Influenza antiviral susceptibility: methods and challenges to detect resistant virus
Brazilian experience

Paola Cristina Resende, PhD
Researcher
National Influenza Center for the Brazilian MoH and WHO
Oswaldo Cruz Institute - FIOCRUZ - Rio de Janeiro

paola@ioc.fiocruz.br

May, 2017
Summary

• To give a brief background on antivirals and methods to measure the drug viral susceptibility

• To update on the compiled data from Brazil
Available antivirals against Influenza viruses

**Neuraminidase inhibitors (NIs)**
- Oseltamivir
- Zanamivir
- Peramivir
- Lanamivir

**M2 inhibitors**
- Amantadine
- Rhimantadine

**Polymerase inhibitors**
- T705 - Favipiravir

Promising drug in advanced clinical trial phases

**Antiviral Resistance**
- High levels of resistance (H1N1 and H3N2, changes in amino acid - L26F, V27A and or S31N)
- Not effective against Influenza B
Neuraminidase inhibitors

NA functions by cleaving the HA from the sialic acid receptor. NA inhibitors are sialic acid analogs which bind the NA active site preventing viral budding.

Conformational changes may occur due to mutations in this site, leading to a lack of interaction with the antiviral drug Zanamivir, Oseltamivir, Peramivir, and Lanamivir. They differ in their chemical structure, bioavailability, and mode of administration.

Adaptado de Nguyen et al, 2012.
METHODS FOR LABORATORY SURVEILLANCE OF ANTIVIRAL RESISTANCE OR REDUCED SUSCEPTIBILITY

GENOTIPIC Assays

PHENOTIPIC Assays
GENOTIPIC Assays

1. **Sanger sequencing – NA partial or full length**
   - Widely used
   - Data can be to address for a number of questions
   - Quantification of mixtures is difficult, slower than screening assays (1-5 days)

2. **SNP detection by Pyrosequencing (PSQ)**
   - Screening of SNPs, fast (5-6 hours)
   - Estimate relative allele proportion (viral subpopulations/quasispecies)
   - Problems in regions with homopolymers (Eg. AAAAA)

3. **SNP detection by Real time RT-PCR**
   - Screening of SNPs, fast (3-4 hours)
   - Need for control virus
   - Difficulties with probes specificity or SNP in the primers site may affect sensibility
   - Lower capacity to distinguish between genotypes that have the same Tm

http://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/nai_genotyping_molecular/en/
## GENOTIPIC Assays

### Advantages

- Relatively simple and rapid.
- Methods can be applied directly with clinical material.
- Methods can be implemented using existing molecular technologies in the laboratory.

### Disadvantages

- Interpretation of mutations is difficult without phenotypic information.
  - Lack of known mutations associated with antiviral resistance is not a guarantee of susceptibility to a particular drug.
  - Interpretation of the significance of novel mutations is difficult.

### Challenges

- Understanding the relationship between virus drug susceptibility phenotype and genotype.
- Determining what proportion, resistant vs sensitive, of a mixed virus population is required to confer resistance.
PHENOTIPIC Assays

**Advantages**
Enable detection of all resistance-causing mutations (including unidentified mutations)

**Disadvantages**
The viral isolation is necessary

Classification of viruses based on their fold differences compared to the median of viruses from the same type/subtype/lineage showing NI.
- Normal inhibition (NI)
- Reduced inhibition (RI)
- Highly reduced inhibition (HRI)

**NA activity and NA inhibition**

Tested with the NIs antiviral drugs: OST, ZAN, PER, LAN

**IC50** – drug capacity of virus inhibition in 50%

<table>
<thead>
<tr>
<th></th>
<th>Sensitive</th>
<th>Resistant</th>
<th>H275</th>
<th>H275Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazilian Isolates</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Reference viruses</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
GENOTIPIC Assays

Types of technology:

Fluorescence (FL)
In-house and commercial kits
Substrate used:
MUNANA (methyl umbelliferone N-acetyl neuraminic acid)
Instrumentation: Fluorimeter

Advantages:
• low cost
• Flexibility

Disadvantages:
• Need to prepare reagents in house
• Requires viruses with higher titers
• No standard "universal" protocol

Chemiluminescent (CL)
Commercial kits NA-Star and NA-XTD
Substrate used:
1,2-dioxetane derivative of neuraminic acid
Instrumentation: Luminometer

Advantages:
• Greater linearity of signal
• Higher sensitivity in measuring NA activity
• Standard protocols by fabricant

Disadvantages:
• More expensive than FL assays

http://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/nai_genotyping_molecular/en/
Brazilian experience with the Influenza antiviral resistance surveillance
Brazilian experience with Influenza antiviral resistance

Diagnostic Microbiology and Infectious Disease 71 (2011) 98–99

Letter

Antiviral resistance surveillance for influenza A virus in Brazil: investigation on 2009 pandemic influenza A (H1N1) resistance to oseltamivir


315 H1N1pdm09 strain analyzed from 2009 to 2010
All strains H275

Brazilian experience with Influenza antiviral resistance

First detection of resistant viruses in Brazil
Community transmission?

