir resistant and zanamivir susceptible.<sup>7</sup> N1 numbering is used in manuscript.

### 2.4.2 | The NA-STAR and NA-XTD chemiluminescent assay

The NA-STAR and NA-XTD chemiluminescent assays (Applied Biosystems, Foster City, CA, USA) were used to determine the susceptibility of NA from influenza A and B virus isolates to oseltamivir and zanamivir. Virus culture supernatant fluid aliquots were retrieved and pre-treated with 10% Triton X-100 solution to inactivate virus.<sup>27</sup> The NA inhibition assays were performed following the manufacturers' protocols as described.<sup>28</sup> Briefly, virus isolates were titrated to determine the signal-to-noise ratio of 40 as NA concentration for use in the inhibition assay. The final NAI concentrations

ranging from 0.028 to 550 nanomolar (nmol/L) and the appropriate virus dilution (signal:noise = 40 or a minimum concentration of NA  $\geq 40\,000$  relative light units [RLU]) were pre-incubated for 30 min at room temperature in white flat-bottom 96-well plates (Microlite™ 2+, Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescence reaction was started with addition of NA-STAR/NA-XTD substrate followed by incubation for 15 min at room temperature. Following incubation, light emission was triggered by addition of accelerator buffer. Plates were read with the Glomax luminometer (Promega Inc, Madison, Wisconsin, USA).

The 50% inhibitory concentration ( $IC_{50}$ ) of oseltamivir and zanamivir was determined with the Robust Statistics (Robosage) MS Excel program add-in from AMC Statistics (<http://www.rsc.org/Membership/Networking/InterestGroups/Analytical/AMC/Software/RobustStatistics.asp>). The quartiles and interquartile ranges (IQRs) of the antiviral concentration in log scale were obtained using box-and-whisker analyses and were subsequently back-transformed to obtain final antiviral concentrations in nmol/L. Statistical cutoffs for identification of virus strains with reduced NAI susceptibility (or outliers) were defined as:  $X_{0.75} + 3IQR$  (with  $X_{0.75}$  = 75th percentile). Outliers were defined as viruses with  $IC_{50}$  values > cutoff and >10 times the median or mean  $IC_{50}$ .<sup>7,28</sup> The NAIs oseltamivir (Tamiflu, the metabolized active form oseltamivir carboxylate was used in our assays) and zanamivir (Relenza) were obtained from Hoffmann-La Roche Ltd (Basel, Switzerland) and GlaxoSmithKline (Stevenage, UK). Observed oseltamivir  $IC_{50}$  values reported for resistant phenotype reference viruses were as follows: seasonal A(H1N1) = 93.24 nmol/L, A(H1N1)pdm09 = 70.63 nmol/L, A(H3N2) = 5.24 nmol/L, and influenza B was 92.97 nmol/L for oseltamivir and 27.90 nmol/L for zanamivir with the NA-XTD assay. Reference strains susceptible to oseltamivir and zanamivir had the following respective  $IC_{50}$  values: A(H1N1) = 0.13 and 0.35 nmol/L, A(H1N1)pdm09 = 0.10 and 0.42 nmol/L, A(H3N2) = 0.21 and 1.01 nmol/L, and influenza B = 0.68 and 2.58 nmol/L.<sup>29</sup>

### 2.5 | Detection of drug resistance mutations and drift in influenza virus neuraminidase

The NA genes of influenza A and B strains from 2009 to 2013 were amplified and sequenced from original clinical specimens. Briefly, the universal influenza A and B primers (uni12 and Buni11) were used for complementary DNA (cDNA) preparation followed by nested PCR.<sup>30,31</sup> Sequencing was performed using the BigDye V3.1 chain terminator sequencing reaction ready mix (Applied Biosystems). Sequence chromatograms were assembled and verified using Sequencher® version 5.4.6 DNA sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA, <http://www.genecodes.com>). Multiple sequence alignment and translation to deduced amino acid sequences were done with the BioEDIT software program.<sup>32</sup> NA sequences of influenza A(H1N1)pdm09, A(H3N2), and influenza B viruses generated were screened for the presence of known molecular markers (N1 numbering) of NAI resistance that demonstrated clinical relevance in influenza A viruses of N1 (I223R,

