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Original Article

Oseltamivir-resistant influenza A 2009 H1N1 virus in immunocompromised patients

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Background First-line treatment of influenza A 2009 H1N1 relies on neuraminidase inhibitors such as oseltamivir. Resistance conferred by the H275Y neuraminidase gene mutation is concerning and likely to increase.

Objectives To characterize oseltamivir resistance in a hospital-based patient population.

Patients and Methods All available respiratory specimens positive for influenza A by direct fluorescent antibody, RT-PCR, or culture from patients at the University of Utah 5/09-12/09 were collected. Specimens were confirmed as 2009 H1N1 by the Utah Department of Health. RT-PCR and pyrosequencing were used to test for the H275Y mutation (CDC protocol). PyroMark Q24 AQ software (Qiagen, Valencia, CA, USA) was used to allow for quantitative H275Y mutation analysis. Medical records of patients with resistant virus were reviewed.

Results We tested 191 influenza A virus-positive samples from 187 unique patients. Fifty (27%) patients were hospitalized. Four patient specimens (2·1%) were found to carry the H275Y mutation. Three patients were hospitalized, representing 6% of inpatient samples tested. Three patients had undergone hematopoietic stem cell transplant in the past year. Two patients died. Their influenza viruses were confirmed to be oseltamivir-resistant at an independent reference laboratory and through the Center for Disease Control and Prevention (CDC). One patient reported no history of prior oseltamivir exposure.

Conclusions Widespread oseltamivir resistance among 2009 H1N1 remains a potential threat. Rapid techniques, such as pyrosequencing, which has the additional benefit of identifying mixed mutant populations of virus, may play a key role in identifying at-risk individuals and potentially unsuspected cases. Targeted surveillance of immunocompromised patients will be critical toward improving future influenza planning and therapy.

Keywords Hematopoietic stem cell transplant, pyrosequencing, surveillance.

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Introduction

The World Health Organization (WHO) declared influenza A 2009 H1N1 (2009 H1N1) a pandemic in June 2009. Like seasonal influenza, infants, young children, those with certain medical conditions, and immunocompromised individuals are at higher risk of developing complications.[1] Early treatment with antiviral drugs appears to reduce the length and severity of symptoms and may reduce complications.[2] Unlike current strains of seasonal H1N1 virus, which are now resistant to oseltamivir,[3] the majority of 2009 H1N1 virus strains remain susceptible. The H275Y mutation in the neuraminidase (NA) gene confers oseltamivir resistance in seasonal H1N1 and 2009 H1N1 viruses.[4]

Despite concerns over the possibility for widespread development of oseltamivir resistance, first-line treatment of patients with 2009 H1N1 continues to rely on NA inhibitors. As of February 2010, the Center for Disease Control and Prevention (CDC) and the WHO had identified globally 225 cases of 2009 H1N1 virus resistant to oseltamivir.[5] Oseltamivir resistance of 2009 H1N1 virus has been sporadically reported worldwide and linked primarily to *de novo* development of the H275Y mutation with exposure to oseltamivir therapy or chemoprophylaxis. We screened 191 2009 H1N1-positive samples and describe four cases occurring in immunocompromised patients in Utah, one of whom was infected with H275Y mutant virus in the absence of prior oseltamivir exposure.

Methods

All available respiratory specimens that were positive for influenza A by direct fluorescent antibody testing (DFA), reverse transcription-polymerase chain reaction (RT-PCR), or viral culture from inpatients and a subset of outpatients at the University of Utah from May to December 2009 were retrospectively screened for oseltamivir resistance by pyrosequencing. Results from this study had no effect on patient management. Subtyping of specimens was performed by the Utah Department of Health (UDoH) or presumed to be 2009 H1N1 virus based on UDoH surveillance data.

Viral nucleic acid was extracted from available patient specimens using the QIAamp Viral RNA extraction kit or the QIAamp Virus BioRobot 9604 (Qiagen, Valencia, CA, USA). The CDC protocol was used for RT-PCR and pyrosequencing to detect the H275Y mutation of 2009 H1N1,[6] except biotinylated amplicons were analyzed using the allelic quantification (AQ) module (PyroMark Q24 AQ software; Qiagen) with a dispensation order of GATCGACTATG. Mutant sequences also underwent dideoxy Sanger sequencing by capillary gel electrophoresis.