Detection of Oseltamivir-Resistant Pandemic Influenza A(H1N1)pdm2009 in Brazil: Can Community Transmission Be Ruled Out?

Thiago Moreno L. Souza¹, Paola C. Resende¹, Natália Flintelman-Rodrigues¹, Tatiana Schaffer Gregianini², Nilo Ikuta⁵, Sandra Blanchini Fernandes³, Ana Luisa Furtado Cury⁴, Maria do Carmo Debur Rosa⁵, Marilda M. Siqueira¹

Table 1. Clinical and virological aspects of patients in which OST-resistant strains of influenza were detected.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutations</th>
<th>Region</th>
<th>Age</th>
<th>Gender</th>
<th>Symptoms date</th>
<th>Collection date</th>
<th>Oseltamivir (Beginning date)</th>
<th>Deceased</th>
<th>Oseltamivir before sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H275Y</td>
<td>Southern</td>
<td>RS 26</td>
<td>M</td>
<td>24-Aug-09</td>
<td>25-Aug-09</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>2</td>
<td>S247N</td>
<td>Southern</td>
<td>RS 3</td>
<td>M</td>
<td>2-Jul-11</td>
<td>2-Jul-11</td>
<td>NI</td>
<td>Yes</td>
<td>NI</td>
</tr>
<tr>
<td>3</td>
<td>H275Y</td>
<td>Southern</td>
<td>SC 36</td>
<td>M</td>
<td>14-May-12</td>
<td>20-May-12</td>
<td>21-Jul-12</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>H275Y</td>
<td>Southern</td>
<td>PR 12</td>
<td>M</td>
<td>21-Jun-12</td>
<td>22-Jun-12</td>
<td>22-Jul-12</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>H275Y</td>
<td>Southern</td>
<td>RS 34</td>
<td>M</td>
<td>28-Jun-12</td>
<td>2-Jul-12</td>
<td>1-Jul-12</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>H275Y</td>
<td>Southern</td>
<td>RS 28</td>
<td>M</td>
<td>22-Jun-12</td>
<td>27-Jun-12</td>
<td>25-Jun-12</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>S247N</td>
<td>Southeast</td>
<td>MG 37</td>
<td>F</td>
<td>1-Jul-12</td>
<td>5-Jul-12</td>
<td>5-Jul-12</td>
<td>Yes</td>
<td>NI</td>
</tr>
</tbody>
</table>

¹ Years old; ² months old; NI – not informed

doi: 10.1371/journal.pone.0080081.t001
Oseltamivir-resistant influenza A(H1N1)pdm2009 strains found in Brazil are endowed with permissive mutations, which compensate the loss of fitness imposed by antiviral resistance

Thiago Moreno Lopes e Souza, Natalia Fintelman-Rodrigues, Paola Cristina Resende, Milene Mesquita, Tatiana Schaffer Gregianini, Fernando A Bozza, Ana Carla Pecego, Sandra Bianchini Fernandes, Ana Luisa Furtado Cury, Irina Nastassja Riediger, Marilda M Siqueira

Viral characteristics and clinical aspects of patients in which oseltamivir (OST)-resistant samples were detected

<table>
<thead>
<tr>
<th>Patients</th>
<th>Mutations</th>
<th>IC_{50} (nM)</th>
<th>Region</th>
<th>State</th>
<th>Age</th>
<th>Gender</th>
<th>Symptoms onset (date)</th>
<th>Sample collection (date)</th>
<th>OST use</th>
<th>Deceased</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H275Y</td>
<td>102</td>
<td>Southeast</td>
<td>RJ</td>
<td>40</td>
<td>F</td>
<td>2 April 2013</td>
<td>5 April 2013</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>H275Y</td>
<td>116</td>
<td>South</td>
<td>RS</td>
<td>26</td>
<td>F</td>
<td>26 March 2013</td>
<td>5 April 2013</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

IC_{50}: the half maximum inhibitory concentration; OST: oseltamivir; RJ: Rio de Janeiro; RS: Rio Grande do Sul.
Brazilian experience with Influenza antiviral resistance

- 31 cases of resistant virus from May to August, 2011 in Newcastle, Australia (Hurt et al 2011a; Hurt et al, 2012)
- 104 cases of resistant virus from September, 2013 to July, 2014 in Sapporo/Hokkaido, Japan (Takashita et al, 2015a)

**Permissive mutations**

V241I e N369K, better fitness for H1N1 resistant viruses (H275Y) (Hurt, 2006; Takashita et al, 2015)

