

SURVEILLANCE OF OSELTAMIVIR-RESISTANT INFLUENZA A(H1N1)pdm09 IN GUANAJUATO STATE, MEXICO FROM 2009 TO 2012

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ABSTRACT

Background: The influenza A(H1N1)pdm09 virus was first identified in Mexico in April 2009, subsequently spreading worldwide. Soon after the WHO declared a pandemic, a series of cases involving oseltamivir-resistant viruses were described, following concerns about the spread of strains resistant to neuraminidase inhibitors that could hamper control measures. To study the prevalence of oseltamivir-resistant influenza A(H1N1)pdm09, we implemented a surveillance program across the state of Guanajuato, Mexico. **Methods:** We collected respiratory samples from patients with confirmed infection with influenza A(H1N1)pdm09 virus between 2009 and 2012 in rural and urban regions in Guanajuato, Mexico. Specimens were screened for the H275Y mutation by Sanger sequencing. **Results:** A total of 1,192 laboratory confirmed influenza A(H1N1)pdm09-positive samples were processed between 2009 and 2012. Using two endpoint real-time polymerase chain reaction, 575 samples were sequenced. Two different clusters, I and II, were identified. The H275Y substitution was found in only one sample from cluster I. **Conclusions:** The prevalence of oseltamivir-resistant influenza A(H1N1)pdm09 2009 viruses during the pandemic period and following years was very low in our State. (REV INVES CLIN. 2015;67:235-9)

Key words: Influenza. H1N1. Oseltamivir. Resistance.

INTRODUCTION

Starting in March 2009, cases of a respiratory disease caused by the novel influenza A(H1N1)pdm09 virus, a new strain carrying genes from North American and Eurasian swine, avian, and human influenza viruses, were identified in Mexico¹⁻⁴. During the following weeks

the virus disseminated worldwide and the World Health Organization (WHO) declared a pandemic⁵. The novel virus was found to be resistant to the matrix 2 blockers, so neuraminidase inhibitors were recommended as treatment for severe illness⁶. Soon after the pandemic started, cases involving oseltamivir-resistant virus were reported, raising concerns about the feasibility

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of control^{7,8}. All identified strains had the H275Y substitution, conferring them resistance to oseltamivir but not to zanamivir^{7,8}. The dissemination of oseltamivir-resistant seasonal influenza A (H1N1) virus was first detected in 2007⁹, becoming the predominant lineage of influenza A (H1N1) virus in humans, which has now been replaced by the new pandemic (H1N1) virus: influenza A(H1N1)pdm09^{9,10}. This finding raised concerns that the H275Y mutation could become dominant in this pandemic influenza A(H1N1)pdm09 virus as well. Thus, we conducted a surveillance study between 2009 and 2012 in primary care centers and hospitals in the state of Guanajuato in Mexico to detect the emergence of oseltamivir resistance among circulating influenza A(H1N1)pdm09 strains. We report here the results of the three-year surveillance.

MATERIAL AND METHODS

Study design, setting, and population

We conducted a cross-sectional study to investigate the emergence of oseltamivir-resistant viruses in Guanajuato, Mexico, between April 2009 and May 2012. During the influenza A(H1N1)pdm09 pandemic, healthcare facilities were specifically designated by the National Directorate of Epidemiology (DGE) of the Ministry of Health in Mexico, as Influenza Surveillance Units (USMI). The USMIs are primary healthcare facilities and general hospitals distributed across the country, designated to, systematically and in a standardized manner, collect samples of persons with influenza-like illness (ILI). The 583 existing USMIs were assigned at a national level based on access, infrastructure, staff, and geographical criteria to achieve national coverage^{11,12}. This study was conducted at the 15 USMIs located across the state of Guanajuato in Mexico.

