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Short Article

Neuraminidase H275Y and hemagglutinin D222G mutations in a fatal case of 2009 pandemic influenza A (H1N1) virus infection

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Oseltamivir-resistant 2009 H1N1 influenza virus infections associated with neuraminidase (NA) H275Y have been identified sporadically. Strains possessing the hemagglutinin (HA) D222G mutation have been detected in small numbers of fatal 2009 H1N1 cases. We report the first clinical description of 2009 H1N1 virus infection with both NA-H275Y and HA-D222G mutations detected by pyrosequencing of bronchioalveolar lavage fluid obtained on symptom day 19. The 59-year-old immunosuppressed patient had multiple conditions conferring higher risk of prolonged viral replication and severe illness and died on symptom day 34. Further investigations are needed to determine the significance of infection with strains possessing NA-H275Y and HA-D222G.

Keywords 2009 H1N1, fatal, oseltamivir, pandemic influenza, pyrosequencing, resistance, severity.

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Introduction

A novel influenza A (H1N1) virus was identified in April 2009 that rapidly spread worldwide. The clinical syndrome associated with 2009 pandemic influenza A (H1N1) [2009 H1N1] virus infection has ranged from asymptomatic infection to rapid respiratory decompensation and death. Some host risk factors such as chronic pulmonary disease, pregnancy, immunosuppression, neuromuscular disease, and morbid obesity among others have been associated with more severe outcomes.[1] Viral factors may also influence disease severity. Recently, a mutation in the hemagglutinin gene at position 222 from aspartic acid to glycine (HA-D222G) in 2009 H1N1 virus was identified in individuals with severe illness or death in Scotland, Norway, and Hong Kong, but not in upper respiratory tract specimens in persons with mild illness, suggesting the possibility of a correlation with more severe clinical outcomes or viral tropism.[2] 2009 H1N1 strains possessing D222G have been shown to have enhanced capacity for binding to alpha2-3 receptors expressed on ciliated cells of the lower respiratory tract (bronchioles, alveoli, type II pneumocytes).[3]

Another viral factor that could affect influenza illness outcomes is antiviral resistance. A single mutation at position 275 (N1 numbering) from histidine to tyrosine in the neuraminidase protein (NA-H275Y) is associated with oseltamivir resistance, but such virus strains are susceptible to zanamivir. The predominant seasonal influenza A (H1N1) virus strains circulating during the 2008–09 Northern Hemisphere influenza season contained the H275Y mutation, but were susceptible to M2 channel blockers. To date, only sporadic reports of oseltamivir resistance have been identified among circulating 2009 H1N1 virus strains, accounting for 1·3% of US isolates.

As part of surveillance for changes in circulating influenza viruses, the Minnesota Department of Health (MDH) Public Health Laboratory began limited partial HA and NA gene sequencing of respiratory specimens collected from 2009 H1N1 fatal cases and identified a single patient whose specimen contained mutations for both NA-H275Y and HA-D222G.

Materials and methods

All persons hospitalized with influenza-like illness and all persons with a critical illness or death of a suspected infectious etiology without an alternate cause are reportable to the MDH as required by state law. Investigations of

reportable diseases are considered public health response and are classified as exempt by the MDH institutional review board. Medical records were reviewed. Original specimens were tested by real-time reverse transcription PCR (rRT-PCR) for 2009 H1N1 viral RNA based on CDC protocol,[4] and pyrosequencing for NA-H275Y and HA-222 variants was performed. Viral RNA was extracted via Corbett Life Science's X-tractor Gene utilizing Qiagen's QIAamp Virus BioRobot 9604 Kit (Qiagen, Hilden, Germany) per manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) amplifications were performed using SuperScript® III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen, Carlsbad, CA, USA) to amplify partial gene regions of both the NA and HA genes. NA amplicons were generated, and the H275Y mutation was detected according to the CDC pyrosequencing protocol[5] with the following modifications: Qiagen's PyroMark™ Q24 system was employed, and a final pyrosequencing primer concentration of 2·0 μm was used. Similarly, HA gene fragments were amplified according to the H275Y RT-PCR parameters.[5] HA amplicons were analyzed on the PyroMark™ Q24 system following the CDC protocol for the detection of HA222 variants utilizing only the customized dispensation order.[6,7]

Surveillance and virologic results

From April 2009 to February 2010, there were 60 deaths reported in Minnesota associated with 2009 H1N1, of which 53 (88%) had respiratory specimens available for testing. Adequate nucleic acid to perform pyrosequencing for H275Y mutational analysis was present in 39 (74%) fatal cases, of which 38 (97%) had wild-type sequence (H275H) and 1 (3%) had the H275Y mutation. Adequate nucleic acid to perform pyrosequencing for HA-222 was present in 43 (81%) fatal cases, of which 38 (88%) had wild-type sequence only (D222D), 4 (9%) had mixed infection with quasi species of 2009 H1N1 strains identified with three sequences (D222D, D222N and D222G), and 1 (2%) had only D222G mutation.