Approval for this study was obtained from the Institutional Review Board of the University of Utah. The electronic medical records of patients with resistant virus were reviewed, and demographic, clinical, and laboratory data were abstracted.

Results

We tested 191 influenza A-positive samples from 187 unique patients: 109 women and 78 men. The ages ranged from 10 months to 84 years. Fifty (27%) patients were hospitalized.

Four patients (2·1%) were found to carry the H275Y mutation (Table 1). All four patients were confirmed to be infected with the 2009 H1N1 virus by RT-PCR. Three patients were hospitalized, representing 6% of inpatient samples tested. Cases 1-3 had all undergone hematopoietic stem cell transplant (HSCT) in the past year. Two patients died (cases 1 and 2). Based on clinical suspicion, these patients were tested for and confirmed to have oseltamivir-resistant influenza A virus by an independent reference laboratory or the CDC.

Case 1

A 69-year-old man with multiple myeloma requiring tandem autologous HSCT was hospitalized 7 months post-transplant with orbital cellulitis, invasive fungal sinusitis, mental status changes, fever, and hypoxia (Figure 1). His maintenance chemotherapy included bortezomib, thalidomide, and dexamethasone. His white blood cell count (WBC) at admission was 4250/μl with an absolute lymphocyte count of 2300/μl.

An admission nasopharyngeal swab was RT-PCR-positive for 2009 H1N1 virus. In addition to treatment for invasive fungal sinusitis, the patient was started on high-dose oseltamivir (150 mg twice daily). His respiratory status progressively declined. The patient was intubated, and a nasogastric tube was placed for nutrition and medication delivery. Chest computed tomography scan demonstrated bilateral lower lobe consolidation with patchy bilateral ground glass opacities throughout. A follow-up bronchoalveolar lavage (BAL) specimen collected after 9 days of oseltamivir remained influenza A culture-positive.

He completed his 10-day course of oseltamivir but clinically continued to do worse. As his clinical status worsened, his renal function also declined with serum creatinine increasing from 1·17 mg/dl on day 10 to a peak of 2·14 mg/dl on day 14. A reference laboratory detected the H275Y mutation in the influenza virus isolate from the BAL sample taken on day 9. Use of intravenous zanamivir was being pursued when the patient developed an acute cerebral infarction. He died of this complication on hospital day 17. During our retrospective testing, the H275Y mutation was not present on admission.

Case 2

A 66-year-old man was presented with a mild respiratory illness (Figure 1). His past medical history included multiple myeloma treated with two autologous HSCTs 5 months before and polycystic kidney disease leading to kidney transplantation 2·5 years before this illness. His immunosuppressive medications included tacrolimus, prednisone, and thalidomide. A nasopharyngeal sample was tested positive for influenza A by DFA. He was treated with 150 mg oseltamivir twice daily as an outpatient. The patient continued to have respiratory symptoms after the end of his first 10-day course of oseltamivir.

He continued to have fever and mild cough 22 days after his initial positive influenza test despite a 10-day course of oseltamivir and a decrease in his immunosuppression regimen. This continued, and he was subsequently admitted to the hospital on day 40. His WBC was 5340/μl with an absolute lymphocyte count of 860/μl. Creatinine upon admission was elevated at 1·31 mg/dl. He was noted to have cytomegalovirus viremia at time of admission, and intravenous ganciclovir therapy was initiated.

