Case report

Emergence of intratreatment resistance to oseltamivir in pandemic influenza A H1N1 2009 virus

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Background: Pandemic influenza A H1N1 2009 virus presents a new challenge to health authorities and communities worldwide. In Argentina, the outbreak was at its peak by the end of June 2009, during the southern winter. A systematic analysis of samples from patients with pandemic H1N1 2009 studied in our laboratory (Virology Laboratory, Hospital de Niños R Gutiérrez, Buenos Aires, Argentina) detected two patients presenting intratreatment emergence of the H275Y neuraminidase mutation, which confers resistance to oseltamivir.
Methods: Complementary DNAs, including the 275 codon, were obtained by reverse transcriptase PCR using viral RNAs extracted from nasopharyngeal or tracheal aspirates. Conventional sequencing and pyrosequencing were performed on each sample. In order to measure the virus susceptibility to oseltamivir, 50% inhibitory concentration determinations were performed by chemiluminescence.
Results: Sequential samples of two paediatric patients under oseltamivir treatment were analysed. Pretreatment samples were composed of 100% oseltamivir-sensitive variants. In case 1, the oseltamivir-resistant variant was found 8 days after the beginning of treatment. In case 2, the viral population became resistant on the second day of treatment, with 83% of the viral population bearing the mutation and this reached 100% on the seventh day.
Conclusions: We describe the intratreatment emergence of oseltamivir resistance in two paediatric patients. Pyrosequencing allowed us to detect variant mixtures, showing the transition of the viral population from sensitive to resistant.

Introduction

Among the neuraminidase inhibitors, oseltamivir remains the most widely prescribed anti-influenza drug and large quantities were stockpiled for use in the H1N1 2009 pandemic. In addition, it is the only drug licensed for use in children ≥1 year old. The most common neuraminidase mutation in oseltamivir-resistant seasonal H1N1 viruses is H275Y, which reduces the inhibitory activity of oseltamivir and can emerge under selective pressure [1]. Recent studies have shown that resistance in seasonal H1N1 had been rather uncommon before 2007, but had spread worldwide 12 months later [2]. Because this mutation can also arise in other influenza A N1 viruses, considerable attention was paid to monitoring for resistance in the pandemic H1N1 2009 virus. A systematic analysis of samples from children with pandemic H1N1 2009 was conducted in our laboratory (Virology Laboratory, Hospital de Niños R Gutiérrez, Buenos Aires, Argentina). We report the emergence of H275Y oseltamivir resistance during treatment in two paediatric patients of that study.

Case 1

On 17 June 2009, a 2-year-old boy with embryonic rhabdomyosarcoma was hospitalized for 24 h for his eighth chemotherapy cycle (ifosfamide, mesna, vincristine and actinomycin D). At the time of admission he had fever (37.7°C) and rhinorrhea. No infiltrates were found on a chest X-ray. Pandemic H1N1 2009 virus was detected in a nasopharyngeal aspirate (NPA).The following day, the patient was discharged after beginning oseltamivir treatment (45 mg twice daily for 5 days, as an outpatient). On 25 June 2009 he was rehospitalized because

of febrile neutropaenia (38.7ºC, 551 neutrophils/mm^3). Respiratory symptoms (rhinorrhea, tachypnea and cough) were present, with bilateral interstitial infiltrate in a chest radiograph. Bacteriological cultures were negative and pandemic H1N1 2009 was still present in NPA samples obtained on 25 and 26 June 2009 (Figure 1A). He received antibiotics, antipyretics and filgrastim, and was discharged on 27 June 2009. By that time he was still symptomatic and the follow-up was made as an outpatient. No further virological studies were performed until the next chemotherapy cycle, 30 days later, in which influenza virus was undetectable.

