Laboratory diagnosis of 2009 H1N1 influenza A virus

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The emergence of 2009 pandemic influenza H1N1 has necessarily led to the rapid evolution of sensitive, specific, and high-throughput molecular diagnostic assays for this virus at the same time that clinical laboratories attempt to cope with increasing demands in the setting of resource limitations. This situation has given rise to testing algorithms focusing on priority, clinical relevance, and appropriate surveillance. We describe the current state of understanding around diagnostic testing and laboratory detection of 2009 H1N1 influenza A virus. ([Crit Care Med 2010; 38[Suppl]:S000–S000])

Key Words: emerging infections; influenza A; polymerase chain reaction; rapid influenza diagnostic test; sensitivity; specificity; swine-origin influenza

Since the emergence of 2009 H1N1 in the spring of 2009, laboratory detection methods have evolved rapidly to meet the ongoing challenges posed by this pandemic strain of novel influenza A virus. Early challenges of identifying the nature of the virus (1) and the timely provision of influenza A subtyping information have since been replaced with the need for algorithmic approaches to diagnostic testing (2), lest laboratories become further overwhelmed with clinical specimens given the widespread circulation of 2009 H1N1. Accordingly, laboratories have sought rapid, sensitive, specific, and scalable high-throughput detection methods to better-cope with the demands of population-level diagnostic testing inherent to a pandemic situation (3). This article reviews the current state of understanding surrounding the approach to diagnostic testing and laboratory detection of 2009 H1N1 influenza A virus.

Who Should Be Tested for Diagnosis of 2009 H1N1 Infection?

For a number of reasons, not the least of which being limited laboratory resources, widespread confirmatory diagnostic testing for 2009 H1N1 has not been a viable strategy since early in the pandemic. Rather, many public health bodies, governments, and institutions have recommended confirmatory diagnostic testing only for patients with influenza-like illness warranting admission to hospital, those with underlying risk factors for complicated infection such as pregnancy, those with worsening clinical status, or those in institutional or other outbreak situations (4, 5). Because most patients with 2009 H1N1 will recover at home uneventfully without antiviral therapy, confirmatory diagnostic testing adds little to their management. Testing should be performed only in circumstances in which the results will alter clinical or infection control management, or as needed for public health surveillance purposes.

The performance characteristics of diagnostic assays for 2009 H1N1 stratified by clinical and demographic data, such as age, gender, severity of illness, and management setting, are scarce. In their evaluation of several testing methods for 2009 H1N1 during the New York City outbreak, Ginocchio et al (2) demonstrated excellent performance of their reverse-transcription polymerase chain reaction (RT-PCR) assay across patient ages ranging from 2 wks to 97 yrs. Sabetta et al (6) demonstrated differential performance of a rapid influenza diagnostic test (RIDT) in patients with RT-PCR–positive nasopharyngeal swabs based on clinical symptoms: those with a Centers for Disease Control and Prevention-defined influenza-like illness (fever plus cough or sore throat) were 50% more likely to have positive RIDT results compared to those without. Finally, in their study of 84 2009 H1N1 RT-PCR–positive specimens, Vasoo et al (7) found that both inpatient or emergency department status were predictive of positive RIDT results, perhaps suggesting that more severely ill patients shed higher quantities of more virus, and thus are more likely to test positive in low-sensitivity diagnostic tests, such as the RIDT. In support of this hypothesis, higher median fluorescence intensity on the RT-PCR assay was also predictive of RIDT positivity. However, other factors thought to be associated with shedding of increased concentrations of virus, such as younger age and shorter duration of symptoms, were not predictive of RIDT positivity in this study (7).

In patients requiring hospitalization or intensive care unit (ICU) admission, several studies have demonstrated that clinical symptoms are poor predictors of a diagnosis of seasonal influenza; only laboratory testing is adequate to diagnose influenza (8–11). This is a general truth about infectious agents causing primarily respiratory illness, and these conclusions are likely true of pandemic influenza as well. Based on what is known about the clinical features of 2009 pandemic H1N1 influenza, ICU clinicians should be testing all patients suspected of having a respiratory infection, patients admitted with exacerbations of underlying lung diseases such as chronic obstructive pulmonary disease or asthma, and all patients with a febrile illness of uncertain origin. Influenza can also cause myocarditis and encephalopathy and should be considered in the differential diagnosis of these conditions during periods of increased pandemic activity.
During Illness, When Should Testing for 2009 H1N1 Be Performed?