Given his prolonged outpatient course and continued respiratory symptoms, the patient was suspected to have resistant oseltamivir H1N1 virus. Although BAL specimens were culture negative for influenza A virus on days 42 and 43, a sample taken on day 22 and positive for influenza A virus by DFA was sent to the CDC where on day 43, results of molecular testing demonstrated the H275Y resistance

Table 1. Characteristics of four immunocompromised patients with oseltamivir-resistant (H275Y) influenza A 2009 H1N1 virus seen at an academic referral center, 5/2009-12/2009

Case	Age Sex	Prior medical conditions	Influenza testing			Oseltamivir use at time of resistant virus detection	Outcome
			Day	Result	Virus population		
1	69 Male	Multiple Myeloma Autologous HSCT ×2	0 1 9 79%	RT-PCR+ Culture + Culture +	100% Wild type 100% Wild type H275Y*	Day 9/10 (150 mg twice daily)	Hospitalized died
2	66 Male	Multiple Myeloma Autologous HSCT ×2	0 2 9 22 43 44 78 79	DFA + RT-PCR+ DFA+ DFA+ Culture – Culture – DFA + RT-PCR +	100% Wild type Not tested 100% Wild type 96% H275Y† N/A N/A 100% H275Y* 100% H275Y	12 days after completing 10 day course (150 mg twice daily)	Hospitalized died
3	36 Female	AML Allogeneic HSCT	0	DFA +	92% H275Y	Day 8/10 prophylaxis (70 mg twice daily)	Outpatient recovered
4	48 Female	Asthma Eczema Cyclosporine	0	RT-PCR +	43% H275Y	None	Hospitalized recovered

HSCT, Hematopoietic stem cell transplant; AML, Acute myelogenous leukemia; RT-PCR, Reverse transcriptase polymerase chain reaction; DFA, Direct fluorescent antibody; N/A, Not applicable.
*Specimen tested and confirmed to have the H275Y mutation at a reference laboratory.
†Specimen tested and confirmed to have the H275Y mutation at the Center for Disease Control and Prevention.

mutation. With this information, oseltamivir therapy was discontinued 21 days after initiation of his second course. Intravenous zanamivir treatment was pursued; however, the patient improved with supportive care alone and was discharged on day 48, 9 days after admission.

Over the next month, the patient complained of no symptoms suggestive of influenza virus infection. On day 78 of his illness, he was readmitted with rapid onset respiratory distress. His WBC was elevated at 16 970/μl with an absolute lymphocyte counts (ALC) of 1350/μL. His creatinine at admission was again elevated at 1·55 mg/dl and peaked at 1·91 mg/dl during this admission. His initial chest radiograph demonstrated areas of consolidation in the left upper and lower lobes. Tacrolimus levels remained steady between 5 and 10 ng/ml throughout the hospitalization. Nasopharyngeal samples were positive for influenza A virus by DFA and RT-PCR. Given his history of oseltamivir-resistant H1N1 virus, inhaled zanamivir was started and eventually transitioned to intravenous administration after 4 days. He refused intubation and died on hospital day 12 of his second admission, day 88 from his initial positive influenza test. We later demonstrated that the influenza virus present during his second admission also had the H275Y mutation.

Case 3

A 36-year-old woman with a history of subtype M4 acute myelogenous leukemia and allogeneic HSCT 10 months prior was presented with a history concerning for influenza. Multiple family members had recently tested positive for influenza A virus and were started on standard treatment courses of oseltamivir. At the same time, she was started on oseltamivir prophylaxis (75 mg once daily). She was seen in clinic on day 8 of her prophylaxis, as directed, complaining of newly developed mild fever and cough. Her WBC was 10640/μl with an ALC of 3300/μl. Her creatinine was also elevated at 1·51 mg/dl although consistent with her baseline. Chest radiograph at presentation showed no evidence of an acute pulmonary process. Her oseltamivir dose was increased to 150 mg twice daily for 10 days after a nasopharyngeal sample tested positive for influenza A virus by DFA. She clinically improved and did not require admission.

Case 4

A 48-year-old woman with severe asthma and eczema on oral cyclosporine therapy was admitted for a severe asthma exacerbation. WBC at admission was 12 300/μl with an ALC of 600/μl. Chest radiograph showed minimal