H275Y) or N2 (E119V/I, R293K, N295S) subtypes or influenza B viruses (R150K, D197E).<sup>28,33-37</sup> NAI resistance markers were also identified through surveillance or reverse genetics studies in N1 (E119V, D199G, I223K, N295S) or N2 (E119D, D151E/V, R224K, E276D, R371K) subtypes and influenza B (E117A/D/G/V, P139S, G140R, D197Y, R292K).<sup>28,34-42</sup>

### 3 | RESULTS

#### 3.1 | Influenza viruses detected and isolated

A total of 15 985 specimens were submitted through VW surveillance from 2007 to 2013, of which 6342 (39.7%) specimens were positive for influenza. Of these, 5070 (79.9%) were influenza A (including 101 dual positives; A(H3N2)/A(H1N1)pdm09: 25, A(H3N2)/B: 47, A(H1N1)pdm09/B: 28, A/B: 1) and 1373 were influenza B (21.6%) (Figure 1, Table 1). Influenza A positives comprised of A(H1N1) 9.0% (454/5070), A(H1N1)pdm09 47.5% (2407/5070), A(H3N2) 41.0% (2079/5070), and A no subtype (subtype or serotype could not be determined) 2.6% (130/5070). Culture supernatant fluids from 1236 influenza virus isolates were tested for viral NA activity (Figure 1).

#### 3.2 | Neuraminidase inhibitor susceptibility of influenza A and B isolates

A total of 307 influenza virus isolates from 2007 to 2013 were tested for susceptibility to NAIs in the NA enzyme inhibition assay, including 135 influenza viruses isolated during 2007-2008 (Table 1). Thirty-nine influenza A(H1N1) isolates from 2007 were susceptible to oseltamivir with median  $IC_{50}$ : 0.08 nmol/L (range 0.01-3.60) and to zanamivir with median  $IC_{50}$ : 0.34 nmol/L (range 0.33-12.60)

(Table 2). Four virus isolates were outliers: 2/4 for oseltamivir and 3/4 for zanamivir.

In 2008, the median  $IC_{50}$  values for oseltamivir increased markedly (median 73 nmol/L; range 1.56-305 nmol/L) with 97.9% (46/47) for virus isolates. There was a 912-fold increase in median  $IC_{50}$  values between isolates from 2007 and resistant isolates from 2008. Fifty-three percent (25/47) of influenza A(H1N1) isolates from 2008 remained susceptible to zanamivir (Table 2). Four sporadic cases of seasonal influenza A(H1N1) were detected in 2009, but NAI susceptibility was not determined (Table 1).

During 2007-2008, all except 1/6 A(H3N2) isolates had low NA activity.  $IC_{50}$  for oseltamivir and zanamivir was 0.05 nmol/L and 0.48 nmol/L, respectively, for this isolate. Influenza B isolates were susceptible to both NAIs (Table 2).

For 2009-2013, 172 influenza virus isolates were tested for susceptibility to NAIs (Table 1). Influenza A(H1N1)pdm09 isolates were susceptible to oseltamivir (98.1%; 103/105) and zanamivir (92.4%; 97/105) with respective median  $IC_{50}$  values of 0.11 nmol/L (range 0.01-0.78) and 0.35 nmol/L (range 0.27-0.533) (Table 2). Influenza A(H3N2) isolates were all susceptible to inhibition by oseltamivir ( $n = 18$ ) and zanamivir ( $n = 20$ ) with median  $IC_{50}$  values of 0.05 nmol/L (range 0.01-0.08) and 0.56 nmol/L (range 0.47-0.66), respectively during 2009-2013 (Table 2). Influenza B virus isolates ( $n = 47$ ) from 2009 to 2013 were susceptible to oseltamivir and zanamivir (Table 2).