The clinical specimen with the H275Y mutation identified by pyrosequencing was the same specimen that was identified with the D222G mutation by pyrosequencing. This was a bronchial alveolar lavage (BAL) fluid specimen collected on symptom day 19 that was tested within 48 hours of collection. Real-time RT-PCR testing of the BAL specimen was positive for influenza A, swine H1, and swine influenza A targets with C_t values all <35. Pyrosequencing was performed on nucleic acid extract from the BAL specimen.

Case report

A 59-year-old male with systemic lupus erythematosus since 2006 presented in November 2009 with 10 days of dry cough and 48 hours of progressive dyspnea on exertion. He had no fever, myalgia, or lower-extremity edema. He was taking 60 mg daily of prednisone, mycophenolate mofetil, trimethoprim/sulfamethoxazole, and levofloxacin. He had been hospitalized briefly 1 month prior for heart failure symptoms and was diagnosed with pulmonary hypertension and biopsy-demonstrated glomerulonephritis. At discharge and several weeks subsequently, he had minimal pulmonary symptoms.

The patient was admitted on illness day 10 with bibasilar crackles but no jugular venous distension. He was admitted to the intensive care unit requiring noninvasive positive airway pressure. Chest X-ray demonstrated bilateral lung infiltrates. His white blood cell count was 5·5 cells/μl (normal, 3·2–10·6), including 5·3 neutrophils/μl (normal, 1·3–7·0) and 0·08 lymphocytes/μl (normal, 0·8–3·1). Direct fluorescent antibody testing of a nasopharyngeal specimen was positive for influenza A, and oseltamivir 75 mg twice daily was started for 5 days (Figure 1). His respiratory status declined, and he was intubated on symptom day 18. Oseltamivir 150 mg twice daily for 1 day was restarted per gastric tube, followed by 75 mg twice daily. Computed tomography of the chest revealed bilateral ground glass densities and superimposed irregular nodules most pronounced in the lower lobes. A BAL was performed on symptom day 19 and tested for influenza rRT-PCR at MDH Public Health laboratory. Bronchial alveolar lavage fluid was also sent for viral culture to a clinical laboratory that grew influenza A virus using centrifuged shell vial technique. This isolate was not available for further testing. The BAL fungal culture grew *Aspergillus* sp. His respiratory status declined with worsening pulmonary infiltrates, bilateral pneumothoraces, refractory hypoxemia, and worsening renal function requiring dialysis. Support was withdrawn on symptom day 34 and the patient expired. No autopsy was performed.

Conclusions

To our knowledge, this is the first clinical description of a patient with 2009 H1N1 virus infection identified with NA-H275Y and HA-D222G. Such mutations have been described in three other instances without clinical details.[8] This patient had multiple underlying medical conditions that increased the risk of severe complications from 2009 H1N1 virus infection. Bacterial co-infection was not detected, but fungal culture of BAL fluid yielded *Aspergillus. Aspergillus* infection associated with severe 2009 H1N1 disease has been described in two other cases.[9]

Oseltamivir resistance may have developed during treatment in this patient as has been previously described; however, initial infection with an oseltamivir-resistant strain cannot be excluded. While there are documented instances

