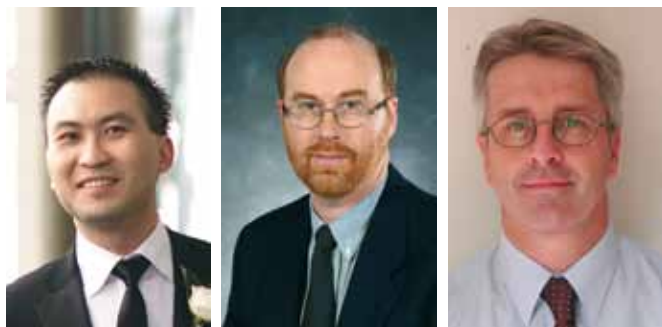# Laboratory diagnosis of influenza and the impact of the pandemic (H1N1) 2009 virus

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Aetiological confirmation of respiratory tract infections in patients facilitates appropriate antimicrobial use and infection control procedures. From a public health perspective, the laboratory confirmation of influenza allows assessment of circulating viruses, community attack rates and the efficacy of vaccination programs, while assisting modelling as part of pandemic preparedness planning. Rapid antigen and immunofluorescent antigen tests are relatively insensitive in detecting pandemic (H1N1) 2009 influenza compared to seasonal subtypes, and influenza subtype-specific nucleic acid amplification tests should be used as the 'gold-standard' for diagnosis. Pathogen-specific serological testing aids the retrospective diagnosis of infection, and is used in seroprevalence studies. Influenza virus isolation is needed for vaccine assessment and formulation. Although some challenges surrounding diagnostic testing during pandemic (H1N1) 2009 have been resolved, others remain; this may test laboratories again in future pandemics.

Pandemic (H1N1) 2009 influenza (hereafter pH1N1), the first influenza pandemic of the 21st century and the first in 41 years, provided a significant challenge to the community and health authorities. The rapid spread of the pandemic required the development of high-throughput pH1N1-specific laboratory tests to provide timely information demanded by clinicians, public health authorities and policy-makers. The laboratories were confronted with an increase in testing demand that far exceeded predictions, and which stretched then overwhelmed the resources of many. Furthermore, some of the most significant problems had not been anticipated in the pre-pandemic planning process and strategies to deal with them could often not be implemented[1,2].

The Australian Health Management Plan for Pandemic Influenza (AHMPPI) is a national health plan that outlines the management of pandemic influenza, including the role of laboratory testing during the different pandemic phases[3]. Each state and territory had developed plans for dealing with the predicted test demand, usually by centralisation of most testing in state reference laboratories, though many were able to use other laboratories that had suitable capacity and expertise. While these arrangements were criticised in some jurisdictions[4,5] due to increased turnaround times, it was clear that there were a number of complex factors contributing to problems[1,6].

There were multiple reasons for the delays, including the overwhelming number of tests requested for surveillance of disease prior to significant community transmission[1,2], and testing of individuals with clinically mild disease during the DELAY and CONTAIN phases of the pandemic. Other issues included the long transit times between the point of collection and receipt in the reference laboratory; inappropriately collected specimens; incorrectly filled out or missing request forms; difficulties in identifying urgent samples; the lag between result validation and notification to public health units; the requirements for increased data collection and reporting; and the lack of flexible electronic data transfer systems.

Despite the recommendation that laboratory testing be confined to those with, or at risk of, severe influenza infection during the PROTECT phase (declared on 17 June 2009), high numbers of specimens requesting pH1N1 testing were still being received in laboratories. As the first pandemic wave coincided with the southern hemisphere's winter season, the sensitive but non-specific clinical case definition used to guide laboratory testing for influenza (for example, fever and cough plus one other respiratory symptom was used in NSW) dictated that a significant number of specimens were tested. Diagnostic testing was also complicated by the fact that the peak of pH1N1 activity coincided with the peak activity of other winter respiratory viruses (including respiratory syncytial virus and seasonal influenza). Different algorithms incorporating various testing modalities such as nucleic acid amplification tests (NAAT), antigen detection tests, pathogen-specific serology and viral cultures were employed throughout the pandemic. Furthermore, there was a need to rapidly develop, optimise and validate new NAAT while assessing the performance of existing seasonal influenza tests in detecting pH1N1.