#### 3.3 | Amino acid substitutions in NA genes of influenza viruses

No seasonal influenza A(H1N1) viruses from 2007 to 2008 were sequenced. A total of 140 partial or near-complete NA gene sequences were generated (43 A(H1N1)pdm09, 38 A(H3N2), 42 B/

**TABLE 1** Number of laboratory-confirmed influenza A and B viruses isolated and tested for susceptibility to neuraminidase inhibitors, 2007-2013, South Africa

	N	2007 n (%)	2008 n (%)	2009 n (%)	2010 n (%)	2011 n (%)	2012 n (%)	2013 n (%)
Influenza A/B	6341	511 (8.1)	389 (6.1)	1753 (27.6)	895 (14.1)	1142 (18.0)	744 (11.7)	907 (14.3)
Influenza subtypes								
A(H1N1)	454	141 (31.0)	309 (68.1)	4 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
A(H1N1)pdm09	2414	—	—	712 (29.5)	211 (8.7)	882 (36.5)	6 (0.2)	603 (25.0)
A(H3N2)	2079	209 (10.1)	13 (6.3)	865 (41.6)	237 (11.4)	159 (7.6)	439 (21.1)	157 (7.6)
<sup>a</sup> A no subtype	130	27 (20.8)	16 (12.3)	59 (45.4)	1 (0.8)	14 (10.8)	10 (7.7)	3 (2.3)
B	1373	134 (9.8)	51 (3.7)	122 (8.9)	468 (34.1)	128 (9.3)	318 (23.2)	152 (11.1)
NA enzyme inhibition								
A(H1N1)	86	39 (45.3)	47 (54.7)	0 (0.0)	—	—	—	—
A(H1N1)pdm09	105	—	—	39 (37.1)	27 (25.7)	26 (24.8)	0 (0.0)	13 (12.4)
A(H3N2)	21	1 (4.8)	0 (0.0)	0 (0.0)	9 (42.9)	2 (9.5)	7 (33.3)	2 (9.5)
B	95	24 (25.3)	24 (25.3)	9 (9.5)	4 (4.2)	20 (21.1)	7 (7.4)	7 (7.4)

N, total number; NA, neuraminidase; (%) =  $n/N \times 100$  for each row; —, means relevant subtype did not circulate.

<sup>a</sup>Subtype or serotype could not be determined.

**TABLE 2** Susceptibility to neuraminidase inhibitors of influenza A and B viruses isolated during 2007-2013 in South Africa

2007-2008		Oseltamivir, IC <sub>50</sub> in nmol/L					Zanamivir, IC <sub>50</sub> in nmol/L					
Influenza type	Year	Isolates tested (N)	Median (range)	IQR	X <sub>0.75</sub>	Statistical cutoff (X <sub>0.75</sub> + 3IQR)	Susceptible	Median (range)	IQR	X <sub>0.75</sub>	Statistical cutoff (X <sub>0.75</sub> + 3IQR)	Susceptible
A(H1N1)	2007	39	0.08 (0.01-3.60)	0.61	0.64	2.47	Yes	0.34 (0.33-12.60)	0.56	0.71	2.39	Yes
	2008	47 <sup>a</sup>	73.00 (1.56-305)	67.18	117.6	319.14	No	0.39 (0.13-1.99)	0.09	0.43	0.70	Yes
A(H3N2)	2007-2008	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Influenza B	2007-2008	48	2.84 (1.76-5.47)	1.70	3.85	8.95	Yes	1.99 (1.27-2.89)	1.29	2.20	6.07	Yes
2009-2013		Oseltamivir, IC <sub>50</sub> in nmol/L					Zanamivir, IC <sub>50</sub> in nmol/L					
Influenza type	Year	Isolates tested (N)	Median (range)	IQR	X <sub>0.75</sub>	Statistical cutoff (X <sub>0.75</sub> + 3IQR)	Susceptible	Median (range)	IQR	X <sub>0.75</sub>	Statistical cutoff (X <sub>0.75</sub> + 3IQR)	Susceptible
A(H1N1) pdm09	2009-2013	105 <sup>b</sup>	0.11 (0.01-0.78)	0.14	0.18	0.60	Yes	0.35 (0.27-0.533)	0.07	0.40	0.61	Yes
A(H3N2)	2009-2013	20 <sup>c</sup>	0.05 (0.01-0.08)	0.03	0.06	0.15	Yes	0.56 (0.47-0.66)	0.12	0.63	0.99	Yes
Influenza B	2009-2013	47 <sup>d</sup>	3.20 (1.56-5.40)	1.47	4.01	8.42	Yes	2.38 (0.91-5.48)	1.35	3.01	7.06	Yes

IC<sub>50</sub>, 50% inhibition concentration; nmol/L, nanomolar; IQR, interquartile range (=75th percentile-25th percentile); X<sub>0.75</sub>, 75th percentile.