**Communitarian Clusters (H275Y)**

- 31 cases of resistant virus from May to August, 2011 in Newcastle, Australia (Hurt et al 2011a; Hurt et al, 2012)
- 104 cases of resistant virus from September, 2013 to July, 2014 in Sapporo/Hokkaido, Japan (Takashita et al, 2015a)

Possibility of emergence of a variant totally resistant to antiviral classes currently available
Brazilian experience with Influenza antiviral resistance

Brazilian samples analyzed for H275Y in A/H1pdm09 from 2009 to 2016

<table>
<thead>
<tr>
<th>Year</th>
<th>A/H1pdm09 cases</th>
<th>Studied A/H1pdm09 cases (for H275Y)*</th>
<th>A/H1pdm09 carrying H275Y (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>5655</td>
<td>315</td>
<td>1 (0.3%)</td>
</tr>
<tr>
<td>2010</td>
<td>140</td>
<td>100</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>2011</td>
<td>163</td>
<td>163</td>
<td>1 (0.6%)</td>
</tr>
<tr>
<td>2012</td>
<td>345</td>
<td>335</td>
<td>5 (1.5%)</td>
</tr>
<tr>
<td>2013</td>
<td>310</td>
<td>208</td>
<td>2 (0.9%)</td>
</tr>
<tr>
<td>2014</td>
<td>89</td>
<td>68</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>2015</td>
<td>47</td>
<td>33</td>
<td>1 (3.0%)</td>
</tr>
<tr>
<td>2016</td>
<td>792</td>
<td>757</td>
<td>2 (0.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>7541</td>
<td>1979</td>
<td>12 (0.6%)</td>
</tr>
</tbody>
</table>

*PSQ or Sanger sequencing
Brazilian experience with Influenza antiviral resistance

Emergence of resistant virus by OST treatment pressure

Resistant case from 2016

1 - Immunocompromised patient (Cancer) – Male 27 years-old

Onset symptoms: 0

Days: 0 1 2 3 4 5 6 7 19

1 dose of OST 75mg/day

S1

RT-PCR: H1N1pdm09
PSQ: 100% H275
iNA:
Normal inhibition (NI)
OST, ZAN, LAN and PER

2 dose of OST 75mg/day

S2

RT-PCR: H1N1pdm09
PSQ: 36% H275 e 64% Y275
iNA:
Normal inhibition (NI) - ZAN and LAN
Highly reduced inhibition (HRI) - OST and PER

Additional phenotypic analyzes with ZAN, LAN and PER were performed by CDC WHOCC, Atlanta

Patient status: Recovered
Brazilian experience with Influenza antiviral resistance

<table>
<thead>
<tr>
<th>Influenza Vírus</th>
<th>Genetic resistant markers against Oseltamivir (A)</th>
<th>Samples PSQ per year (N)</th>
<th>Samples Sanger per year (N)</th>
<th>N of samples containing genetic resistance markers</th>
</tr>
</thead>
</table>

<sup>(A) Reduced inhibition (RI), Highly reduced inhibition (HRI)</sup>
<sup>(B) Numbering N2</sup>
<sup>* SNP detected by Pirosequencing (PSQ)</sup>

✔ Phenotypc assay NIs: 111 were tested in 2016
  • 1 presented **Highly reduced inhibition (HRI) to the OST.**
  • 110 presented **Normal inhibition to the OST.**
Conclusions

✓ Considering the risk of emergence and spread of resistant viruses to the class of antiviral currently available it is important to strengthen viral surveillance and also to encourage and to carry out studies for new antiviral drugs.

New challenges for the study of antiviral resistance markers:

✓ New antiviral drugs that act on other viral targets and in host factors

✓ Strategies for monitoring emergence of resistant strains can change soon
Antiviral susceptibility surveillance

Useful websites:

WHO – Antiviral susceptibility surveillance
http://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/en/

ISIRV – Antiviral Group
https://www.isirv.org/site/index.php/special-interest-groups/antiviral-group-home

Annual Review:

Acknowledgments

NIC, FIOCRUZ team
Marilda Siqueira
Aline Matos
Milene Mirada
Cristiana Garcia
David Brown
Paola Resende
Fernando Motta
Prisicila Born
Braulia Caetano
Maria de Lourdes

Brazilian MoH
Dr. Sérgio Nishioka
Walquiria Almeida
Influenza team
Surveillance Network
States Central Labs

CDC Influenza Division - WHOCC
Dr. Larissa Gubareva

Australian - WHOCC
Dr. Aeron Hurt

PAHO Influenza team
Dr. Rakhee Palekar
Dr. Juliana Leite

Thank you!
Muchas Gracias!
Obrigada!

paola@ioc.fiocruz.br
aline.matos@ioc.fiocruz.br
mmsiq@ioc.fiocruz.br