Procedures

Samples from nasopharyngeal swabs or bronchial aspirates were obtained from patients with ILI, or with severe acute respiratory infection (SARI) of ≤ 7 days of symptom onset, according to the national Influenza Surveillance System case definitions and using standardized procedures^{11,12}. Samples were collected from all patients with severe manifestations, and from one in every three consecutive patients with mild or moderate symptoms. All samples were processed and

stored at -70°C , to be later analyzed at the Public Health Laboratory of Guanajuato State using real-time reverse transcription-polymerase chain reaction (RT-PCR). We followed the CDC's protocol for detection and characterization of influenza viruses, in accordance with the recommendations of the Ministry of Health of Mexico¹¹⁻¹³. Viral RNA was extracted from samples positive for influenza A(H1N1)pdm09 using MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Rotkreuz, Switzerland). Two endpoint real-time RT-PCRs were performed in all samples. The first test used the primers 5'-CGATGGACCAAGGATGGACAGGCC-3' and 5'-CWACCCAGAARCAAGGTCTTATG-3', and the amplicon, corresponding to the target fragment as template. A second endpoint real-time RT-PCR was performed using the primers FWD1: 5'-GGGGAAGATTGTAAATC-AGTYGA-3' and REV: 5'-CWACCCAGAARCAAGGTCTTATG-3' to obtain appropriate amplicons to be sequenced. Amplicons generated from real-time RT-PCR were submitted to Sanger sequencing using the capillary sequencer ABI 3730XL (Applied Biosystems). Resultant sequences containing 377 nucleotides were aligned to identify the H275Y substitution.

Study definitions

Influenza-like illness, SARI, and suspected and confirmed influenza cases were defined according to definitions used by the DGE for the National Influenza Surveillance System¹¹. Briefly, ILI was considered in patients with fever ($\geq 38^{\circ}\text{C}$), cough, and headache in addition to any of the following symptoms: nasal discharge, arthralgia, myalgia, chest or abdominal pain, sore throat, or malaise. SARI was considered in all patients with dyspnea, fever ($\geq 38^{\circ}\text{C}$) and cough in addition to any of the following: malaise, chest pain, tachypnea, or acute respiratory distress syndrome. Suspected cases were those who met these criteria, and confirmed cases were those with at least one sample positive for influenza.

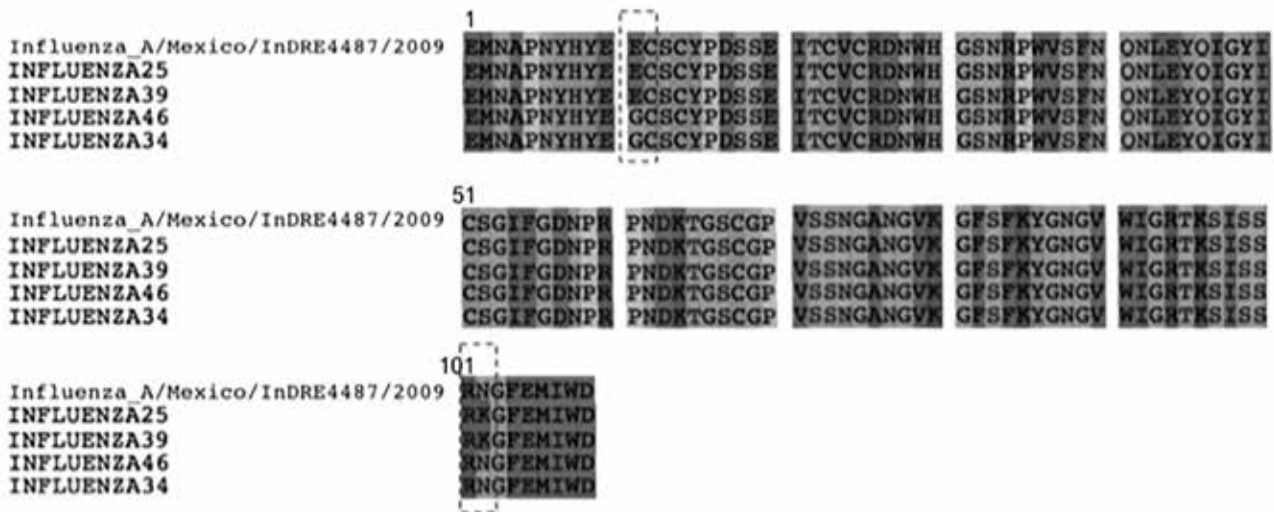
RESULTS

We collected 3,066 respiratory samples during the study period, of which 1,409 (46%) were confirmed to have influenza A(H1N1)pdm09 virus; 1,192 (85%) of these were suitable for further analysis. A(H1N1)pdm09 viruses were identified by RT-PCR in 575 (41%) samples. Demographic and clinical characteristics of the patients with sequenced samples were similar to