Reasons for discrepant number of isolates assayed for oseltamivir vs zanamivir: <sup>a</sup>H1N1 (zanamivir): 25/47 tested, 22 insufficient specimens; <sup>b</sup>H1N1pdm09: 103/105, two failed for both drugs; zanamivir: 97/103, six failed (one showed no inhibition as IC<sub>50</sub> could not be calculated, five insufficient); <sup>c</sup>H3N2 (oseltamivir): 18/20 included, two failed; <sup>d</sup>B(zanamivir): 46/47 included, one insufficient sample. Italic font: outliers with increased IC<sub>50</sub> values (>10x median and >statistical cutoff) present in data.

Victoria, and 17 B/Yamagata lineage) for 2009-2013. Deduced NA amino acid sequences showed the absence of the oseltamivir resistance mutation H275Y in influenza A(H1N1)pdm09 isolates compared to A/California/07/2009 as reference (Figure 2a). Amino acid mutations were observed at positions 44, 48, 106, 200, 241, 248, 260, 299, 351, 369, 382, and 407 when compared to the reference strain, A/California/07/2009. Some amino acid mutations became fixed: V241I, N248D, and Y351F. The N369K mutation first observed in 2010 viruses also became fixed. None of these mutations are associated with reduced susceptibility to NAIs.

Amino acid substitutions associated with continued evolutionary changes in the NA gene of influenza A(H3N2) viruses were observed at positions 26, 40, 43, 81, 93, 143, 147, 215, 221, 238, 258, 329, 331, 335, 367, 372, 402, and 416. The D147N, I215V, S367N mutations became fixed from 2009 to 2013 compared to the A/Brisbane/10/07 reference strain (Figure 2b). These mutations were not associated with resistance to NAIs.

No amino acid substitutions associated with reduced susceptibility to oseltamivir and zanamivir were observed in deduced NA

protein sequences of influenza B lineages, specifically at positions 150 and 197 (Figure 2c and 2d). Drift amino acid substitutions were observed at several amino acid positions in 2010-2012 for B/Victoria and 2010-2013 for B/Yamagata viruses.

## 4 | DISCUSSION

In this study, we confirm change in circulation of oseltamivir-susceptible seasonal influenza A(H1N1) strains in 2007 to almost 100% resistant phenotype strains in 2008 in South Africa. During the same period, Cameroon reported resistant phenotype strains for 70% of A(H1N1) viruses.<sup>43</sup> During the same period, a single influenza A(H3N2) and all influenza B virus isolates from our study remained susceptible to both NAIs.

Influenza A(H1N1)pdm09 and A(H3N2) virus isolates from 2009 to 2013 and 2007 to 2013, respectively, also remained susceptible to inhibition by both NAIs despite the observed increased IC<sub>50</sub> values for some isolates. No resistance was detected for influenza B viruses. Since 2009, seasonal influenza A(H1N1) has not

(A) Amino acid Position	34	40	44	48	106	200	223	241	247	248	260	264	270	275	299	321	351	369	382	386	388	394	407	432
CAL09	I	L	N	T	V	N	I	V	S	N	K	V	N	H	S	I	Y	N	G	N	S	V	V	K
2009 (29)					I			I		D							F					T		
2010 (17)				I				I		D							F	K						
2011 (26)					I			I		D	R				A		F	K						F
2012 (0)								I		D							F	K						
2013 (17)			S			S		I		D							F	K	E					

(B) Amino acid Position	26	40	43	81	93	119	143	147	215	221	222	238	258	275	329	331	335	367	372	402	416
BRIS07	I	Y	N	L	D	E	V	D	I	E	I	T	E	H	N	S	S	S	L	N	S
2009 (8)	T	C/F						N	V									N	F		T
2010 (6)			Y					N	V									N			
2011 (11)			H	P	G			N	V	D		A	K		T	R	G	N		D	N
2012 (8)			H					N	V	D		A				R	G	N		D	N
2013 (5)				P	G		M	N	V									N		D	