Case 2

A previously healthy 1-year-old girl weighing 10.6 kg was hospitalized for pneumonia and empyema on 15 July 2009; 5 days before admission she had presented fever and progressive respiratory symptoms. On admission to the intensive care unit, she was hypoxic and febrile, with consolidation in the upper right pulmonary lobe, and required mechanical ventilation. *Streptococcus pneumoniae* bacteriaemia was detected. Although tracheal aspirate (TA) was negative for influenza virus, oseltamivir (30 mg twice daily), with ceftriaxone and vancomycin, was administered for 4 days. Dexamethasone treatment (0.6 mg/day) was added on 18 July 2009 and continued for 9 days, replaced then by hydrocortisone (60 mg/day), which continued until discharge. On 20 July 2009, the lung disease worsened, and the culture of pleural drainage recovered *Klebsiella pneumoniae*. At the same time, pandemic H1N1 2009 was detected in TA, thus oseltamivir treatment was restarted (30 mg twice daily) for 8 days (Figure 1B). Her condition worsened in the following days, with infiltrates involving the other lung. She also had haemodynamic compromise, requiring inotropics. Serum immunoglobulins and complement, in addition to most blood lymphocyte subpopulations, that is, CD3$^+$, CD4$^+$ and CD8$^+$ subsets, were within the normal range, although the CD3$^-$/CD56$^+$/CD16$^+$ natural killer subset was low (186 cells/mm^3) and the CD20$^+$ B-lymphocyte subset was high (5,472 cells/mm^3). She experienced prolonged disease, remaining in the intensive care unit until 8 August 2009, when the virus became undetectable. Following right upper lobectomy because of bronchopleural fistula, she was discharged on 23 September 2009.

Methods

Specimens

The two cases of resistance development during treatment presented here were detected while performing a systematic oseltamivir resistance analysis of 291 pandemic H1N1 2009 positive samples. Usually, we receive one sample of each patient, but if a patient's condition worsens, clinicians send additional samples in order to determine the presence of persistent infection or other respiratory viruses. NPA samples for case 1 and TA samples for case 2 were used.

Real-time reverse transcriptase PCR, sequencing and pyrosequencing

Viral RNA was extracted directly from patients' NPAs or TAs using either QIAamp Viral RNA (Qiagen GmbH, Hilden, Germany) or PureLink Viral RNA/DNA kit (Invitrogen, Carlsbad, CA, USA) and used as a template for real-time reverse transcriptase (RT)-PCR, sequencing and pyrosequencing. Pandemic H1N1 2009 virus diagnosis was made following the US Centers for Disease Control and Prevention (CDC) Real-time RT-PCR Protocol for Detection and Characterization of Swine Influenza [3] (materials kindly provided by the Influenza Branch, CDC, Atlanta, GA, USA).

A 620-bp amplicon including the neuraminidase 275 codon was obtained directly from NPAs following the World Health Organization sequencing protocol (neuraminidase fragment 4) [4]. Purified DNA fragments were sequenced using the DYEnamicTM ET Terminator Cycle Sequencing kit (GE Healthcare, Little Chalfont, UK) in an automated capillary sequencer (MegaBACE 1000; GE Healthcare, Piscataway, NJ, USA).

Pyrosequencing was performed following the World Health Organization influenza A (H1N1) NA-H274 protocol [5] using the PSQTM96 MA platform (Biotage AB, Uppsala, Sweden). Relative proportions of sensitive and resistant variants were determined with the Pyromark ID version 1.0 software (Biotage AB) following allele quantitation analysis.