Table 1. Demographic and clinical characteristics of patients with confirmed pandemic influenza A (H1N1) 2009 virus infection in the state of Guanajuato, Mexico between 2009-2012

| Characteristics | Influenza A (H1N1) 2009 infected patients with non-sequenced samples (n = 617) | Influenza A (H1N1) 2009 infected patients with sequenced samples (n = 575) | p |
|-----------------------|--|--|------|
| Male, n (%) | 307 (49.7) | 289 (50.2) | 0.89 |
| Age, mean (\pm SD) | 21.02 (\pm 17.1) | 23 (\pm 17.09) | 0.14 |
| Fever, n (%) | 599 (97.08) | 561 (97.5) | 0.68 |
| Cough, n (%) | 554 (89.7) | 527 (91.6) | 0.22 |
| Dyspnea, n (%) | 188 (30.4) | 173 (30.1) | 0.81 |

Figure 1. Alignment of some representative sequences corresponding to clusters I and II compared with the reference strain. The first (upper) line corresponds to the reference strain, the second and third lines correspond to strains 25 and 39, which are representatives of cluster I (with Lys replacing Asn at position 376), and fourth and fifth lines correspond to strains 46 and 34, which are representatives of cluster II (with Gly replacing Glu at position 276).



those of the patients with non-sequenced samples (Table 1). We identified two different clusters, I and II. Compared with the reference strain (Influenza_A/Mexico/IndRE4487/2009), the cluster I sequences had a lysine-asparagine replacement at position 376, and the cluster II sequences had a glycine replacing glutamate at position 276 (Fig. 1).

Except for one sample, all sequences analyzed corresponded to the wild-type influenza A(H1N1)pdm09 virus, with no mutations detected at position 275 in the neuraminidase gene, providing no evidence for oseltamivir-resistant influenza A(H1N1)pdm09 circulating strains in the state of Guanajuato, Mexico, during the study period. The H275Y substitution was found only in one sample from cluster I (Fig. 2). This specimen was obtained from an otherwise healthy

14-year-old girl who sought care on January 26, 2012. Her symptoms began the previous day, with malaise, fever, cough, headache, sore throat, and myalgia. She was diagnosed with ILI and samples were collected. No signs of severity were present. She had never received oseltamivir, had no known contacts with other influenza cases, nor had she recently traveled. She denied recent contact with poultry or farm animals. She received oseltamivir for five days and did well, with total resolution of the symptoms in the following days. No other cases of influenza were identified among her relatives.