(C) Amino acid Position	35	42	45	46	73	87	106	125	150	197	285	295	329	340	345	358	395	401
BRIS08 (VIC)	D	P	I	T	L	C	T	N	R	D	K	S	N	N	S	E	A	V
2007 (2)													D					
2008 (0)																		
2009 (0)																		
2010 (8)	G			I	F			D			E			G			T/V	
2011 (1)			T		F													
2012 (31)	G	T/S	T	I	F	W	N	K				R	D/E	D		K	I	
2013 (0)																		

(D) Amino acid Position	42	46	65	68	71	106	125	128	150	186	197	248	295	340	463	465
FLOR06 (YAM)	Q	T	R	A	V	T	T	K	R	K	D	I	S	D	D	A
2007(1)							K									
2008 (0)																
2009 (0)																
2010 (3)	R			T			K			R				N	N	T
2011 (0)																
2012 (8)	R		H	T	M	I	K	R		R		V	R	N	N	T
2013 (5)		I					I					V	R			

**FIGURE 2** Polymorphism of amino acids in the neuraminidase of (A) influenza A(H1N1)pdm09 viruses aligned with A/California/07/2009 reference (CAL09); (B) influenza A(H3N2) viruses aligned with A/Brisbane/10/07 reference (BRIS07); (C) influenza B viruses of Victoria lineage (VIC) aligned with B/Brisbane/60/2008 reference (BRIS08); and (D) influenza B viruses of Yamagata (YAM) lineage aligned with B/Florida/2006 reference (FLOR06), 2009-2013, South Africa. Amino acid is designated using standard one letter code. Numbers in parenthesis indicate the number of viruses' sequences included in analysis



been detected during seasonal epidemics and has been replaced by A(H1N1)pdm09.<sup>44</sup> Influenza viruses (H1N1 and H1N1pdm09) carrying the H275Y mutation and associated with clinical resistance to oseltamivir displayed > 400-fold increase in  $IC_{50}$ .<sup>9,45</sup>

No seasonal influenza A(H1N1) NAs were sequenced in this study. However, in 2008 the H275Y substitution was reported in resistant strains from South Africa.<sup>9</sup> Circulation of resistant phenotype seasonal influenza A(H1N1) was aborted with emergence of the influenza A(H1N1)pdm09 strain. Since 2009, we did not detect amino acid mutations associated with reduced susceptibility to NAIs. However, the V241I and N369K mutations in resistant influenza A(H1N1)pdm09 isolates are thought to increase the stability of neuraminidases with H275Y substitutions.<sup>46</sup> The I215V NA substitution was observed in A(H3N2) viruses that circulated in the 2013-2014 season and caused borderline reduction in susceptibility to oseltamivir and zanamivir.<sup>10</sup> These observations highlight the need for continued surveillance even if NAIs are not widely used in South Africa.

Resistance to inhibition by oseltamivir was reported at frequencies of 0.2% (2/900 adults) to 1.1% (2/190 children) for influenza A(H3N2) from combined studies including clinical trials conducted in Japan.<sup>47</sup> Oseltamivir resistance was mostly associated with E119V and R292K mutations.<sup>47</sup> In global surveillance, data from 2013 to 2014 H3N2-resistant phenotype isolates were reported at 0.3%.<sup>10</sup> We observed that most A(H3N2) isolates displayed low NA activity, and therefore, NA susceptibility to antivirals could not be assessed. Our inability to obtain A(H3N2) isolates with high levels of NA activity could be related to changes in phenotypic characteristics of A(H3N2) viruses.<sup>48,49</sup> During virus isolation, only immunofluorescence and hemagglutination assays were routinely used to detect virus growth, which may further account for low number of isolates subsequently identified with high NA levels.

Despite observed continued drift in B lineages in this study, these mutations were not associated with NAI resistance or reduced susceptibility. The influenza B/Memphis/20/96 strain carrying the R150K mutation associated with highly reduced resistance to both zanamivir and oseltamivir and used in this study as reference resistant strain was initially isolated from an immune-compromised patient in the United States following zanamivir treatment.<sup>50,51</sup> Susceptibility to NAIs was determined for influenza virus isolates from 24 patients [2009: 13 A(H1N1)pdm09 and 2 influenza B; 2012: 9 A(H3N2)] enrolled through the severe acute respiratory illness (SARI) surveillance.<sup>18,19</sup> Influenza isolates from SARI cases were susceptible to the NAIs oseltamivir and zanamivir (data not shown).