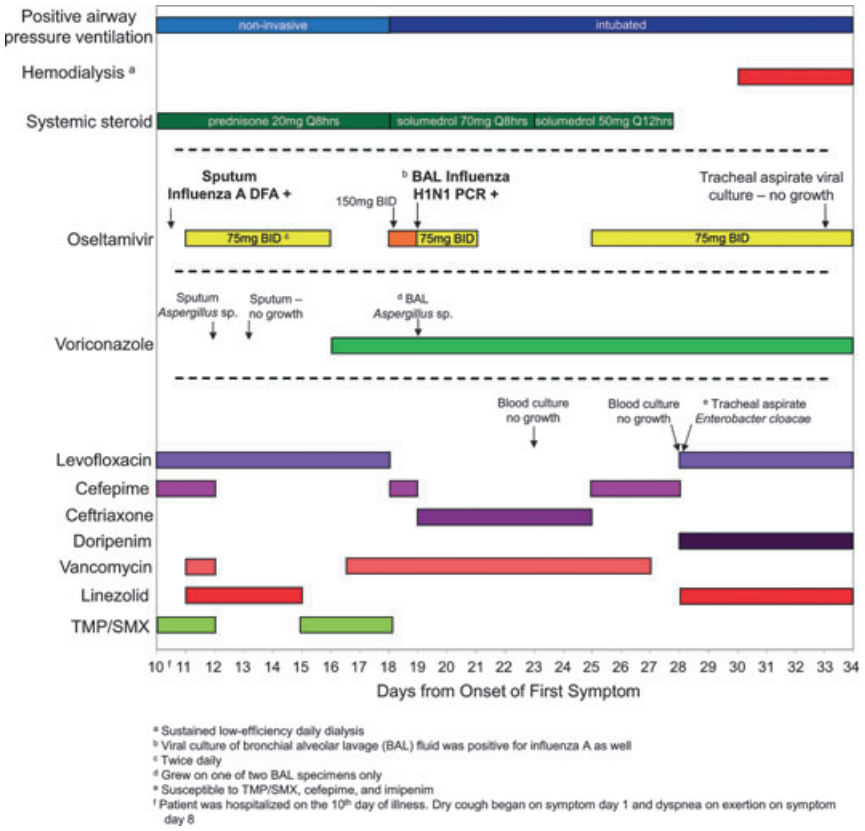

Figure 1. Treatment and testing following hospitalization. The patient was hospitalized beginning on day 10 after onset of first respiratory symptoms. No influenza antiviral medication was administered prior to hospitalization.

of non-sustained person-to-person transmission of 2009 H1N1 virus strains containing H275Y, these appear to be rare events.[10]

It is possible that oseltamivir resistance may impact clinical outcomes particularly among patients with immunosuppression and prolonged influenza viral replication.[11] A comparison of patients infected with oseltamivir-resistant versus susceptible strains found that immunosuppression was the only risk factor significantly associated with oseltamivir resistance.[12] Because of this increased risk of development of oseltamivir resistance during or after oseltamivir treatment, clinicians should consider the use of zanamivir as primary therapy for immunosuppressed patients with influenza, and especially for patients with suspected or 0-confirmed oseltamivir resistance.[13,14]

Recombinant clones of 1918 pandemic H1N1 virus with a hemagglutinin mutation at position 222 (225 in H3 numbering system) from aspartic acid to glycine were found to change viral tropism from alpha2-6 sialic acids to dual tropism for alpha2-3 and alpha2-6 sialic acids, suggesting the potential importance of a single substitution at the receptor-binding site of the hemagglutinin.[15] Recent reports have suggested a link between the HA-D222G mutation in 2009 H1N1 virus infection with severe and fatal outcomes.[16]

If the HA-D222G mutation results in greater affinity for receptors with alpha2-3 sialic acids found predominantly in the human lower respiratory tract, it is possible that a HA-D222G subpopulation would be preferentially selected by lower respiratory tract sampling from a heterogeneous influenza pool, especially in persons with severe lower respiratory tract disease and with prolonged viral replication. In a recent study of paired nasal swab and BAL specimens, D222G mutants accounted for 40% of the lower respiratory tract viral population compared with only 10% in the upper respiratory tract.[17]

Influenza virus mutations have also been identified more frequently among isolates obtained after growth in cell culture compared with primary clinical specimens, suggesting that replication of virus in tissue cell culture can lead to selection of virus variants with altered receptor specificity.[18] Therefore, it is essential that molecular testing for influenza viruses is performed on original specimens and not viral culture isolates to limit this possibility.

As this was not a study and with only a single isolate available for testing, it is not possible to ascertain whether the combination of NA-H275Y and HA-D222G mutations contributed to illness severity. This case illustrates the importance of further research regarding viral factors impacting severity. Future studies should prospectively

collect the following specimens from patients for influenza testing by rRT-PCR, genetic sequencing, and other molecular analyses: (i) multiple, serial respiratory specimens from close to illness onset or early in the clinical illness, prior to or at the time of initiation of antiviral therapy, following antiviral treatment, and later in the clinical course if possible; and (ii) paired (obtained at the same time) clinical specimens from both the upper and lower respiratory tract if possible, especially for critically ill patients. Sampling should be carried out for persons with mild uncomplicated influenza illness and for persons with severe complications requiring hospitalization and critical care management. Integration of clinical (including information on antiviral treatment), epidemiological, and virologic data in such comparative studies will facilitate interpretation and contribute to improved understanding of viral changes that might lead to severe clinical outcomes of influenza virus infection.

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