Oseltamivir-resistant influenza A(H1N1)pdm09 virus in southern Brazil

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The neuraminidase (NA) genes of A(H1N1)pdm09 influenza virus isolates from 306 infected patients were analysed. The circulation of oseltamivir-resistant viruses in Brazil has not been reported previously. Clinical samples were collected in the state of Rio Grande do Sul (RS) from 2009-2011 and two NA inhibitor-resistant mutants were identified, one in 2009 (H275Y) and the other in 2011 (S247N). This study revealed a low prevalence of resistant viruses (0.8%) with no spread of the resistant mutants throughout RS.

Key words: influenza A(H1N1)pmd09 - resistance - oseltamivir

Since April 2009, the influenza virus referred to as A(H1N1)pdm09 has spread worldwide. The primary treatment for infected patients is the antiviral drug oseltamivir, a neuraminidase inhibitor (NAI) (Hurt et al. 2011a). Oseltamivir-resistant influenza A(H1N1)pdm09 is rare; nevertheless, 570 cases were reported around the world as of 24 August 2011 (WHO 2011). The most common mutation associated with oseltamivir resistance is the amino acid change H275Y in the neuraminidase (NA) protein (Sheu et al. 2008, Kiso et al. 2010, Renaud et al. 2011*)*. However, other mutations have been described in seasonal influenza viruses (Sheu et al. 2008). The circulation of oseltamivir-resistant viruses in Brazil has not been reported previously. The aim of this study was to detect resistant influenza A(H1N1)pdm09 isolates in the state of Rio Grande do Sul (RS) and to assess the possible spread of resistant viruses.

More than 30,000 patients with severe acute respiratory syndrome (SARS), which is characterised by fever, cough, dyspnoea, myalgias, arthralgias, gastrointestinal symptoms and malaise (SS-RS 2011), were reported in Brazil in 2009. RS reported 5,286 SARS cases, which was the most reported by any Brazilian state, among which 2,109 were confirmed by reverse transcription-polymerase chain reaction (RT-PCR). The A(H1N1) pdm09 virus was not detected in 2010, but reappeared in 2011, with 106 confirmed cases in the winter season between June-August in RS (Gregianini et al. 2011).

The present study analysed 306 clinical samples from RS collected between 2009-2011; 200 samples from 2009 were randomly selected from 931 independent clinical samples from SARS patients that were collected between July-November 2009 and all 106 samples from 2011 were included. The clinical samples (nasopharyngeal aspirate or swabs) were obtained from the Central Laboratory of Public Health (a government laboratory involved in pandemic H1N1 diagnosis in RS). All of the samples had been previously confirmed to be positive for SARS by real-time RT-PCR detection according to the procedure recommended by the Centers for Disease Control and Prevention (Atlanta, GA, USA).

This research was approved by the Ethical Committee of Lutheran University of Brazil (protocol 2010-048H, 20/03/2010).

To determine whether the virus isolates harboured genetic markers associated with resistance to antiviral drugs, the sequence of the NA gene was determined. Briefly, viral RNA was extracted using either the QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany) or the NewGene Prep/PreAmp Kit (Simbios, Cachoeirinha, Brazil).

RT-PCR was performed in a 30 µL reaction volume containing MMLV buffer (Promega), 200 µM dNTPs (GE Healthcare), 40 U RNAseOut (Promega), 20 U MMLV RT (Promega), 1 U Taq DNA polymerase (Cenbiot) and 0.25 µM of the primers (IDT) NAF 121 (5'-GGGAATCAAAATCAGATTGAAACA-3') and NAR 970 (5'-CTCCGAAAATCCCACTGCAT-3'). Nested amplification was performed in a 30 µL reaction volume containing Taq buffer, 1,5 mM $MgCl_2$, 200 µM dNTPs (GE Healthcare), 1 U Taq DNA polymerase (Cenbiot) and 0,25 µM of the nested primers (IDT) NAF 203 (5'-CATCAGCAACACCAACTTTGC-3') and NAR 882 (5'-ATCCCTGCACACACATGTGATT-3'). The amplification programmes were as follows: (i) RT-PCR: 1 cycle of 30 min at 37ºC and 3 min at 95ºC, 20 cycles of 20 sec at 95ºC, 40 sec at 55ºC and 1 min at 72ºC and 1 final cycle of 5 min at 72ºC and (ii) nested PCR: 3 min at 95ºC, 35 cycles of 20 sec at 95ºC, 40 sec at 55ºC and 1 min at 72ºC and 1 final cycle of 5 min at 72ºC.

All amplicons (680 bp) were analysed by 2% agarose gel electrophoresis. The sequencing reaction was performed using 4 µL of BigDye Terminator version 3.1 Cy-

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cle Sequencing Kit (Applied Biosystems by Life Technologies Co, Foster City, CA, USA), 3,2 pmol of internal primers and 5-20 ng of DNA. The NA PCR product was sequenced using an ABI PRISM 3130 *xl* DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The consensus sequences were obtained using the programme SeqMan (Lasergene, DNASTAR, Madison, WI). The nested RT-PCR yielded NA amplicons for 261 samples, 156 from 2009 and 105 from 2011.