Limitations to our study were as follows: (a) No severe cases were included; (b) virus culture supernatant fluids used were retrieved from freezers. Strains were tested at passages 1-2 to limit mutations introduced through repeated passaging. We are unsure of the effects of long-term storage and possible freeze-thawing on NA concentrations but others used inactivated viruses in the  $IC_{50}$  assay<sup>27</sup>; and (c) only a small proportion of influenza-positive cases were tested annually.

This study provides baseline data on influenza A and B viruses with oseltamivir-susceptible phenotypes in South Africa.

Guidelines for the prevention and treatment of influenza in South Africa are published electronically.<sup>52</sup> However, although oral oseltamivir and inhaled zanamivir are available in South Africa, they are not routinely prescribed.<sup>14,53</sup> This is also observed for hospitalized patients enrolled through our pneumonia surveillance program in public hospitals (C. Cohen, unpublished data). Oseltamivir is prescribed more frequently by private healthcare practitioners, but no data are available on the extent of use in South Africa. Given the very low frequency of resistance reported globally, the use of NAIs to treat patients at high risk of developing severe illness should be promoted.

## CONFLICT OF INTEREST

The authors do not declare financial or personal conflict of interest related to this study.

## DISCLAIMER

The findings and conclusions in this report do not necessarily represent the official position of the US Centers for Disease Control and Prevention. The corresponding author has full access to all data and final responsibility for the decision to submit this publication.

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## REFERENCES

1. WHO. Influenza (Seasonal) fact sheet. <http://www.who.int/media-centre/factsheets/fs211/en/>. Accessed July 6, 2017.
2. Simonsen L, Spreeuwenberg P, Lustig R, et al. Global mortality estimates for the 2009 Influenza Pandemic from the GLaMOR project: a modeling study. *PLoS Med*. 2013;10(11):e1001558.
3. Iuliano AD, Roguski KM, Chang HH, et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. *Lancet*. 2017;391(10127):1285-1300.
4. FDA. Relenza. 1999. [https://www.accessdata.fda.gov/drugsatfda\\_docs/nda/99/21036.cfm](https://www.accessdata.fda.gov/drugsatfda_docs/nda/99/21036.cfm). Accessed May 9, 2018.
5. FDA. Tamiflu. 1999. [https://www.accessdata.fda.gov/drugsatfda\\_docs/nda/99/21087\\_Tamiflu.cfm](https://www.accessdata.fda.gov/drugsatfda_docs/nda/99/21087_Tamiflu.cfm). Accessed May 9, 2018.
6. Hurt AC, Barr IG, Hartel G, Hampson AW. Susceptibility of human influenza viruses from Australasia and South East Asia to the neuraminidase inhibitors zanamivir and oseltamivir. *Antiviral Res*. 2004;62(1):37-45.
7. Okomo-Adhiambo M, Sleeman K, Ballenger K, et al. Neuraminidase inhibitor susceptibility testing in human influenza viruses: a laboratory surveillance perspective. *Viruses*. 2010;2(10):2269-2289.
8. Hurt AC, Ernest J, Deng YM, et al. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Res*. 2009;83(1):90-93.
9. Besselaar TG, Naidoo D, Buys A, et al. Widespread oseltamivir resistance in influenza A viruses (H1N1), South Africa. *Emerg Infect Dis*. 2008;14(11):1809-1810.