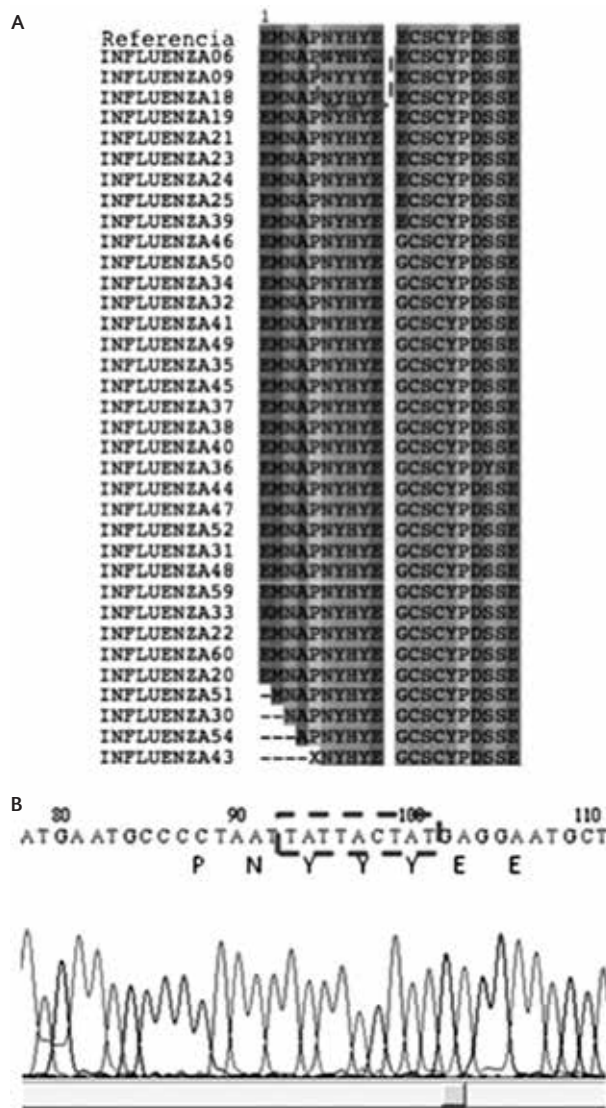
DISCUSSION

Despite the widespread use of oseltamivir during the 2009 influenza pandemic, and the concern about

oseltamivir-resistance, we only identified one sample (0.002%) containing the H275Y single-nucleotide substitution mutation among 575 sequenced samples of influenza A(H1N1)pdm09 virus in the state of Guanajuato, Mexico. Samples were obtained from a geographically representative sample of medical units across the state. This low prevalence of resistance is concordant with a previous study in Mexico with a similar sample size, where among 692 randomly selected respiratory specimens from hospitalized patients from all the states, only one had the H275Y mutation¹⁴. Similarly, in a large multicenter prospective global surveillance study of resistance to neuraminidase inhibitors in both hemispheres during the same study period, no resistance to oseltamivir was detected at baseline in 899 patients with confirmed influenza A(H1N1)pdm09 virus¹⁵. Moreover, only 17 (1.9%) of these patients, none of them hospitalized, developed oseltamivir resistance during a 10-day follow-up period¹⁵. In contrast, all cases with seasonal influenza A (H1N1) (n = 47) were oseltamivir-resistant at baseline and conserved the resistance throughout follow-up¹⁵. Similarly, in a study in a single third-level hospital in Mexico City, no oseltamivir-resistant strains were identified in 95 samples of influenza A(H1N1)pdm09 virus-infected patients¹⁶. Thus, our findings are consistent with the reports from Mexico^{14,16} and other parts of the world^{15,17,18} of a low potential of emergence of oseltamivir resistance among influenza A(H1N1)pdm09 virus strains.

Despite these findings, it is important to emphasize that active surveillance of the prevalence of oseltamivir resistance is necessary to guide policies that help prevent its widespread dissemination and select for appropriate antivirals. In Mexico, as in many other countries, the abuse of antibiotics prescribed for viral respiratory infections is common¹⁷. This practice is useless and may have contributed to the serious increase in antibiotic resistance in Latin America in recent years¹⁸. Paradoxically, patients with an illness compatible with influenza, including those with a high risk for complications, rarely, if ever, receive antivirals for several reasons¹⁹. Even though this may account for the low prevalence of oseltamivir-resistant pandemic H1N1 strains, our results appeal for further policies for ILI treatment, including openhanded use of antivirals in high-risk patients, and always under clear follow-up guidelines to prevent dissemination of resistance. Such a policy also could help reduce the indiscriminate use of antibiotics.

Figure 2. Sequence alignment of the neuraminidase. **A:** The generated sequences were aligned at the amino acid level. The sequence from sample 9 showed the H275Y substitution that confers resistance to oseltamivir. **B:** The presence of the mutation was confirmed by sequencing the cloned amplicon. Sequence chromatograms comparing a wild-type strain and the influenza 09 strain with the H275Y mutation.



A limitation of this study is that we could not rule out the presence of other mutations related with resistance to oseltamivir and other neuraminidase inhibitors since the sequencing was only restricted to a small fragment of the neuraminidase gene and no *in vitro* neuraminidase inhibition assays were performed.

In conclusion, the prevalence of oseltamivir-resistant influenza A(H1N1)pdm09 virus in the state of Guanajuato during the virus emergence, pandemic

dissemination, and subsequent years was very low, similar to that reported worldwide. Maintaining and strengthening surveillance systems for oseltamivir-resistant influenza A(H1N1)pdm09 virus at the local level is feasible and relevant, and could potentially have an impact on clinical guidelines for the management of ILI and antibiotic prescription policies.

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