Enhanced Diagnosis of Pandemic (H1N1) 2009 Influenza Infection Using Molecular and Serological Testing in Intensive Care Unit Patients with Suspected Influenza

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During the 2009 outbreak of pandemic (H1N1) 2009 influenza (pH1N1) in Australia, acute and convalescent serum specimens were collected from 33 patients with severe respiratory disease admitted to intensive care units. Using hemagglutination inhibition of pH1N1, 29 paired serum samples showed significant increases in specific antibody titers. Of these 29 patients, 18 had pH1N1 RNA detected by routine nucleic acid testing. These results indicate that up to one-third of pH1N1 cases may not have laboratory confirmation of infection unless serological testing is included for suspected cases.

During the period from 1 June through 31 August 2009 (ie, the southern hemisphere winter), 722 people were admitted to intensive care units (ICUs) in Australia and New Zealand with pandemic (H1N1) 2009 influenza virus (pH1N1) infection. Among these patients, infection was diagnosed by use of a nucleic acid test (NAT) for 717 patients and serology testing for 5 patients [1]. However, additional NAT-negative patients, clinically indistinguishable from those with NAT-proven pH1N1, were admitted to ICUs with severe influenza-like illness [2]. Factors that may influence the sensitivity of NATs include delay in sample collection following the onset of illness, technically inadequate sampling, initiation of antiviral treatment, presence of inhibitors of NATs in the sample, and the use of specimens collected from the upper respiratory tract (as opposed to the lower respiratory tract) in patients with pneumonia [3–5]. Our study reports the retrospective diagnosis of pH1N1 with serological testing, using a hemagglutination inhibition (HI) assay for a group of patients with influenza-like illness admitted to participating ICUs with suspected or proven pH1N1.

Methods. The study group was formed from 33 adult patients admitted to an ICU from the community or from another location in the hospital (ie, transfer patients with respiratory failure following an influenza-like illness). Patients were included in our study on the basis of the criteria of admission to an ICU following an influenza-like illness and the availability of paired acute and convalescent serum samples taken a minimum of 7 days apart. Specimens obtained during the period from 3 July through 23 September 2009 were referred for pH1N1 serology testing to South Eastern Area Laboratory Services in Sydney, Australia, from 4 reference laboratories that serve 8 ICUs in 3 Australian states (New South Wales, Queensland, and Western Australia). Of the 33 patients, 32 had been previously tested for pH1N1 using an NAT. A total of 56 specimens were tested by use of an NAT, including 9 bronchoalveolar lavage specimens, 34 combined nose and throat swab specimens, 5 nasopharyngeal aspirate specimens, 4 sputum specimens, 4 endotracheal tube aspirate specimens, and 1 cerebrospinal fluid specimen. Multiple specimens were tested in 20 of 33 patients, with 17 patients having both upper and lower respiratory tract specimens tested and 3 patients having only upper respiratory tract specimens tested.

The HI assay that was used was modified from established techniques [6]. The HI assay used the reference strain A/California/7/2009 as the assay antigen (obtained from the World Health Organization [WHO] Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia). All paired serum samples were tested in parallel at South Eastern

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Area Laboratory Services. Patient serum samples were initially treated with a receptor-destroying enzyme (Denka Seiken) at a ratio of 4 parts receptor-destroying enzyme to 1 part serum and incubated overnight at 37°C. Five parts of 1.6% sodium citrate was then added, and the treated serum sample was incubated at 56°C for 30 min. After titration of the treated serum samples in phosphate-buffered saline containing 0.8% bovine serum albumin, the antigen was added. After 1 h of incubation, fresh human group O red blood cells from a single donor were added and incubated for an additional 3 h. Positive and negative control serum and antiserum samples were included in each testing run. Two independent operators read the plate to determine the HI titer, and no discordance occurred. The endpoint titer was taken as the highest dilution of serum completely inhibiting agglutination. An antigen titration was performed in duplicate with each run to confirm the presence of 4 units of hemagglutinin. Recent pH1N1 infection was identified serologically by a minimum 4-fold increase in specific HI titer between acute and convalescent serum samples.

Results and discussion. All 18 pH1N1 cases identified as positive by use of an NAT met the serological criteria for pH1N1 infection, evidenced by a minimum 4-fold increase in HI titer between serum samples taken during the acute and convalescent phase of illness (Table 1). However, 9 (69%) of 13 patients with severe respiratory illness in an ICU who tested negative by use of an NAT had HI titer levels that would indicate a diagnosis of recent pH1N1 infection (Table 1). An additional 2 patients with diagnostically elevated HI titers had no NAT performed on respiratory tract specimens. One patient had a cerebrospinal fluid specimen tested by use of an NAT, and 1 was tested only by immunofluorescence on a respiratory specimen (data not shown). In our study, NAT results for influenza A (matrix A target) and pH1N1 were generally the same (ie, either both negative or both positive), with a single patient who tested positive for influenza A and negative for pH1N1 (Table 1). This patient had no serological evidence of pH1N1 infection, indicating that this patient was infected with seasonal, rather than pandemic, influenza. Thus, a significant number of critically ill patients infected with pH1N1 did not have the infection detected by the routine laboratory tests (eg, by use of an NAT) used most frequently during the pandemic. It should be noted that our study was performed on a distinct subset of patients—those admitted to an ICU with influenza-like illness progressing to respiratory failure. The degree to which these results can be generalized to the wider population has not been determined.

