CASE REPORT

Oseltamivir-resistant influenza A pandemic (H1N1) 2009 virus in Northern Greece

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Abstract
Resistance to oseltamivir was observed to influenza A pandemic (H1N1) 2009 virus strains, isolated from two patients in North Greece. Investigations showed resistant viruses with the neuraminidase (NA) 275Y genotypes. Pandemic A (H1N1) 2009 virus should be closely monitored for emergence of resistant variants. Hippokratia 2011; 15 (3): 272-274

Keywords: oseltamivir-resistant, pandemic influenza A (H1N1) 2009 virus, Northern Greece

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During April 2009, a novel H1N1 virus was detected in epidemiologically unrelated cases of influenza-like illness in California and was subsequently recognized to be the cause of a major outbreak of respiratory disease in Mexico that had been already ongoing for some weeks[1]. This emergence prompted the World Health Organization (WHO) to declare a pandemic caused by this virus on June 11, 2009. Although most cases of infection are mild or even asymptomatic, 16.713 fatal cases were reported to WHO as of March 2010[2].

Initial testing of the 2009 pandemic influenza A (H1N1) virus found it susceptible to neuraminidase inhibitors (oseltamivir and zanamivir) and resistant to adamantanes (amantadine and rimantadine)[3]. Oseltamivir and zanamivir block the function of NA, thus inhibiting the spread of newly formed viral particles[4].

Since the first report of oseltamivir-resistant pandemic A (H1N1) 2009 virus in June 2009[5], a total of 267 oseltamivir-resistant cases have been reported and confirmed worldwide[2].

All these viruses have the same mutation in the neuraminidase gene (H275Y), conferring resistance to oseltmivir but not to zanamivir. There is also no evidence of reassortment between pandemic (H1N1) 2009 and other seasonal influenza A (H1N1) viruses, which have shown a high prevalence of oseltamivir resistance[6].

This finding raises strong concerns that the 275Y resistant mutation in pandemic A (H1N1) 2009 virus might circulate and dominate.

In Greece, an enhanced surveillance system for pandemic influenza A (H1N1) virus was set up by 30th of April 2009. Specimens were sent to one of two reference laboratories, one in Athens (Hellenic Pasteur Institute) covering southern Greece and one in Thessaloniki (Aristotle University of Thessaloniki, Second Microbiology Laboratory) covering northern Greece[7].

All cases investigated for pandemic H1N1 2009 were notified directly to HCIDC (http://www.keelpno) on an individual basis, both by hospital clinicians and by the reference laboratories and to WHO.

If the sample was PCR-positive for pandemic H1N1 2009 and the clinical manifestations were severe, the patients were hospitalized and specific treatment was administered. In the beginning of the outbreak, antiviral drugs were given to all suspected cases and their contacts, but the following months, antiviral drugs were given only to symptomatic high-risk groups[8].

In the present study, we report virologic investigation of the emergence of oseltamivir resistance in two viruses from two patients in Northern Greece.

A 7.5 year old girl with Dravet syndrome, hypothyroidism, hypogammaglobulinemia of IgG2 subclasses and psychomotor retardation had a fever and difficulty in breathing on January 1, 2010. On January 3, 2010, physical examination showed a temperature of 38,5°C, a blood pressure of 116/75mmHg, a pulse rate of 94 beats/min, and a oxygen saturation of 85% at room air. Due to *status epilepticus* on the same day the patient was intubated and was submitted to the pediatric intensive care unit for mechanical ventilation and further treatment.

She was started receiving oseltamivir (Tamiflu) at day 4 after the initial symptoms had started, but despite the therapy, her health condition deteriorated due to *Pseudomonas aeruginosa* pneumonia. Two months ago, the girl was vaccinated against seasonal influenza and pneumoniococci but failed to vaccinate against the pandemic influenza A (H1N1).

Diagnosis of pandemic influenza A (H1N1) 2009 virus

was established 4 days after the initial symptoms had started at January 5, 20101. 17 days later, her clinical condition gradually improved and she was transferred from the PICU to the pediatric clinic. Oseltamivir was discontinued after 20 days. Her hospitalization lasted for 1 month; no illness was reported among her family members.

