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Detection of a Transient R292K Mutation in Influenza A/H3N2 Viruses Shed for Several Weeks by an Immunocompromised Patient

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We describe the case of an immunocompromised patient, positive for influenza A virus (H3N2), in whom the neuraminidase R292K mutation was transiently detected during oseltamivir treatment. The R292K mutation was identified by direct testing in 3 of 11 respiratory specimens collected throughout the patient's illness but in none of the cultures from those specimens.

CASE REPORT

A 58-year-old male presented to our institution on day 96 following a cord blood allogeneic stem cell transplant for treatment of chemo-refractory chronic lymphocytic leukemia. His symptoms included abdominal pain, weight loss, and increased stool frequency, and he was subsequently readmitted for further management. Despite negative *Clostridium difficile* PCR tests, colonoscopy revealed mucosal ulceration and exudates throughout the colon and the patient was diagnosed with pseudomembranous colitis. He was started on a course of metronidazole and vancomycin. The patient remained hospitalized with persistent diarrhea, bloody at times, and intermittent human herpesvirus 6 (HHV-6) viremia.

On day 133 posttransplantation (21 February 2012), the patient was noted to have a cough consistent with influenza-like illness, and a nasopharyngeal swab (NPS) was collected and submitted for respiratory virus PCR (FilmArray RP; BioFire Diagnostics, Salt Lake City, UT). The sample tested positive for influenza A/H3N2 virus, and a 5-day course of oseltamivir was initiated that day. An NPS collected on 28 February 2012, following completion of the course of oseltamivir, was still positive for influenza A/H3N2 virus. At this time, the patient had no respiratory symptoms, but as a precaution, a second 5-day course of oseltamivir was administered starting on 28 February 2012. Despite the two courses of treatment, repeated NPSs still tested positive for influenza A/H3N2 virus (Table 1). At this point, the patient developed a persistent, nonproductive cough, which continued to worsen. Samples that were positive following several days of antiviral treatment were forwarded for assessment of their antiviral resistance profiles and tested as described below. A chest X ray on 16 March 2014 showed a small pleural effusion, and a follow-up computed tomography (CT) scan revealed new cavitary lesions. A bronchoscopy was performed, and bronchoalveolar lavage (BAL) fluids and bronchial washing specimens (BW) were submitted for viral, bacterial, and fungal cultures. Influenza A virus, adenovirus, and human herpesvirus 6 (HHV-6) were detected in the BAL fluid, with influenza A virus detected by culture in both BAL fluid and BW. The patient was not given any further antiviral treatment and continued to shed the virus for an additional 8 weeks. The first NPS to return a negative result was collected on 4 June 2012, followed by a second negative sample on 11 June 2014. The patient's health continued to deteriorate due to multiple comorbidities, and he expired on 1 July 2012.

A FilmArray respiratory panel and viral culture were performed on NPS specimens and BAL fluid/BW, respectively, as previously reported (1).

RNA extraction. Total nucleic acid was extracted from primary respiratory samples using an easyMAG instrument (bioMérieux, Durham, NC) according to the manufacturer's instructions.

RT-PCR for pyrosequencing. The Qiagen one-step reverse transcription (RT)-PCR kit was used throughout. For pyrosequencing, each reaction mixture contained 10 μl 5× buffer, 2 μl 10 mM deoxynucleoside triphosphates (dNTPs), 10 units RNase inhibitor, 0.2 μM concentrations of the forward and reverse primers, 2 μl enzyme, and 5 μl of total nucleic acid, in a 50-μl total volume. RT-PCR thermocycling was performed as follows: 50°C for 30 min; 95°C for 15 min; 45 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min; and 10 min at 72°C, with a final 4°C hold. RT-PCR products were purified as previously described (2).

NA pyrosequencing. Centers for Disease Control and Prevention (CDC)-developed, variant-specific, pyrosequencing assays were used to screen for the E119V, R292K, and S294N variations in the neuraminidase (NA) gene product (3, 4). Samples were sequenced in sequence analysis (SQA) mode (the cyclic dispensation of the deoxynucleosides was in a predetermined order) as previously described (2) using the CDC-modified sequencing primers. This was followed by allelic discrimination in single nucleotide polymorphism (SNP) mode (directed dispensation) to measure wild-type (WT) and variant virus percentages in mixed populations (3, 4).