Comprehensive data on the optimal timing of specimen collection for the diagnosis of 2009 H1N1 are limited. Viral titers in respiratory specimens are highest during the first 3 days of illness; however, it is recognized that children typically shed more virus for more prolonged periods than adults (12). Through quantitative RT-PCR analysis of serially collected nasopharyngeal swabs, we have found that viral shedding appears to be greatest on the first and second day of illness, declining significantly thereafter (13, Smieja et al unpublished data). However, in a small proportion of patients, viral detection in those not receiving antivirals persists up to 9 or 10 days after the onset of symptoms (13–15). In their convenience sample of 84 2009 H1N1 RT-PCR–positive specimens, Vasoo et al (7) found that the mean interval between onset of symptoms and specimen collection was 2.8 days (range, 1–9 days). A full three-quarters of patients in their study presented for specimen collection within 3 days of symptom onset (7). Interestingly, in their study of two school outbreaks of 2009 H1N1, Sabetta et al (6) demonstrated that patients with specimens collected within 36 hrs of symptom onset were no more likely to have a positive RIDT result than those whose specimens were collected >36 hrs after symptom onset, perhaps illustrating that a 36-hr cutoff is still well within the timeframe of peak viral shedding.

Other factors that may influence the quantity of virus in a clinical specimen, and theoretically affect the test performance, include the type of specimen collected and pre-analytical transport and storage (12). Accordingly, optimization of the timing, transportation, and storage of clinical specimens would be expected to improve the diagnostic yield, especially using lower-sensitivity tests such as RIDT or direct fluorescent antigen (DFA).

In patients requiring admission to the ICU, clinical judgment is required to determine whether a second test is warranted to assess response to therapy and need for ongoing isolation precautions. In patients who are improving, repeat testing is not helpful, and additional precautions for prevention of transmission of infection can be based on empirical protocols (e.g., precautions until 7 days after the onset of symptoms). However, optimal management for patients with persisting or worsening symptoms is more difficult to determine. In patients who are apparently responding to therapy, PCR often will remain positive until after day 7, and occasionally for longer (10, 15). Anecdotal experience suggests the viral culture is rarely positive in this circumstance (10), but viral culture results require several days. Deterioration inpatient condition may be attributable to selection for viral resistance, as has been documented with other influenza (16) and pandemic H1N1 (17, 18), but is more likely to be to a complicating hospital-acquired disease, such as ventilator-associated pneumonia or pulmonary embolus. In this setting, it seems reasonable to follow PCR at 3- to 4-day intervals until either PCR is negative or clinical improvement is apparent. Quantitative PCR is useful, if available, because viral concentrations will increase in those with evolving viral resistance on therapy (16) or in severely compromised patients who cannot control viral replication even in the presence of specific antiviral therapy (18–21) but remain low and stable in patients with deterioration attributable to other causes.

What Specimen Should Be Collected for Detection of 2009 H1N1?

The ideal clinical specimens for the detection of 2009 H1N1 are thought to be nasopharyngeal swabs or aspirates (4, 5, 22). These swabs have a synthetic tip made of polyester or Dacron, and a plastic or aluminum shaft. They are packaged with or without viral transport media and are sterile. Throat swabs or other swabs with cotton tips or wooden shafts should not be used (5, 22). Nasal aspirates, nasal swabs, and oropharyngeal swabs also may be used for specimen collection, although these are not preferred (5, 22). Expectorated sputum may be used for diagnosis; for patients who are intubated, bronchoalveolar lavage or endotracheal aspirates are acceptable. Eye swabs and stool are not considered appropriate specimens for the detection of 2009 H1N1 and will likely be rejected by the testing laboratory. Sterile tubes of pink viral transport medium containing buffer, protein, antibiotics, and antifungals should be brought to the bedside for specimen collection if not packaged along with the nasopharyngeal swab. Detailed video instructions on how to collect a nasopharyngeal swab are freely available (23, http://content.nejm.org/cgi/content/full/NEJMe0903992/DC1). It is important to remember that careful hand hygiene should be performed and appropriate barrier precautions used during the specimen collection process (5, 22). Once the specimen is collected and placed into the viral transport medium, it should be sent to the laboratory immediately (22).