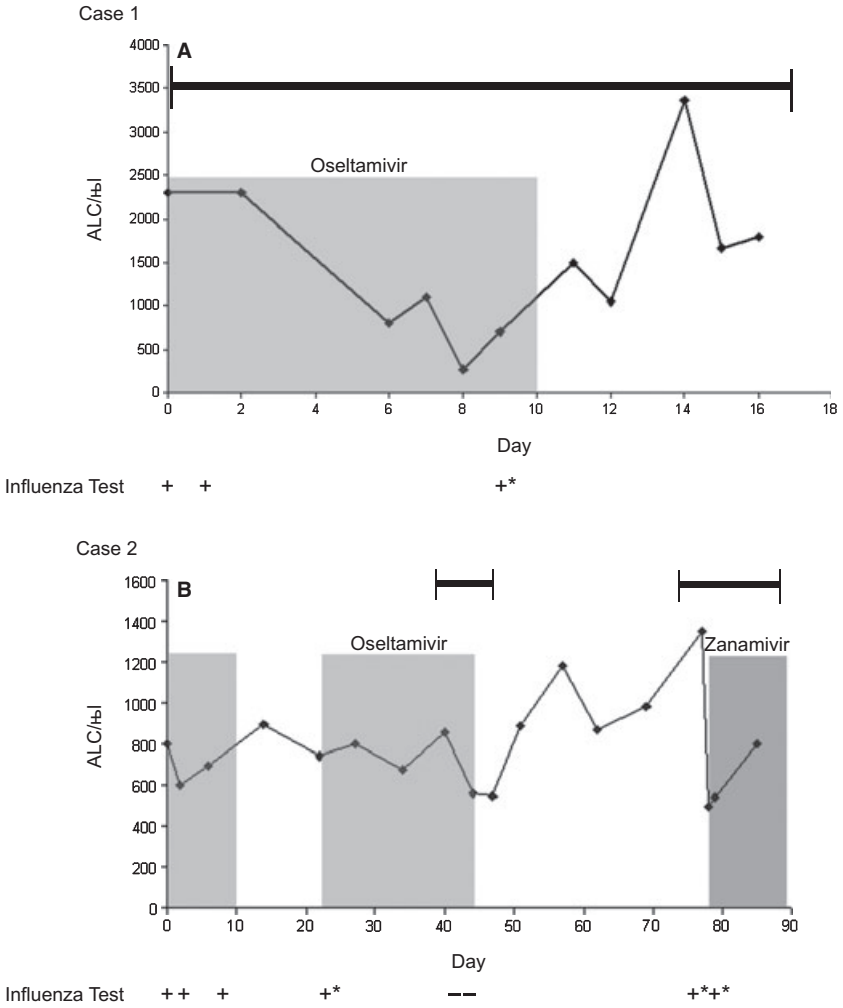

Figure 1. The course of antiviral treatment and absolute lymphocyte counts (ALC) over time are shown for Cases 1 and 2. Hospitalization is indicated by the black bar. (A) Case 1: Day 0 was the first positive test for influenza virus. Subsequent influenza virus tests are with (+). Resistant influenza virus (+*) was detected on day 9. The patient died on day 17. (B) Case 2: First positive influenza virus test was on day 0. Subsequent tests were performed on day 2, 9, 22, 42, 43, 77, and 78 and marked as (+, +*, or –). The sample from day 22 was submitted for resistance testing (+*), and results were reported on day 43 leading to discontinuation of oseltamivir therapy. The patient died on day 88.

subsegmental bibasilar atelectasis. Her creatinine was mildly elevated at 1·13 mg/dl. Her nasopharyngeal sample tested positive for influenza A virus by RT-PCR at admission. This influenza virus had an oseltamivir-resistant viral population of 43%, which was confirmed upon multiple repeats of nucleic acid extraction and pyrosequencing. Sanger sequencing was unable to detect the H275Y mutant population. She reported no prior exposure to oseltamivir. She could not identify any close contacts with individuals with influenza-like illness or who were on oseltamivir. She completed a 5-day course of oseltamivir (75 mg twice daily) and a steroid burst of prednisolone 60 mg by mouth daily for 2 weeks followed by a slow taper to a baseline dose of 10 mg daily. She remained on cyclosporine throughout her course and was discharged home on hospital day 8.