10. Takashita E, Meijer A, Lackenby A, et al. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2013-2014. *Antiviral Res.* 2015;117:27-38.
11. Green RJ, Feldman C, Schoub B, et al. Influenza guideline for South Africa—update 2008. *S Afr Med J* 2008;2:224-230.
12. Feldman C. Antiviral agents for prevention and/or treatment of influenza virus infections: old and new. *Contin Med Educ.* 2003;21(6):318-321.
13. Walaza S, Cohen C. Recommendations pertaining to the use of influenza vaccines and influenza antiviral drugs, 2016. *S Afr Med J.* 2016;106(3):251-253.
14. Zar HJ. Influenza in children. *SAJCH.* 2009;3(2):35-37.
15. Holgate SL, Bekker A, Rabie H, Cotton MF. Oseltamivir use in low-birth weight infants during the 2009 nH1N1 influenza outbreak in the Western Cape, South Africa. *J Trop Pediatr.* 2012;58(2):102-106.
16. Aspinall S, Pretorius B, Vally T, Sebopa B, Turner-Babb C. An observational study of influenza A(H1N1)pdm09 viral shedding and resistance under standard-duration oseltamivir treatment. *South Afr J Epidemiol Infect.* 2013;28(2):122-125.
17. Ntshoe GM, McAnerney JM, Tempia S, et al. Influenza epidemiology and vaccine effectiveness among patients with influenza-like illness, viral watch sentinel sites, South Africa, 2005-2009. *PLoS ONE.* 2014;9(4):e94681.
18. Pretorius MA, Madhi SA, Cohen C, et al. Respiratory viral coinfections identified by a 10-plex real-time reverse-transcription polymerase chain reaction assay in patients hospitalized with severe acute respiratory illness—South Africa, 2009-2010. *J Infect Dis.* 2012;206(Suppl 1):S159-S165.
19. Budgell E, Cohen AL, McAnerney J, et al. Evaluation of two influenza surveillance systems in South Africa. *PLoS ONE.* 2015;10(3):e0120226.
20. Jernigan DB, Lindstrom SL, Johnson JR, et al. Detecting 2009 pandemic influenza A (H1N1) virus infection: availability of diagnostic testing led to rapid pandemic response. *Clin Infect Dis.* 2011;52(Suppl 1):S36-S43.
21. Lassauniere R, Kresfelder T, Venter M. A novel multiplex real-time RT-PCR assay with FRET hybridization probes for the detection and quantitation of 13 respiratory viruses. *J Virol Methods.* 2010;165(2):254-260.
22. Barr IG, Komadina N, Durrant C, Sjogren H, Hurt AC, Shaw RP. Circulation and antigenic drift in human influenza B viruses in SE Asia and Oceania since 2000. *Commun Dis Intell Q Rep.* 2006;30(3):350-357.
23. Fox A, le Mai Q, le Thanh T, et al. Hemagglutination inhibiting antibodies and protection against seasonal and pandemic influenza infection. *J Infect.* 2015;70(2):187-196.
24. Besselaar TG, Botha L, McAnerney JM, Schoub BD. Phylogenetic studies of influenza B viruses isolated in southern Africa: 1998-2001. *Virus Res.* 2004;103(1-2):61-66.
25. Besselaar TG, Botha L, McAnerney JM, Schoub BD. Antigenic and molecular analysis of influenza A (H3N2) virus strains isolated from a localised influenza outbreak in South Africa in 2003. *J Med Virol.* 2004;73(1):71-78.
26. Seleka M, Treurnicht FK, Tempia S, et al. Epidemiology of influenza B/Yamagata and B/Victoria lineages in South Africa, 2005-2014. *PLoS ONE.* 2017;12(5):e0177655.
27. Jonges M, Liu WM, van der Vries E, et al. Influenza virus inactivation for studies of antigenicity and phenotypic neuraminidase inhibitor resistance profiling. *J Clin Microbiol.* 2010;48(3):928-940.
28. Sheu TG, Deyde VM, Okomo-Adhiambo M, et al. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. *Antimicrob Agents Chemother.* 2008;52(9):3284-3292.
29. Murtaugh W, Mahaman L, Healey B, et al. Evaluation of three influenza neuraminidase inhibition assays for use in a public health laboratory setting during the 2011-2012 influenza season. *Public Health Rep.* 2013;128(Suppl 2):75-87.
30. Baillie GJ, Galiano M, Agapow PM, et al. Evolutionary dynamics of local pandemic H1N1/2009 influenza virus lineages revealed by whole-genome analysis. *J Virol.* 2012;86(1):11-18.
31. WHO. 2011. [www.who.int/influenza/gisrs\\_laboratory/manual\\_diagnosis\\_surveillance\\_influenza/en/](http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/). Accessed July 6, 2017.
32. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser.* 1999;41:95-98.