The sequence analysis showed that the H275Y mutation in NA was absent in all samples except for one sample from a male patient collected in August 2009. This patient was 26 years old and lived in the city of Gravataí. The clinical record mentioned that he was an immunosuppressed patient with fever and dyspnoea during his hospitalisation (A/Rio Grande do Sul/4144/2009(H1N1) (GenBank accession JQ283443).

The NA amino acid changes S79G, V116A, I117M/V, E119V/G/A/D, H126N, D151G/A/N/V, D199G/E, R222Q, I223M/V/R, V234M, S247G/N and H274N have also been shown to be associated with oseltamivir resistance in seasonal influenza viruses (Sheu et al. 2008, Deyde et al. 2010, Hurt et al. 2011a). The analysis of these NA mutations in Brazilian patients revealed that the S247N mutation was present in one isolate collected in 2011. The clinical record associated with this sample indicated that the patient was a three-month-old boy from the city of Guaíba who died after hospitalisation. The patient had Down syndrome and exhibited fever, cough and dyspnoea during hospitalisation (A/Rio Grande do Sul/665/2011(H1N1) (GenBank accession JQ283444). The S247N mutation has been identified in clinical specimens and isolates of seasonal H1N1 and influenza A(H1N1) pdm09 viruses from the Asia-Pacific region (Sheu et al. 2008, Hurt et al. 2011c). This mutation is associated with reduced NAI sensitivity and was previously described in pandemic influenza A(H1N1). When associated with the H275Y mutation, the S247N mutation results in an extremely high level of oseltamivir resistance (Sheu et al. 2008, Boltz et al. 2010, Hurt et al. 2011c).

Zanamivir is another NAI that is recommended for the treatment of influenza. Mutations associated with zanamivir resistance occur predominantly in seasonal influenza. Examples of these mutations include E119V/G/A/D, H126N, Q136K, D151G/A/N/V, D199G/E and G249R (Sheu et al. 2008, Deyde et al. 2010, Hurt et al. 2011a). In this study, zanamivir resistance mutations were not observed in any of the 261 samples.

The NA inhibitors zanamivir and oseltamivir are marketed for the treatment and prophylaxis of influenza and have been stockpiled by many countries for use during a pandemic. Although recent surveillance has identified a striking increase in the frequency of oseltamivir-resistant seasonal influenza viruses in Europe, the USA, Oceania and South Africa, there are no reports of significant zanamivir resistance among H1N1 viruses (Hurt et al. 2009).

Clusters of oseltamivir-resistant A(H1N1)pdm09 infections have been described in hospitalised immunocompromised patients and among immunocompetent patients in the community (Le et al. 2010), demonstrating the potential person-to-person spread of resistant strains. The clinical impact of resistant influenza does not appear to be significant for immunocompetent patients, but could be a major threat for immunocompromised patients (Renaud et al. 2011). These resistant viruses readily emerge in such patients because these viruses can replicate to higher titres and for longer periods in immunocompromised hosts than in immunocompetent hosts (Ison et al. 2006).

NAIs were introduced in 1999 and the oseltamivir resistance rate of seasonal influenza isolates was very low (0.5%) until the 2007-2008 influenza season. In the following years (2008-2009), many countries reported extremely high rates of oseltamivir resistance in seasonal H1N1 isolates (95-100%) (Dharan et al. 2009). This resistance was caused by the H275Y amino acid substitution in the NA proteins of these viruses (Meijer et al. 2009) and this mutation became common despite the low use of oseltamivir.

In 2009, A(H1N1)pdm09 spread throughout the world, almost replacing seasonal influenza viruses. Subsequently, low rates of oseltamivir-resistant A(H1N1) pdm09 were reported in many countries (0.5-1%) (Hurt et al. 2011b, 2012, Renaud et al. 2011, Storms et al. 2012). However, higher frequencies were detected in studies in several countries, including Spain (1.93%, all of them detected in patients with severe Influenza A) (Ledesma et al. 2011), Australia (1.3%) and Singapore (3.1%). The overall frequency of oseltamivir resistance in the Asia-Pacific region was 1.1% (Hurt et al. 2011a). In our study, we found similar rates of oseltamivir resistance (0.6% in 2009 and 1% in 2011). The overall prevalence of oseltamivir-resistant viruses was 0.8% (2/259) in our study, indicating that the A(H1N1)pdm09 virus with the H275Y mutation has not spread in RS.

This study identified one A(H1N1)pdm09 isolate with the H275Y mutation and one with the S247N mutation in the NA protein. The prevalence of these mutants was low in RS in 2009 and 2011. There is no evidence that oseltamivir and zanamivir-resistant mutants are spreading throughout RS, suggesting that these antiviral drugs are appropriate for the treatment of A(H1N1)pdm09.

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