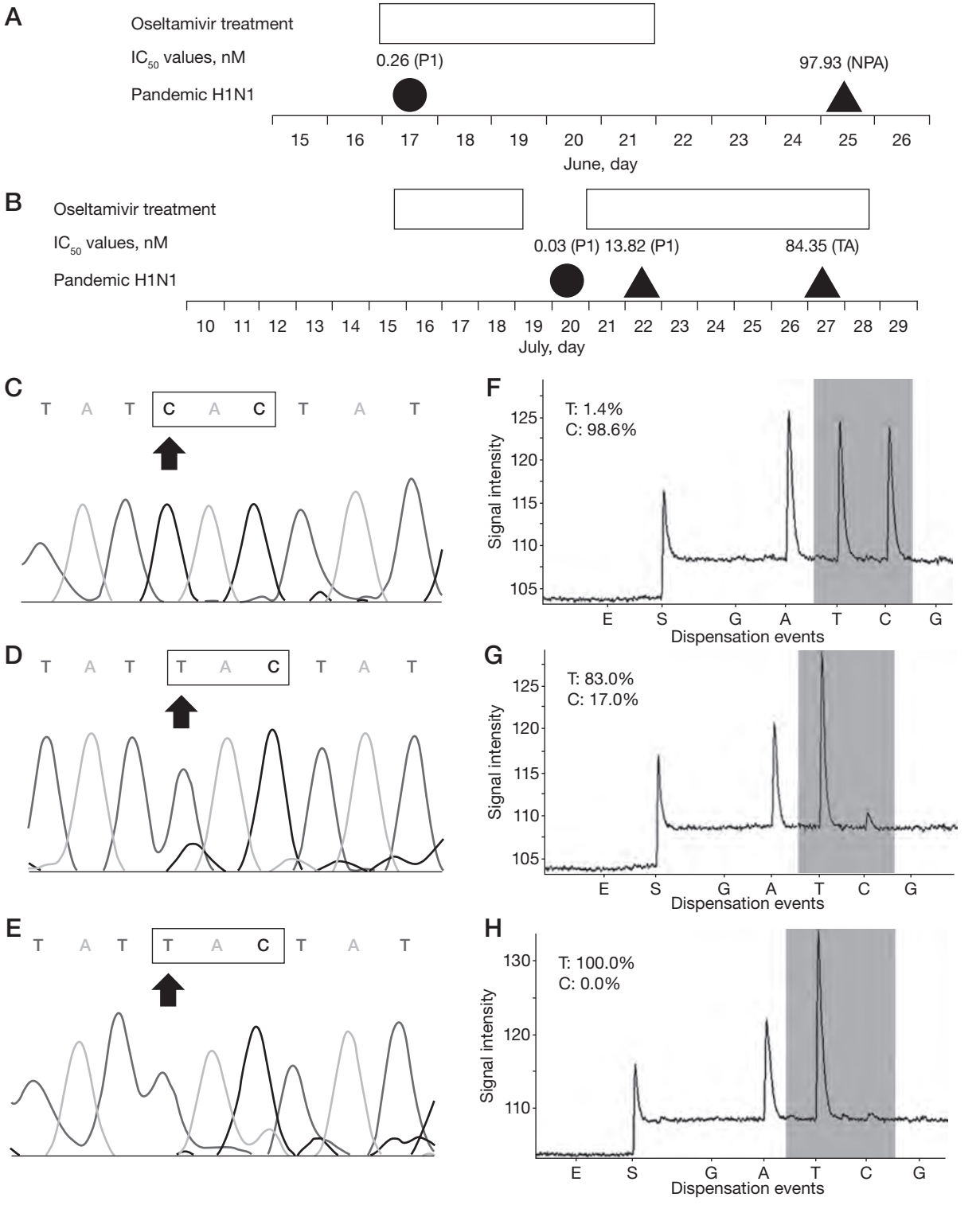
Neuraminidase inhibition assays

Susceptibility of influenza viruses to oseltamivir was assessed by chemiluminescence using the NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Prior to the assay, oseltamivir phosphate (50 μM; kindly provided by Bio Sidus SA, Buenos Aires, Argentina) was activated by incubation with rat plasma at 37°C for 30 min [6] and then diluted in a half-log series (range 0.03–1,000 nM). The tests were performed directly on NPA dilutions. In cases when the original aspirate was no longer available or suitable for analysis, first passage isolates were used. Oseltamivir 50% inhibition concentration (IC_{50}) was calculated by using non-linear curve fitting (GraphPad Prism® 5.02; GraphPad Software, Inc., La Jolla, CA, USA).

Virus culture

Viral isolation was performed by inoculating the amniotic cavity of pathogen-free embryonated hen eggs (Inmuner, Concepción del Uruguay, Argentina).

Figure 1. Emergence of intratreatment resistance to oseltamivir



Time courses of **(A)** case 1 and **(B)** case 2 are shown. The open box indicates days of oseltamivir treatment. Sensitive (circle) and resistant (triangle) pandemic H1N1 2009 viruses and their 50% inhibitory concentration (IC_{50}) values are shown. Type of samples used were nasopharyngeal aspirate (NPA), tracheal aspirate (TA) and first passage of the isolate (P1). Electropherograms of **(C)** sensitive, **(D)** mixed and **(E)** resistant variants for case 2 are shown. Residue 275 encoded by the 3-nucleotide codon is indicated in boxes and the nucleotide substitution (C→T for amino acid change H→Y) is indicated by arrows. Lines are usual lines obtained as a result of basecalling after automated DNA sequencing. Pyrograms of **(F)** sensitive, **(G)** mixed and **(H)** resistant variants for case 2 including percentages of sensitive (C, %) and resistant (T, %) variants calculated from peak intensities in grey areas are shown. E, enzyme; S, substrate.