RT-PCR for conventional sequencing. RT-PCR was per-

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TABLE 1 Analysis of sequential specimens collected from our patient[a]

Mo/day of 2014	Sample	Specimen type	FA RP PCR result	Viral culture result	RT-PCR C_T value (M) in Flu A assay	Pyrosequencing result (% of population)	Dideoxy sequencing result (% of population)	IC_{50}s (nM) of oseltamivir/ zanamivir	No. of days following last dose of oseltamivir
02/21	1	NPS	H3N2	ND	22.3	WT (100)	WT (100)	0.18/0.23	0[b]
02/28	2	NPS	H3N2	ND	18.6	WT (89.3) R292K (10.7)	WT (70) R292K (30)	0.18/0.24	3
03/03	3	NPS	H3N2	ND	25.9	WT (75.4) R292K (24.6)	WT (30) R292K (70)	0.29/0.25	1[c]
03/12	4	NPS	H3N2	ND	21.4	WT (94.2) R292K (5.8)	WT (60) R292K (40)	0.19/0.23	10
03/19	5	NPS	H3N2	ND	26.9	WT (100)	WT (100)	0.14/0.21	17
03/27	6	BAL fluid	ND	Flu A	ND	ND	ND	ND	25
03/27	7	BW	ND	Flu A	ND	ND	ND	ND	25
04/05	8	NPS	H3N2	ND	30.6	NA	NA	NA	34
04/14	9	NPS	H3N2	ND	27.9	NA	WT (100)	NA	43
04/23	10	NPS	H3N2	ND	26.6	NA	WT (100)	NA	52
05/01	11	NPS	H3N2	ND	21.9	WT (100)	NA	0.22/0.29	60
05/07	12	NPS	H3N2	ND	18.8	WT (100)	NA	0.13/0.23	67
05/14	13	NPS	H3N2	ND	30.2	NA	WT (100)	NA	74
05/21	14	NPS	H3N2	ND	ND	ND	ND	ND	81
05/28	15	NPS	H3N2	ND	ND	ND	ND	ND	88
06/04	16	NPS	Neg	ND	ND	ND	ND	ND	96
06/11	17	NPS	Neg	ND	ND	ND	ND	ND	103

[a] FA RP, FilmArray respiratory panel; RT-PCR, real-time RT-PCR; M, matrix gene; NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage; BW, bronchial washings; ND, not determined; Flu A, positive for influenza A virus; NA, no available data; Neg, negative; WT, wild-type influenza A H3N2 virus.
[b] Day 1 of the first course of oseltamivir.
[c] Counting started over with the last dose of the second course of oseltamivir.

formed as described above with the following modifications: primers were used at 0.6 μM, and cycling was changed to 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Following amplification and electrophoresis on 1% agarose, bands were purified with a Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA) or PCR products were treated with ExoSAP-IT to remove unincorporated dNTPs and primers (Affymetrix, Santa Clara, CA).

Real-time RT-PCR for influenza detection. Influenza virus confirmation was performed by using the CDC human influenza virus real-time RT-PCR diagnostic panels with an influenza virus A/B typing kit (IVD; catalog no. FluIVD03-1) and by using the CDC human influenza virus real-time RT-PCR diagnostic panel with the influenza A virus subtyping kit (IVD; catalog no. FluIVD03-2) provided to public health labs through the Influenza Reagent Resource according to the package insert.

Neuraminidase dideoxy sequencing. Multiple in-house RT-PCR and dideoxy sequencing assays were used to obtain a nearly complete NA gene sequence to confirm pyrosequencing variants and to detect other known or previously uncharacterized variants involved with NA inhibitor (NAI) antiviral resistance. Sequencing of PCR products was performed with an ABI 3130 or 3730 DNA analyzer (Applied Biosystems, Foster City, CA) using previously described procedures (5).

Viral culture. According to a published WHO protocol (6), all samples were inoculated into tubes of Madin-Darby canine kidney-ATL cells (MDCK cells; courtesy of the CDC) without influenza antiviral drug pressure and maintained for 2 weeks. MDCK cells were monitored for cytopathic effect (CPE) every day postinfection. Cells and fluid were harvested when CPE was observed over 50% or more of the cell monolayer.