Much of the additional workload fell upon a relatively small number of staff, especially senior staff with the expertise

required to oversee the combined burdens of test development and delivery, while also interacting with clinicians and health authorities. Due to the special skills required, it was difficult to find external staff who were able to assist, or to find time to train those without the skills. Staff fatigue due to excessive work hours and absenteeism during a period of high stress further compromised the workflow within the laboratories.

NAAT were used as the definitive test for pH1N1 as these had been determined to be the preferred test for novel influenza A subtypes[3]. Early data indicated that the antigen detection tests were insensitive, and later studies confirmed the decreased sensitivity of rapid antigen tests (RAT) and direct immunofluorescent antigen tests (IFA) in detecting pH1N1 (17.8%–53.4% and 46.7%–93% respectively)[7-10]. The sensitivity of both RAT and IFA was affected by patient age and sample type[11]; improved RAT sensitivity was noted in the paediatric population (66.2% in combined nasopharyngeal swabs and 84.1% in nasopharyngeal aspirates), suggesting that RAT may be a 'reasonable' test to exclude pH1N1 in nasopharyngeal swabs collected from young children in non-critical situations[12]. Nose and/or throat swabs are easier and more comfortable to collect compared to nasopharyngeal aspirates, and have been shown to be equivalent or superior to nasopharyngeal aspirates for the detection of respiratory viruses by NAAT, including pH1N1[13,14]. Table 1 compares the performance characteristics of the different tests used in detecting pH1N1. Despite the development of new pH1N1-specific RAT that have improved the sensitivity of pH1N1 detection to 77%[15], NAAT remains the test of choice in diagnosing acute pH1N1.

Various NAAT were employed, usually real-time assays directed at non-subtype-specific influenza matrix gene targets and/or pH1N1 specific haemagglutinin gene[16], as well as commercial multiplex-tandem PCR assays targeting influenza and other respiratory viruses. Although RAT were significantly less sensitive than NAAT, they were used by some public and private laboratories to screen for pH1N1 due to their high positive predictive value, particularly during the PROTECT phase when there was widespread community transmission. NAAT were required for confirmation and subtyping of RAT-positive specimens, and for excluding pH1N1 in RAT-negative specimens.

However, even NAAT on upper respiratory tract samples were negative in up to 40% of critically ill patients with pH1N1 pneumonia and positive lower respiratory tract samples[17,18]. Also, in these patients, at least 23% had bacterial and/or viral co-infections[19]. Multiplex-tandem RT-PCR allowed simultaneous detection of other co-circulating respiratory viruses; bacterial co-infections were suggested by high C-reactive protein or procalcitonin levels[20] and confirmed by positive cultures (blood, sputum, bronchoalveolar lavages and/or endotracheal aspirates) or urinary antigen detection tests to guide antimicrobial use following initial empirical broad-spectrum antibiotic therapy[21].

pH1N1-specific haemagglutination inhibition (HI) assays performed on paired acute and convalescent sera may also assist the retrospective diagnosis of severe influenza in NAAT-negative cases[22]. Although influenza viral culture was performed during the pandemic, it is no longer the 'gold-standard' for diagnosing influenza infection as it is less sensitive than NAAT, has longer turnaround times, poses biosafety and biocontainment challenges, and requires specialised laboratory equipment and personnel[23]. However, isolates are needed to monitor antigenic drift, assess potential vaccine changes, and to monitor antiviral resistance. Worldwide, this is undertaken by the World Health Organization (WHO) Global Influenza Network, which consists of five Collaborating Centres and some 110 National Influenza Centres (NIC). Australia is well-served by this system, with a WHO Collaborating Centre in Melbourne and three NIC – in Sydney (ICPMR, Westmead), Melbourne (VIDRL) and Perth (PathWest). These, other laboratories in the Australian Public Health Laboratory Network (PHLN) and a number of other laboratories in Australia and in the Asia-Pacific region contribute influenza strains to the WHO Collaborating Centre in Melbourne[24].

The true incidence of pH1N1 during the first pandemic wave in Australia remains uncertain. In 2009, there were 37,636 laboratory confirmed cases and 191 deaths from pH1N1; 6767 cases and 22 deaths were noted in 2010 (up to 5 November)[25]. However, this underestimates the true community attack rate as laboratory confirmation of suspected influenza was not universal, particularly during the PROTECT phase. Two seroprevalence studies using HI tests undertaken after the first pandemic wave have suggested an overall community rate of 28.4% and 22% in NSW and Australia respectively. This varied according to different age groups and geographic locations[26,27]. Higher infection rates were observed in the younger population and urban centres. However, the pre-pandemic seroprevalence rate of pH1N1 in both studies was 12.8% and 12%, suggesting a "true" population infection rate of 10–15.6%. This is similar to the seroconversion rate of 13% and 14.6% in the Singaporean general community[28] and in the HIV-infected population in Western

Table 1. Sensitivity and minimum turnaround times of tests for pandemic (H1N1) 2009 influenza.