Allantoid fluid was harvested after 5 days of incubation at 37°C.

Results

In both cases, wild-type neuraminidase sequence was present early in the infection (illustrated for case 2 in Figure 1C). While on treatment, the oseltamivir-resistant strain containing the H275Y mutation emerged, as shown in Figure 1D, which later completely replaced the wild-type strain (Figure 1E; GenBank accession numbers are CY053466 and CY053467 for case 1 and CY053468, CY053470 and CY053469 for case 2).

To further analyse the viral population in the samples, pyrosequencing was performed and confirmed that only the wild-type virus was present in the initial sample (illustrated for case 2 in Figure 1F) and the relative proportion of resistant over-sensitive variants increased progressively over time (Figure 1G and 1H). Interestingly, the increase was very fast, with the resistant virus becoming dominant (83% of virus population) at 48 h of treatment. In both cases, the last influenza-positive sample was 100% resistant.

In case 1, a 377-fold increase in oseltamivir IC_{50} was found in the sample obtained 4 days after the end of treatment, when the entire virus population was found to be composed of the H275Y variant (Figure 1A). A similar outcome was observed in case 2, where a 2,636-fold increase in IC_{50} was measured (Figure 1B).

Discussion

We report the emergence of intratreatment oseltamivir resistance in two paediatric cases of pandemic H1N1 2009 by direct sequencing, pyrosequencing and phenotypic activity of neuraminidase. Because mutations can arise from events related to culture conditions, we searched for mutations by both sequencing and pyrosequencing the original clinical samples. Pyrosequencing also allowed us to detect variant mixtures, showing the transition of the viral population which changed from sensitive to resistant during oseltamivir treatment. These findings were consistent with the increase in IC_{50} values for oseltamivir.

The H275Y mutation had already been present worldwide during the seasonal outbreaks prior to the 2009 pandemic [7–9]. In children with seasonal influenza, exposure to oseltamivir leads to resistance more rapidly in those infected with influenza A H1N1 than influenza A H3N2 or influenza B [10]. Paediatric patients are more prone to develop oseltamivir resistance than adults. Putative explanations include that young children are probably suffering a primary influenza infection, thus being immunologically naive to the virus and that the prescribed antiviral dose might not reach the effective concentration. Both conditions would permit higher viral replication, increasing the chances of the emergence of resistant variants [10–12].

Although some resistant mutants have reduced *in vitro* and *in vivo* fitness, they have also been associated with morbidity and mortality in high-risk patients [13,14]. In this report, a previously healthy patient (case 2) suffered a life-threatening disease related to the early emergence of intratreatment resistance. Interestingly, this patient received two treatment cycles with oseltamivir – the initial cycle was stopped prematurely after 4 days and the second lasted for 9 days, during which resistance emerged very rapidly. Although we have no direct evidence, premature treatment withdrawal might have favoured the emergence of resistance.

The H275Y mutation does not confer resistance to zanamivir and can be considered as a treatment option for patients with severe illness caused by oseltamivir-resistant virus. In this study, oseltamivir resistance was detected retrospectively, after the patients had been discharged. The detection of resistance during hospitalization could hypothetically have led to a shift in antiviral treatment, although at the time of patients' hospitalization no alternative treatment was available in our hospital (Hospital de Niños R Gutiérrez). Currently, the available option worldwide is zanamivir, although it is not recommended for children under the age of five because of its administration by inhalation. Another alternative would be intravenous zanamivir, which has been already used as an emergency investigational drug [15–17].

The use of neuraminidase inhibitors as one of the best antiviral measures during pandemics should be coupled with thorough monitoring of resistance in the circulating viruses. Viral isolates should also be evaluated, when possible, in seriously ill patients not responding to therapy.

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Disclosure statement

The authors declare no competing interests.

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