Discussion

The concern for the development of 2009 H1N1 oseltamivir-resistant virus remains high. A small number of reports describe resistance in the setting of chemoprophylaxis and in immunocompromised patients.[7–10] We describe four new cases of oseltamivir resistance in 2009 H1N1 virus after screening 191 specimens using pyrosequencing based on the CDC protocol. All of these cases occurred in immunocompromised patients and illustrate the utility of resistance testing for high-risk

patients. Surveillance also identified an unsuspected case of oseltamivir resistance, which raises the possibility of community transmission.

Sporadic cases of the oseltamivir-resistant seasonal H1N1 virus were reported prior to the 2007–2008 influenza season. However, beginning in 2007–2008, oseltamivir-resistant H1N1 rapidly emerged and became the predominant strain of seasonal influenza.[3] Many experts feel that a similar emergence of resistance in 2009 H1N1 virus is possible given the widespread use of antiviral drugs.[7–11] Sustained transmission of oseltamivir-resistant virus has not yet been observed, although a small outbreak in Vietnam has been described.[10]

The development of antiviral drug resistance has been described in patients with compromised immunity. They often have prolonged viral shedding despite antiviral therapy. Poor absorption of oseltamivir in patients with diarrhea may also play a role. Case 1 developed resistance after 9 days of high-dose oseltamivir therapy, the rapidity of which is concerning because it has been suggested that higher doses of oseltamivir might forestall the development of resistance.

There are a few reports of oseltamivir resistance identified in 2009 H1N1 virus from previously untreated patients.[10,11] Case 4 from this study did not have oseltamivir exposure, and no direct contacts on oseltamivir were identified. Resistant virus constituted 43% of her total influenza virus population, and she recovered without clinical suspicion of resistance. It is unclear whether this represents a *de novo* mutation or community acquisition, but it raises the possibility of limited ongoing transmission of resistant virus in the community. Continued surveillance will be necessary.

The pyrosequencing method described here rapidly identifies and quantifies subpopulations of resistant virus. Relative quantification of resistant versus susceptible virus populations is not possible with other commonly used molecular approaches such as RT-PCR or Sanger sequencing. This may provide additional insights into the development of and selection for oseltamivir-resistant virus. Additional data are needed to better understand the kinetics of drug resistance development in vulnerable patients.

Current recommendations for testing and treating patients at risk for infection with oseltamivir-resistant 2009 H1N1 influenza virus are to perform viral susceptibility testing if the patient does not respond after 5–10 days of oseltamivir treatment.[12–14] Patients with a history of recent transplantation, both hematopoietic stem cell and solid organ, are at highest risk and should be followed with a high index of suspicion for the development of resistance. Treatment guidelines state that 75 mg of oseltamivir twice daily remains the standard therapy, but this dose may be increased to 150 mg twice daily for critically ill patients.[12,13] Patient in neither in case 3 nor case 4 was clinically suspected to have oseltamivir-resistant influenza virus. Our finding of patients with 2009 H1N1 influenza virus harboring the H275Y mutation with apparently low suspicion for developing resistance is concerning. This raises the question of whether all patients with immunosuppression should be routinely tested for oseltamivir resistance; however, we still feel that clinical judgment and targeted surveillance remain the best way to identify oseltamivir-resistant 2009 H1N1.

Our study highlights the potential utility of real-time oseltamivir resistance detection in high-risk immunocompromised patients. Four (2·1%) of 191 isolates of 2009 H1N1 influenza were oseltamivir-resistant, comparable to the 1·3% rate reported by the CDC.[5] Some limitations should be recognized. Our study population was potentially biased toward sicker patients seen in a referral hospital system. Systematic prospective influenza testing in defined populations would provide an unbiased estimate of the prevalence of resistance. We focused on the H275Y neuraminidase mutation, which is currently of greatest concern. Other resistant mutations were not assessed, but these have been rare.[15] Furthermore, we did not use a phenotypic assay that would detect novel mutations.

Conclusions

Widespread oseltamivir resistance among 2009 H1N1 virus remains a potential threat. Rapid techniques, such as pyrosequencing, which has the additional benefit of identifying mixed mutant populations of virus, may play a key role in identifying at-risk individuals and potentially unsuspected cases. Targeted surveillance of immunocompromised patients will be critical toward improving future influenza planning and therapy.

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Conflicts of interest

All authors have no conflicts of interest.

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