As part of the northern Greek NIC surveillance of hospitalized and severe cases of pandemic influenza, the respiratory specimen was retrospectively analyzed for molecular markers for antiviral resistance. The virus in the above specimen had the H275Y mutation in the neuraminidase gene, known to confer resistance to oseltamivir. Furthermore, haemagglutinin sequencing of the HA1 region of the above strain (A/Thessaloniki/225/10) revealed another common variation, D222G, which has been observed to severe and fatal cases, as well as, mild cases of pandemic virus infections. Similar cases have been reported to WHO[9].

The second case was identified through routine surveillance, rather than active antiviral susceptibility testing because of treatment failure in a severe case. The patient was a 13.5 year old previously healthy girl, who did not receive oselatmivir and developed mild ILI symptoms. The patient experienced cough, sore throat and headache with fever (39.5°C). Her symptoms started 3 days before the specimen collection and the laboratory confirmed the presence of 2009 pandemic influenza A (H1N1) virus by real-time RT-PCR, on October 2. One week later, her fever resolved and she was asymptomatic. No illnesses were identified among close contacts potentially exposed during her staying at home. Laboratory testing detected H275Y mutation as well.

In both cases, RNA extraction from clinical samples was achieved using the Viral RNA mini kit (Qiagen, Germany), according to the manufacturer's instructions.

One step real-time RT-PCR was performed at an ABI 7500 platform according to the CDC protocol for the identification of pandemic influenza A (H1N1) viruses[10]. The reagents and positive control material is kindly provided by CDC.

RNA from positive samples where then reverse transcribed and NA amplification was achieved with one-step RT-PCR, using Superscript III One-step RT-PCR kit (Invitrogen, UK).

PCR products were purified using the Chargeswitch PCR cleanup (Invitrogen, UK) according to the manufacturer's instructions. Purified PCR products were sequenced at an ABI 310 platform using the appropriate primer pair for each amplified product. Chromograms were analysed using Chromas software and NA sequences from representative strains were added to GenBank (Accession no for the first case: CY056386, we are waiting the Accession no for the second case).

This report describes confirmed oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infection in two patients. This is the first report of oseltamivir resistance in Greece. These viruses isolated from two totally different cases. The first case was a 7 year old girl with Dravet syndrome, hypothyroidism, hypogammaglobulinemia and psychomotor retardation, who received Tamiflu for 10 days, while the second one was a 13,5 year old previously healthy girl, who did not receive oselatmivir at all. Therefore, it is unlikely that the 275Y mutation was drug-induced. It is also worth mentioning that no illness was reported among their family members.

In a similar study conducted in Hong Kong, China, an oseltamivir-resistant influenza A (H1N1) 2009 virus was found in a 16-year-old previously healthy girl, who was not treated with oseltamivir. Detection of mixed population of 275Y and 275H in the NPA specimen of the above patient suggests that the mutation occurs naturally, either before or during infection. Although no experimental data exists that show the growth properties of this resistant variant, examination of the quasi-species population in the cell culture-propagated virus isolate showed that the 275Y variant has become the dominant population. This finding implies that the 275Y mutation does not compromise replication of pandemic A (H1N1) 2009 virus in vitro[11].

The number of reported cases of oseltamivir-resistant pandemic A (H1N1) 2009 influenza virus remains low despite the large scale of pandemic, widespread oseltamivir use and extensive monitoring of susceptibility[12,13]. Although there is no evidence of general community circulation of such resistant viruses, there is clear evidence of limited person-to-person transmission in several epidemiological settings[6].

On the other hand, lack of general immunity to pandemic A (H1N1) 2009 virus in the human population, combined with the inherent adamantine resistance of the virus, indicates that NA inhibitors constitute the primary treatment regimen for susceptible patient groups and those in whom severe diseases develop during the current pandemic. There is great concern that an oseltamivir-resistant variant of pandemic A (H1N1) 2009 virus may emerge and circulate in a manner similar to oseltamivir-resistant seasonal influenza A (H1N1) virus[14].

Therefore, active surveillance for antiviral resistance in pandemic strains needs to be maintained by clinicians, laboratories and agencies. Knowledge of this virus is still limited and characterization of transmission properties of this resistant variant in in vitro and in vivo models is needed. Moreover, pandemic A (H1N1) 2009 virus should be closely monitored for emergence of resistant variants.

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