NA-Fluor phenotypic testing. Testing of NA's susceptibility to drug inhibitors was performed using the NA-Fluor influenza neuraminidase assay kit (Applied Biosystems, Foster City, CA) as previously described (7) on influenza isolates harvested from MDCK cultures. The NA inhibitor antivirals oseltamivir and zanamivir were kindly provided, respectively, by Roche and GlaxoSmithKline.

Real-time RT-PCR testing was performed to confirm the presence of a single type and subtype of influenza virus in the patient samples and to assess the relative viral loads. All samples were confirmed positive for influenza A/H3N2 virus, with no other influenza viruses detected. The threshold cycle (C_T) values in the influenza A virus matrix target gene assay ranged from 18.6 to 30.6. There was no evident trend for these values.

Genotypic and phenotypic influenza antiviral susceptibility testing was completed on a total of 11 nasopharyngeal samples from this patient (Table 1). Pyrosequencing for antiviral resistance mutations was performed on all primary influenza virus specimens, and results are summarized in Table 1. Of the 11 samples tested, 4 contained no detectable variations from the wild-type sequence at amino acid positions 119, 292, and 294 (samples 1, 5, 11, and 12) and 3 contained a small percentage of viral sequences with changes at position 292 (samples 2, 3 and 4) (mixed-virus populations), while the other 4 samples either did not produce analyzable sequence or gave little to poor signal (samples 8, 9, 10, and 13). The mixed populations with changes at amino acid position 292 were due to a single nucleotide change, from AGA to AAA, with a consequent amino acid change from R to K. When samples 1 through 4 were sequenced by pyrosequencing in SNP mode, sample 1 was 100% wild type, sample 2 was a 89.3%

G/10.7% A mix, sample 3 was a 75.4% G/24.6% A mix, and sample 4 was a 94.2% G/5.8% A mix.

Sanger dideoxy sequencing was also performed on all primary samples, and summarized results are included in Table 1. Of the 11 samples, 5 were found to have wild-type sequence (samples 1, 5, 9, 10, and 13), 3 contained a mixed population of the wild-type sequence and the R292R/K sequence (samples 2, 3, 4), 2 did not sequence through the 292 region of the neuraminidase, and 1 failed to produce NA sequence. Mixed-population samples had varied mixtures of the wild-type sequence and the sequence with an antivirus-resistant variation at amino acid position 292, based on a review of the signal intensities of the resulting chromatograms. Again, the amino acid change from R to K was due to a single nucleotide change in the codon from AGA to AAA. Sample 2, collected on the first day of the second round of oseltamivir treatment, appeared to have approximately a 70% G/30% A mix. Sample 3, collected 1 day after completion of the second round of oseltamivir treatment, had a 30% G/70% A mix, and sample 4 collected 10 days after oseltamivir treatment contained a 60% G/40% A mix.

Additionally, primary samples were inoculated into MDCK cells to propagate influenza virus for neuraminidase inhibition studies. CPE was identified and confirmed by real-time RT-PCR in 7 (samples 1 through 5, 11, and 12) of the 11 samples. Viral harvests were tested in the NA-Fluor influenza virus neuraminidase assay kit with the FDA-approved NAIs oseltamivir and zanamivir. None of the samples had an exceptionally elevated 50% inhibitory concentration (IC_{50}) compared to the IC_{50}s for a known wild-type virus of the same type (A/H3N2). The wild-type, or antivirus-sensitive, A/H3N2 virus (A/Washington/01/2007, kindly provided by the CDC) used for testing resulted in IC_{50}s of 0.1 for oseltamivir and 0.15 for zanamivir. Thus, using criteria established by the World Health Organization expert working group on surveillance of influenza antiviral susceptibility, IC_{50}s of 1 or greater for oseltamivir and 1.5 or greater for zanamivir would have been required for the virus to be classified as having reduced susceptibility to neuraminidase inhibitors (8).