Several factors may account for failure to detect pH1N1 using a laboratory NAT (Table 1). In our study, 4 of the 9 patients with a negative NAT result and a positive serological test result had an NAT performed only on upper respiratory tract specimens (nasopharyngeal swab or nasopharyngeal aspirate specimens). Blyth et al [5] reported that, for patients admitted to the ICU with severe respiratory failure secondary to influenza infection, an NAT on upper respiratory tract samples was 19% less sensitive than concurrently collected lower respiratory tract samples. However, the other 5 patients had at least 1 negative NAT result from a lower respiratory tract specimen (bronchoalveolar lavage or washing specimen). Studies have shown that nasopharyngeal aspirate and swab specimens have equivalent sensitivity for detection of respiratory viruses [7, 8], but it has been suggested that lower respiratory tract specimens are more sensitive because of prolonged viral shedding, with detection of virus in tracheal specimens but not nasopharyngeal swab specimens [9].

In our study, the initial pH1N1 NATs were performed after admission to hospital (0–9 days after admission), but the time elapsed between onset of symptoms and testing was much longer. These data include nucleic acid testing as a result of community surveillance prior to admission to hospital. In 4 of 9 patients with negative NAT results who had a significant increase in HI titer, nucleic acid testing was performed 7–21 days after onset of symptoms, compared with an average of 5 days after onset of symptoms in NAT-positive patients (0–30 days after onset, with a single patient reporting onset of symptoms 30 days before hospitalization and initial nucleic acid testing). Therefore, many patients were symptomatic for a significant period of time prior to hospitalization. Although the majority of discordant cases were tested for pH1N1 using an NAT promptly after admission to hospital, this was generally >7 days after onset of symptoms. We surmise that the likelihood of positive NAT results using respiratory swab specimens is potentially lower at this time (>7 days) after acute infection with pH1N1. Oseltamivir therapy was given to 4 patients for 1 day prior to nucleic acid testing; 2 patients subsequently tested positive by use of an NAT, whereas the other 2 patients subsequently tested negative by use of an NAT. An additional 2 NAT-positive patients received oseltamivir therapy on the same

| Table 1. Comparison of Serological and Nucleic Acid Test Results for 31 Patients with Paired Serum Samples Who Were Admitted to an Intensive Care Unit in Australia during the 2009 Influenza Pandemic |
|---------------------------------|-----------------|-----------------|-----------------|
| Recent pH1N1 infection diagnosed by increasing HI titer level? | pH1N1 detected by use of NAT? | Total |
| (Yes) | (Yes) | (No) | |
| 18 | 9 | 27 |
| 0 | 4 | 4 |
| Total | 18 | 13 | 31 |

NOTE. Data are no. of patients. HI, hemagglutination inhibition; pH1N1, pandemic (H1N1) 2009 influenza.

a Recent pH1N1 infection was identified serologically by a minimum 4-fold increase in specific HI titer between acute and convalescent serum samples.
b One patient in this group tested positive for the influenza A matrix gene but negative for pH1N1, by use of the NAT.
day as nucleic acid testing (data not shown). Therefore, a decrease in pH1N1 viral load as a result of oseltamivir therapy does not appear to be a sufficient explanation for the discordant specimens.

Our results show that the use of nucleic acid testing did not detect all patients admitted to ICUs who were infected with pH1N1. A number of factors contribute to this conclusion, including prolonged time to sampling after acute infection and onset of symptoms, and possibly the lack of excreted virus in infected upper respiratory tract cells [4]. Nucleic acid testing was performed at 4 reference laboratories that serve 8 ICUs in 3 Australian states, with some variation among assay protocols such as use of in-house methods [10, 11] or Respiratory Pathogens Easy-Plex (AusDiagnostics). Although nucleic acid testing was performed at different sites, it is unlikely that this would have influenced the results, because all 4 of the laboratories are state reference laboratories that participate in a national proficiency and continuous improvement program for the NAT, which was previously reported by Stelzer-Braid et al [12].

Our results strongly suggest, in cases of influenza-like critical illness during a known influenza epidemic, influenza treatment should be initiated prior to receiving laboratory confirmation. In cases for which the NAT does not detect pH1N1, the clinical symptoms should determine whether treatment for influenza should be initiated or continued, regardless of the NAT result. All NAT results, in conjunction with all serological test results, should also be considered, as demonstrated in our study by the confirmation of seasonal influenza in a patient who tested positive for influenza A and negative for pH1N1 by use of an NAT. A serological test negative for pH1N1 indicates that the patient has seasonal influenza, not that the test failed to detect pH1N1. The WHO recommends additional laboratory testing by alternative methods for patient who have symptoms strongly suggestive of influenza [13]. Our study demonstrates that serological testing (in this case, the HI assay) is an effective method for diagnosing influenza during a pandemic.

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