33. Baz M, Abed Y, Papenburg J, Bouhy X, Hamelin ME, Boivin G. Emergence of oseltamivir-resistant pandemic H1N1 virus during prophylaxis. *N Engl J Med.* 2009;361(23):2296-2297.
34. Nguyen HT, Fry AM, Loveless PA, Klimov AI, Gubareva LV. Recovery of a multidrug-resistant strain of pandemic influenza A 2009 (H1N1) virus carrying a dual H275Y/I223R mutation from a child after prolonged treatment with oseltamivir. *Clin Infect Dis.* 2010;51(8):983-984.
35. Yen HL, Hoffmann E, Taylor G, et al. Importance of neuraminidase active-site residues to the neuraminidase inhibitor resistance of influenza viruses. *J Virol.* 2006;80(17):8787-8795.
36. Escuret V, Frobert E, Bouscambert-Duchamp M, et al. Detection of human influenza A (H1N1) and B strains with reduced sensitivity to neuraminidase inhibitors. *J Clin Virol.* 2008;41(1):25-28.
37. Zurcher T, Yates PJ, Daly J, et al. Mutations conferring zanamivir resistance in human influenza virus N2 neuraminidases compromise virus fitness and are not stably maintained in vitro. *J Antimicrob Chemother.* 2006;58(4):723-732.
38. Pizzorno A, Bouhy X, Abed Y, Boivin G. Generation and characterization of recombinant pandemic influenza A(H1N1) viruses resistant to neuraminidase inhibitors. *J Infect Dis.* 2011;203(1):25-31.
39. Mishin VP, Hayden FG, Gubareva LV. Susceptibilities of antiviral-resistant influenza viruses to novel neuraminidase inhibitors. *Antimicrob Agents Chemother.* 2005;49(11):4515-4520.
40. Jackson D, Barclay W, Zurcher T. Characterization of recombinant influenza B viruses with key neuraminidase inhibitor resistance mutations. *J Antimicrob Chemother.* 2005;55(2):162-169.
41. Fujisaki S, Imai M, Takashita E, et al. Mutations at the monomer-monomer interface away from the active site of influenza B virus neuraminidase reduces susceptibility to neuraminidase inhibitor drugs. *J Infect Chemother.* 2013;19(5):891-895.
42. Pizzorno A, Abed Y, Bouhy X, et al. Impact of mutations at residue I223 of the neuraminidase protein on the resistance profile, replication level, and virulence of the 2009 pandemic influenza virus. *Antimicrob Agents Chemother.* 2012;56(3):1208-1214.
43. Njouom R, Mba SA, Noah DN, et al. Circulation of human influenza viruses and emergence of Oseltamivir-resistant A(H1N1) viruses in Cameroon, Central Africa. *BMC Infect Dis.* 2010;10:56.
44. Majanja J, Njoroge RN, Achilla R, et al. Impact of influenza A(H1N1) pdm09 virus on circulation dynamics of seasonal influenza strains in Kenya. *Am J Trop Med Hyg.* 2013;88(5):940-945.
45. Chen LF, Dailey NJ, Rao AK, et al. Cluster of oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infections on a hospital ward among immunocompromised patients—North Carolina, 2009. *J Infect Dis.* 2011;203(6):838-846.
46. Hurt AC, Hardie K, Wilson NJ, et al. Characteristics of a widespread community cluster of H275Y oseltamivir-resistant A(H1N1)pdm09 influenza in Australia. *J Infect Dis.* 2012;206(2):148-157.
47. Aoki FY, Boivin G, Roberts N. Influenza virus susceptibility and resistance to oseltamivir. *Antivir Ther.* 2007;12(4 Pt B):603-616.
48. Medeiros R, Escriou N, Naffakh N, Manuguerra JC, van der Werf S. Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. *Virology.* 2001;289(1):74-85.

49. Asaoka N, Tanaka Y, Sakai T, Fujii Y, Ohuchi R, Ohuchi M. Low growth ability of recent influenza clinical isolates in MDCK cells is due to their low receptor binding affinities. *Microbes Infect.* 2006;8(2):511-519.
50. Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *J Infect Dis.* 1998;178(5):1257-1262.
51. Wetherall NT, Trivedi T, Zeller J, et al. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. *J Clin Microbiol.* 2003;41(2):742-750.
52. NICD-NHLS. Influenza- NICD recommendations for the diagnosis, prevention, management and public health response. 2017. <https://tinyurl.com/y75yj9s2>. Accessed December 15, 2017.
53. Duque J, McMorro ML, Cohen AL. Influenza vaccines and influenza antiviral drugs in Africa: are they available and do guidelines for their use exist? *BMC Public Health.* 2014;14:41.

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