Influenza viruses can be associated with increased mortality in hematopoietic stem cell transplant (HSCT) recipients (9). Oseltamivir and zanamivir are the only FDA-approved NA inhibitors (NAIs) for influenza viruses. NAIs prevent the cleavage of sialic acid and inhibit the release and spread of new virions from the cell surface, thus preventing infection of additional cells. Ideally, treatment should be administered within 12 to 72 h of illness onset (10). Generally, influenza viruses with an N2 NA are more sensitive to oseltamivir than other types or subtypes (11); this patient was infected with such a virus. Mutations affecting amino acids at the substrate binding site or the NA active site can result in reduced drug efficacy, most commonly for oseltamivir (12–15). The most common mutation associated with oseltamivir resistance is H275Y in A/H1N1pdm09 viruses, which prevents binding of oseltamivir to the active site of the NA gene (15). This mutation causes resistance in A/H1N1pdm09 viruses; however, A/H3N2 viruses with this same mutation remain susceptible (16). Only a few resistance-associated mutations have been identified in clinical isolates of A/H3N2 influenza viruses. A recent review has summarized these to include mutations at positions 119, 222, 292, and 294 (17).

Sequence changes at the 292 position of NA can result in a conformational change of the sialic acid binding site (18). Antiviral drugs in the NAI classification are designed to mimic sialic acid, and an R292K change results in resistance to both oseltamivir and zanamivir, as well as the intravenously administered NAI peramivir, which is yet to be FDA approved in the United States (19–21). The R292K variant has been infrequently detected in clinical samples. Kiso et al. detected R292K mutations in 6 of 9 pediatric patients who developed resistance after 4 days or more of treatment with oseltamivir. An NAI assay showed a decrease in sensitivity to oseltamivir that was as high as 100,000-fold for viruses isolated from patients with the R292K mutation in comparison with the sensitivity of the wild-type virus (22). In another study, including 64 children with influenza virus infection, 34 of whom were found to be infected with A/H3N2 influenza virus, the R292K mutation was identified in only 1 patient: a 15-month-old baby following oseltamivir treatment for more than 4 days. There was no evidence of prolonged illness or increased disease severity in this patient (23). More recently, Piralla et al. identified the R292K mutation in two immunosuppressed patients, an HSCT recipient and a leukemia patient, who had been on oseltamivir treatment for 8 days and 7 days, respectively (24). Like the patient in the current report, these two immunocompromised patients shed influenza A/H3N2 virus for a prolonged period (19 to 22 days) and were unresponsive to treatment. While one patient died, the other patient had an uncomplicated course and recovered. Unlike the current case, however, each of these patients had additional mutations (N294S, E119V, and a deletion of amino acids 247 to 250) detected either concomitantly or prior to and following the detection of the R292K mutation.

In the current report, the R292K mutation was detected in a few of the patient's primary specimens with genotypic testing methods; however, upon cell culture amplification and phenotypic testing for influenza antiviral drug susceptibility in the cultures, the resulting IC_{50}s were comparable to those of the wild-type, drug-susceptible virus. Upon follow-up sequencing investigations, the cultured viruses showed evidence of nearly 100% reversion to drug susceptibility. Culturing A/H3N2 influenza viruses has been shown to alter the influenza antiviral drug susceptibility profile as assessed in NA inhibition assays. When the NA of cultured virus is subsequently sequenced, reduced or additional variant populations may be observed (25, 26). This highlights one of the difficulties in assessing the influenza antiviral susceptibility profiles of patients undergoing antiviral treatment and emphasizes the importance of the direct analysis of specimens with multiple testing methods (27).

Of interest, the R292K mutation was detected early following completion of oseltamivir treatment (Table 1) but was not detected after week 4. Only wild-type virus was detected 17 days after completion of the second oseltamivir course and until the last week of life. The reason for the extended viral shedding after completion of two courses of oseltamivir is unclear, although the inability to resolve viral infections has been reported for many viruses, including influenza virus, in immunosuppressed patients (7). Further, the patient may have continued to harbor resistant virus at very low levels that were undetectable by the methods used here. He had several coinfecting pathogens, including adenovirus and HHV-6, which may have contributed to the persistently wan-

ing immune response and prolonged influenza virus shedding. The R292K mutation may have contributed to the patient's inability to clear the infection due to the early emergence of a resistance mutation at a time when antiviral drug treatment might be expected to otherwise assist in lowering viral load, which would contribute to improved chances of clearing the infection. Since detectable levels of the mutation were present while the patient was on oseltamivir, if this knowledge had been available at the time, a change in therapy might have resulted in a more rapid viral clearance. This case report emphasizes the need for continued surveillance and monitoring for antiviral resistance in influenza A virus infections, especially in immunocompromised patients, who tend to have prolonged periods of shedding and disease (9).

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