Type of test	Sensitivity	Specificity	Minimum turnaround time (post-receipt in the laboratory)
Rapid antigen test	18%–77%	100%	15–30 minutes
Immunofluorescent antigen test	47%–93%	97%	2–4 hours
Nucleic acid amplification test	98%	100%	4–6 hours
Viral isolation	89%	100%	2–7 days
Serology on convalescent serum	Up to 100%	Up to 100%	18–24 hours

Sydney respectively[29]. The ideal way to determine true pH1N1 seroincidence is to demonstrate individual seroconversion as in these studies, but appropriate specimens are usually only available fortuitously and in small numbers in retrospective studies. Another serosurvey of children and pregnant women in Western Australia (subtracting the pre-pandemic from post-pandemic seroprevalence rates) identified that 25.4% of children aged 1–4, 39.4% of children aged 5–19 and 10.2% of pregnant women aged 21–45 years were infected with pH1N1[30].

Leading into the southern hemisphere's influenza season of 2010, immunity to pH1N1 in the Australian population was at least 40.1–46.5% (post-pandemic seroprevalence of 22–28.4% plus the estimated pH1N1 monovalent vaccine uptake of 18.1% up to the end of February 2010[31]). This may in fact be higher if one includes the uptake of the pH1N1-containing trivalent seasonal influenza vaccine (available as of mid-March 2010). Elevated pH1N1-specific antibodies were most likely to be demonstrated in the elderly Australian population, with 37.5% of individuals aged between 60 and 101 years having HI titres ≥1:40. The prevalence of cross-reacting antibody was highest in the oldest age-group (≥85 years), with more than 60% having HI titres of ≥1:40[32].

The observation that pH1N1 significantly displaced seasonal influenza A/H1N1 and A/H3N2 to become the dominant influenza strain following the first pandemic wave[33] was further confirmed from typing data this year. Of 11,317 cases of influenza in 2010 (up to 5 November), 6767 (60%) were pH1N1, although 3247 (29%) untyped influenza A cases are also likely to be pH1N1 given the negligible activity of other influenza A subtypes[25]. Pre-existing immunity in the population from prior pH1N1 infection and vaccination is likely to have accounted for the decreased number of clinical and laboratory-confirmed cases in 2010.

WHO has reported only 304 oseltamivir-resistant (but zanamavir-susceptible) pH1N1 viruses worldwide (up to 18 August 2010)[34], with all but one virus possessing the H275Y mutation (confirmed by either neuraminidase [NA] allele-specific gene RT-PCR or sequencing of the NA gene product), or by phenotypic testing to determine the oseltamivir IC_{50}. There have been at least 11 cases of oseltamivir-resistant pH1N1 detected in Australia since the beginning of the pandemic[35]. Resistant viruses have been isolated from immunocompromised patients (solid organ transplants or haematological malignancies) receiving prolonged oseltamivir treatment or primary prophylaxis[36-38]. It is important to continue monitoring with both genotypic and phenotypic testing for oseltamivir-resistant pH1N1 given the recent experience with the previous seasonal A/H1N1 strain[39].

In conclusion, accurate and rapid identification of pH1N1 significantly impacted individual patient and public health management during the first pandemic wave. Good sample collection, together with rapid and reliable testing methodologies, remains the cornerstone for accurate diagnosis. Precise epidemiologic data is crucial for pandemic preparedness planning to limit the impact of pandemic influenza.

References

1. Catton, M.G. *et al.* (2009) Australia's influenza containment plan and the swine flu epidemic in Victoria. *Med. J. Aust.* 191, 288.

2. Weeramanthri, T. *et al.* (2010) Response to pandemic (H1N1) 2009 influenza in Australia – lessons from a state health department perspective. *Aust. Health Rev.* 34, 477–486.

3. Australian Government Department of Health and Ageing. (2010) Australian Health Management Plan for Pandemic Influenza (Updated December 2009) (Accessed 9 November, 2010 at http://www.health.nsw.gov.au/internet/panflu/publishing.nsf/Content/4910C697CAEAC460CA2577CB00158570/$File/ahmppi-2009.pdf).