What Test Should Be Used for Detection of 2009 H1N1?

Four main platforms form the backbone of influenza testing in the clinical laboratory: rapid antigen testing (RIDT), DFA testing, viral culture, and molecular testing such as RT-PCR. Serologic testing offers little in the diagnosis of influenza. Each method has inherent limitations and advantages for influenza testing. RIDT provide a rapid turnaround time and require little technical expertise, but they lack sensitivity compared to viral culture and molecular methods (2, 24) and do not distinguish between the various types of influenza A (e.g., 2009 H1N1 vs. seasonal H1N1 vs. seasonal H3N2) (2, 6, 7, 12, 24). Viral culture and DFA are more sensitive (2) but require technical expertise to perform and have greater laboratory infrastructural requirements. Both DFA and viral culture have the added of advantage of identifying other respiratory viruses that commonly circulate during influenza season, such as parainfluenza, adenovirus, respiratory syncytial virus, and human metapneumovirus (2), although neither DFA nor viral culture can distinguish subtypes of influenza A. Furthermore, both DFA and viral culture require expertise in microscopy, and viral culture has a long turnaround time. The most sensitive test is RT-PCR. The only platform that can specifically and reliably distinguish 2009 H1N1 from other influenza A viruses is molecular testing, usually using an RT-PCR assay.

The Table 1 summarizes the reported performance characteristics of various diagnostic tests used to detect 2009 H1N1 from clinical specimens. The performance of RIDT demonstrated interassay and interstudy variability, with sensitivities for detecting 2009 H1N1 ranging from 17.8% to 53.4% (Table 1). While most RIDT had reasonable positive predictive values, their poor negative predictive values underscore the recommendation of the Centers for Disease Control and Prevention to avoid clinical decision-
making during the pandemic based on negative RIDT assays (25). Again, RIDT cannot distinguish 2009 H1N1 from any other circulating influenza A strain. Only one study reported the performance characteristics of DFA for 2009 H1N1, which appear to be comparable or superior to those of the RIDT (Table 1). R-Mix viral culture performed well compared to RIDT and DFA, with a sensitivity of 88.9% and specificity of 100% (2). Although viral culture remains the reference standard for influenza diagnosis, both the increased time to results (2–7 days) and the need for downstream subtyping of a positive culture render it much less attractive and efficient than nucleic acid-based tests, most of which can be multiplexed and yield a specific result in a single step. Thus, the rapid, sensitive, and specific nucleic acid testing methods are clearly the optimal choice for diagnosis of the 2009 pandemic strain of H1N1.

On the emergence of 2009 H1N1, such authoritative bodies as the Centers for Disease Control and Prevention, World Health Organization, and Public Health Agency of Canada acted quickly to provide detailed methods for RT-PCR detection of the virus (26–28), which had been identified in other conventional molecular assays as “influenza A unsubtypeable.” A common approach for the confirmation and subtyping of influenza A before the emergence of the pandemic strain would be to perform an initial screening RIDT, DFA, culture, or RT-PCR targeting influenza A matrix gene (the latter of which would detect all influenza A subtypes), followed by an RT-PCR assay specific for different influenza A hemagglutinin genes, which would discriminate between pandemic H1, seasonal H3, and seasonal H1 viruses (29). The US Food and Drug Administration-approved respiratory virus panel is a multiplex RT-PCR assay with a microfluidic array detection on the Luminex 100 (Luminex, Austin, TX) instrument that detects ≥10 viruses, including parainfluenza 1 through 3, respiratory syncytial virus, adenovirus, human metapneumovirus, and influenzas A and B (30–32); it is another molecular approach to detecting and subtyping influenza A. In addition to detecting the influenza A matrix gene, it will discriminate between seasonal H3 and H1 based on hemagglutinin sequences; 2009 H1N1 is identified in this assay as an influenza A that fails to subtype as either seasonal H1 or seasonal H3. The manufacturers of this system, and other commercial systems detecting influenza by PCR, are working on the development of 2009 H1N1-specific assays (3, 26–28).

In addition to qualitative RT-PCR assays, quantitative methods increasingly are being adapted (9, 33, 34), although more for surveillance and investigational purposes rather than for initial clinical diagnosis. Both qualitative and quantitative RT-PCR assays are reportedly up to 500-times more sensitive than viral culture (33), with quantitative detection limits of approximately 5000 viral genome copies per sample (33, 34). It has been reported, and has been our experience, that qualitative RT-PCR has greater sensitivity than quantitative assays (33, Smieja et al, unpublished)

<table>
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<tr>
<th>Reference</th>
<th>Number of Specimens</th>
<th>Platform Evaluated</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV, %</th>
<th>NPV, %</th>
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<tbody>
<tr>
<td>Vasoo et al (7) 84 RIDT (Directigen EZ Flu A+B; Becton, Dickinson, Franklin Lakes, NJ)</td>
<td>46.7 (34.6–59.1) 100 (86.2–100) 100 89.6</td>
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<td>RIDT (BinaxNOW Influenza A&amp;B; Inverness Medical Innovations, Bedford, UK)</td>
<td>38.3 (27.1–51) 100 (86.2–100) 100 88.2</td>
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<td>RIDT (QuickVue Influenza A+B; Quidel, San Diego, CA)</td>
<td>53.3 (40.9–65.4) 100 (86.2–100) 100 90.8</td>
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<td>Sabetta et al (6) 63 RIDT (Xpect Flu A&amp;B; Remel, Lenexa, KS)</td>
<td>47 86 92 32</td>
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<td>Fouad et al (38) 273 RIDT (QuickVue Influenza A+B)</td>
<td>51 99 — —</td>
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<td>Kok et al (24) 174 RIDT (QuickVue Influenza A+B)</td>
<td>53.4 100 100 76.2</td>
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<tr>
<td>Ginocchio et al (2) 288 RIDT (BinaxNOW A&amp;B or 3M Rapid Detection Flu A+B; 3M, St. Paul, MN)</td>
<td>17.8 93.6 77.4 47.9</td>
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<td>DIA</td>
<td>46.7 94.5 91.3 58.9</td>
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<td>R-Mix viral culture (Diagnostic Hybrids, Athens, OH)</td>
<td>88.9 100 100 87.9</td>
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<td>RT-PCR (Luminex RVP; Luminex, Austin, TX)</td>
<td>97.8 100 100 97.3</td>
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<td>Pabbaraju et al (3) 35 Conventional RT-PCR and sequencing after RVP</td>
<td>92.9 (80.5–98.5)</td>
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<td>CPC</td>
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<td>In-house HA RT-PCR</td>
<td>95.2 (83.8–99.4) — —</td>
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<td>In-house M2 RT-PCR</td>
<td>90.5 (77.4–97.3) — —</td>
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<td>In-house M2 RT-PCR</td>
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<tr>
<td>Mahony et al (32) 20 RT-PCR (Luminex RVP)</td>
<td>100 100 — —</td>
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DFA, direct fluorescent antigen; HA, hemagglutinin; M1/M2, matrix gene; NPV, negative predictive value; PPV, positive predictive value; RIDT, rapid influenza diagnostic test; RT-PCR, reverse-transcription polymerase chain reaction; RVP, respiratory virus panel; —, XXXX.
data); however, efforts to optimize the performance of quantitative RT-PCR are being undertaken.

CONCLUSION

In summary, the most common diagnostic algorithms to be used when seasonal influenza may be co-circulating with 2009 H1N1 includes either a commercial RT-PCR that subtypes influenza A or a pan-influenza A matrix gene RT-PCR plus RT-PCR differentiating between H1 and H3 subtypes, followed by one of the 2009 H1N1-specific assays targeting matrix, nucleoprotein, or neuraminidase genes (26–28). In the absence of other circulating influenza (i.e., outside of regular influenza season), subtyping may be deemed unnecessary, thus simplifying the algorithm to include any test that detects influenza A by PCR. Nucleic acid-based approaches to the detection of 2009 H1N1 are clearly superior to conventional nonmolecular-based tests for influenza (such as RIDT, DFA, and culture) because of their rapidity, excellent sensitivity, specificity, scalability, and ability to distinguish between seasonal and pandemic influenza A, often in a single step. Although RIDT and DFA are rapid and inexpensive, they are insensitive and do not differentiate among influenza A subtypes.

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