4. Beaman, M.H. & Leung, M.J. (2010) Pandemic influenza testing at the coalface: time for reassessment? *Med. J. Aust.* 192, 102–104.

5. Grayson, M.L. & Johnson, P.D.R. (2009) Australia's influenza containment plan and the swine flu epidemic in Victoria. *Med. J. Aust.* 191, 150.

6. Smith, D.W. *et al.* (2010) Pandemic influenza testing at the coalface: time for reassessment? *Med. J. Aust.* 192, 541–542.

7. Ginocchio, C.C. *et al.* (2009) Evaluation of multiple test methods for the detection of the novel 2009 influenza A (H1N1) during the New York City outbreak. *J. Clin. Virol.* 45, 191–195.

8. Kok, J. *et al.* (2010) Comparison of a rapid antigen test with nucleic acid testing during cocirculation of pandemic influenza A/H1N1 2009 and seasonal influenza A/H3N2. *J. Clin. Micro.* 48, 290–291.

9. Boggild, A.K. & McGeer, A.J. (2010) Laboratory diagnosis of 2009 H1N1 influenza A virus. *Crit. Care Med.* 38, S1–S5.

10. Pollock, N.R. *et al.* (2009) Ruling out novel H1N1 influenza virus infection with direct fluorescent antigen testing. *Clin. Infect. Dis.* 49, e66–e68.

11. Kok, J. *et al.* (2010) Age and specimen type affect the performance of antigen tests in pandemic H1N1 (2009) influenza. Poster presented at *ASID Annual Scientific Conference, Darwin.* 25.

12. Andresen, D.N. & Kesson, A.M. (2010) High sensitivity of a rapid immunochromatographic test for the detection of influenza A virus 2009 H1N1 on nasopharyngeal aspirates from young children. *J. Clin. Micro.* 48, 2658–2659.

13. Esposito, S. *et al.* (2010) Comparison of nasopharyngeal nylon flocked swabs with universal transport medium and rayon-bud swabs with a sponge reservoir of viral transport medium in the diagnosis of paediatric influenza. *J. Med. Microbiol.* 59, 96–99.

14. de la Tabla, V.O. *et al.* (2010) Comparison of combined nose-throat swabs with nasopharyngeal aspirates for detection of pandemic influenza A/H1N1 2009 virus by real-time reverse transcriptase PCR. *J. Clin. Micro.* 48, 3492–3495.

15. Choi, Y.J. *et al.* (2010) Evaluation of new rapid antigen test for detection of pandemic influenza A/H1N1 2009 virus. *J. Clin. Micro.* 48, 2260–2262.

16. Chidlow, G. *et al.* (2010) Duplex real-time reverse transcriptase PCR assays for rapid detection and identification of pandemic (H1N1) 2009 and seasonal influenza A/H1, A/H3, and B viruses. *J. Clin. Micro.* 48, 862–866.

17. Blyth, C.C. *et al.* (2009) Rapid-test sensitivity for novel swine-origin influenza A (H1N1) virus in humans. *N. Engl. J. Med.* 361, 2493.

18. Mulrennan, S. *et al.* (2010) Pandemic influenza (H1N1) 2009 pneumonia: CURB-65 score for predicting severity and nasopharyngeal sampling for diagnosis are unreliable. *PLoS One.* 5, e12849. DOI:10.1371/journal.pone.0012849.

19. Blyth, C.C. *et al.* (2010) Bacterial and viral coinfections in severe influenza infections 2009. Poster presented at *50th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston.* V–448b.

20. Ingram, P.R. *et al.* (2010) Procalcitonin and C-reactive protein in severe 2009 H1N1 influenza infection. *Intensive Care Med.* 36, 528–532.

21. Wright, P.F. *et al.* (2009) When to consider the use of antibiotics in the treatment of 2009 H1N1 influenza-associated pneumonia. *N. Engl. J. Med.* 361, e112.

22. Iwasenko, J.M. *et al.* (2010) Enhanced diagnosis of pandemic (H1N1) 2009 influenza infection using molecular and serological testing in intensive care unit patients with suspected influenza. *Clin. Inf. Dis.* 51, 70–72.

23. Playford, E.G. & Dwyer, D.E. (2002) Laboratory diagnosis of influenza virus infection. *Pathology* 34, 115–125.

24. Dwyer, D.E. *et al.* (2006) Laboratory diagnosis of human seasonal and pandemic influenza virus infection. *Med. J. Aust.* 185, S48–S53.

25. Australian Government Department of Health And Ageing. 2010. Australian influenza surveillance summary report No. 44, Reporting period: 30 October – 5 November 2010 (Accessed 22 November, 2010 at: http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-ozflu-no44-10.htm/$File/ozflu-no-44-2010.pdf).

26. Gilbert, G.L. *et al.* (2010) Prevalence of influenza A (H1N1) 2009-specific antibodies amongst residents of New South Wales, Australia, after the first wave of the 2009 pandemic in the southern hemisphere. *PLoS ONE* 5, e12562. DOI:10.1371/journal.pone.0012562.

27. McVernon, J. *et al.* (2010) Seroprevalence of 2009 pandemic influenza A(H1N1) virus in Australian blood donors, October – December 2009. *Euro Surveill.* 15, pii=19678 (Accessed 9 November, 2010 at: http://www.eurosurveillance.org/images/dynamic/EE/V15N40/art19678.pdf).

28. Chen, M.I. *et al.* (2010) 2009 Influenza A(H1N1) Seroconversion rates and risk factors among distinct adult cohorts in Singapore. *JAMA* 303, 1383–1391.

29. Kok, J. *et al.* (2011) Pandemic (H1N1) 2009 influenza virus seroconversion rates in HIV-infected individuals. *JAIDS* 56, 91–94.

30. Dowse, G.K. *et al.* (2011) Incidence of pandemic (H1N1) 2009 influenza infection in children and pregnant women during the 2009 influenza season in Western Australia – a seroprevalence study. *Med. J. Aust.* 194, 68–72.

31. Australian Institute of Health and Welfare. 2010. 2010 Pandemic Vaccination Survey summary results August 2010 (Accessed 12 November, 2010 at: http://aihw.gov.au/publications/phe/128/12096.pdf).

32. Booy, R. *et al.* (2011) Cross-reacting antibodies against pandemic (H1N1) 2009 virus in elderly Australians. *Med. J. Aust.* 194, 19–23.

33. Blyth, C.C. *et al.* (2010) The impact of the pandemic influenza A(H1N1) 2009 virus on seasonal influenza A viruses in the southern hemisphere, 2009. *Euro Surveill.* 15, pii=19631 (Accessed 8 November, 2010 at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19631).

34. WHO. (2010) Weekly update on oseltamivir resistance to influenza A(H1N1) 2009 viruses (Accessed 9 November, 2010 at: http://www.who.int/csr/disease/swineflu/oseltamivirresistant20100820.pdf).

35. Wang, B. *et al.* (2010) Detection of the rapid emergence of the H275Y mutation associated with oseltamivir resistance in severe pandemic influenza virus A/H1N1 09 infections. *Antiviral Res.* 87, 16–21.

36. Speers, D.J. *et al.* (2010) Oseltamivir-resistant pandemic (H1N1) 2009 influenza in a severely ill patient: the first Australian case. *Med. J. Aust.* 192, 166–168.

37. Tramontana, A.R. *et al.* (2010) Oseltamivir resistance in adult oncology and haematology patients infected with pandemic (H1N1) 2009 virus, Australia. *Emerg. Inf. Dis.* 16, 1068–1075.

38. Baz, M. *et al.* (2011) Emergence of oseltamivir-resistant pandemic H1N1 virus during prophylaxis. *N. Engl. J. Med.* 361, 2296–2297.

39. Hurt, A.C. *et al.* (2009) Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Res.* 83, 90–93.

Biographies

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Development and testing of the Australian pandemic influenza vaccine – a timely response

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In April 2009 a novel virus strain appeared which would cause the first influenza pandemic of the 21st century. This pandemic was the first to occur in an era where bioinformatic technologies contributed to the response to this virus; still, the creation of a vaccine relied largely on existing egg-based technology. The ongoing threat of a H5N1 pandemic spurred the development of strategies to rapidly produce a pandemic vaccine. These plans were implemented and allowed CSL and Australia to conduct the first clinical trials and produce one of the first 2009 pandemic vaccines. However, new candidate **influenza vaccine viruses often present challenges to manufacturing a new vaccine. This pandemic virus was no exception. Being in the post-pandemic phase, it is important to review lessons learned to improve our response to future pandemics. In hindsight, the production of a pandemic vaccine is similar to that of seasonal influenza vaccines, yet the urgency of the pandemic response compresses timelines. This report explores those timelines and implications for producing a pandemic vaccine for Australia.**