CLINICAL AND VACCINE IMMUNOLOGY, Mar. 2011, p. 520–522 Vol. 18, No. 3
1556-6811/11/$12.00 doi:10.1128/CVI.00449-10

Evaluation of Serological Diagnostic Methods for the 2009 Pandemic Influenza A (H1N1) Virus▽

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Received 1 October 2010/Returned for modification 12 November 2010/Accepted 3 January 2011

Serology improves influenza diagnosis by capturing cases missed by reverse transcriptase PCR (RT-PCR). We prospectively evaluated microneutralization and hemagglutination inhibition assays for 2009 influenza A (H1N1) virus diagnosis among 24 RT-PCR-confirmed cases and 98 household contacts. Compared to hemagglutination inhibition, microneutralization demonstrated a higher level of concordance with RT-PCR (kappa = 0.69 versus kappa = 0.60) and greater sensitivity (83% versus 71%; *P* = 0.016).

The emergence of a novel pandemic influenza A (H1N1) virus (here referred to as pH1N1) in April 2009 has required clinical virology laboratories to adapt influenza detection assays to this new strain (16). Although reverse transcriptase PCR (RT-PCR) is the preferred diagnostic modality for influenza (7), false-negative RT-PCR results occur, especially if sampling was performed late in the illness or if the patient had received antiviral therapy (1, 7, 12, 17). In the clinical diagnostic setting, influenza serological testing cannot inform treatment decisions because of the requirement for paired (acute and convalescent) blood samples; however, serology improves influenza diagnosis by capturing cases missed by RT-PCR (3, 9, 11, 17). Furthermore, influenza serology provides important public health data and is a valuable research tool.

To date, the performance of serological methods such as hemagglutination inhibition (HAI) and microneutralization (MN) for detection of pH1N1 has not been extensively validated. Our study aimed to assess the diagnostic accuracy of HAI and MN in RT-PCR-confirmed cases and their household contacts.

Clinical data and samples for laboratory testing were prospectively collected during serial household visits over 3 to 4 weeks in a study evaluating pH1N1 transmission among community cases and their household contacts in May to July 2009 (11). Of note, there was no concurrent seasonal influenza circulation in the province of Quebec at the time of the study, and none of the participants received antiviral therapy or prophylaxis. Nasopharyngeal secretions were obtained from all subjects during the first household visit by flocked swab (Copan Innovation, Brescia, Italy) and tested by conventional RT-PCR tests comprising a specific pH1N1 assay for the hemagglutinin gene and a universal influenza A virus assay targeting the matrix gene (4, 5, 10, 11). Blood for serological evaluation of pH1N1 infection was drawn from subjects ≥7 years old at the initial visit (acute sample) and 3 to 4 weeks later (convalescent sample). Sera were tested by HAI and MN according to WHO standard protocols with minor modifications (11, 15). Positive- and negative-control sera were included in each testing run. Seroconversion was defined as an acute-phase serum titer of <1:10 with a convalescent titer of ≥1:40 (or ≥1:20 during preliminary analyses) or a significant increase (≥4-fold) in antibody titers between the two sera. Paired sera that met all of the following criteria were excluded from the present analyses: acute-phase serum drawn >7 days after onset of illness, acute-phase serum titer of ≥1:10, and absence of seroconversion.

MN. Sera were first inactivated for 30 min at 56°C. Beginning with a 1:10 dilution, 2-fold serial dilutions of sera were mixed with an equal volume of medium (Dulbecco's modification of Eagle's medium with L-glutamine, 4.5 g/liter glucose, and sodium pyruvate) containing 100 PFU of A/Quebec/147023/2009 (pH1N1) virus. After a 2-h incubation at 37°C in a 5% CO_2 humidified atmosphere, the residual infectivity of the virus-serum mixture (50 μl) was determined by infecting confluent MDCK cells. Neutralizing antibody titers were defined as the reciprocal of the highest dilution of serum that completely neutralized the infectivity of the virus as determined by the absence of cytopathic effect at day 4 postinfection.

HAI. Nonspecific inhibitors were removed from serum by overnight treatment with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan). Physiologic saline solution was then added to achieve a 1:10 dilution, followed by incubation with packed turkey red blood cells (TRBC) at 4°C for 60 min to remove nonspecific agglutinins (Lampire Biological Laboratories Inc., Pipersville, PA). Treated serum was serially diluted in 25 μl of phosphate-buffered saline (PBS) and then mixed with an equal volume of PBS containing 4 hemagglutinin units of the A/Quebec/147023/2009 (H1N1) virus. After 30 min of incubation at room temperature, 50 μl of 0.7% TRBC solution was added to the mixture and then incubated for 30 to 45 min before evaluation of hemagglutination. The HAI titer was recorded as the reciprocal of the last dilution that inhibited hemagglutination.

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▽ Published ahead of print on 12 January 2011.

TABLE 1. Comparison of HAI and MN assay results[a]

MN test and result	No. of results			
	$HAI_{1:40}$[b]		$HAI_{1:20}$[c]	
	+	−	+	−
$MN_{1:40}$[b]				
+	38	19	44	13
−	1	64	4	61
$MN_{1:20}$[c]				
+	38	24	44	18
−	1	59	4	56

[a] +, evidence of seroconversion. −, absence of seroconversion. Convalescent titer cutoffs for positivity were 1:40 or 1:20 in pH1N1 index cases ($n = 24$) and their household contacts ($n = 98$). HAI, hemagglutination inhibition assay. MN, microneutralization assay. $MN_{1:40}$ versus $HAI_{1:40}$, kappa = 0.66; $MN_{1:40}$ versus $HAI_{1:20}$, kappa = 0.72; $MN_{1:20}$ versus $HAI_{1:40}$, kappa = 0.59; $MN_{1:20}$ versus $HAI_{1:20}$, kappa = 0.64.

[b] Seroconversion was defined as an acute-phase serum titer of <1:10 with a convalescent titer of ≥1:40 or a significant increase (≥4-fold) in antibody titers between the two sera.

[c] Seroconversion was defined as an acute-phase serum titer of <1:10 with a convalescent titer of ≥1:20 or a significant increase (≥4-fold) in antibody titers between the two sera.

TABLE 2. Comparison of HAI and MN assays to RT-PCR for pH1N1 diagnosis[a]

Assay and result	% Sensitivity[b] (95% CI)	No. of cases with RT-PCR result	
		Positive	Negative
$MN_{1:40}$[c]	82.8 (70.1, 91.0)		
+		48	9
−		10	55
‡$MN_{1:20}$[d]	84.5 (72.1, 92.2)		
+		49	13
−		9	51
$HAI_{1:40}$[c]	60.3 (46.6, 72.7)		
+		35	4
−		23	60
$HAI_{1:20}$[d]	70.7 (57.1, 81.5)		
+		41	7
−		17	57

[a] HAI, hemagglutinin inhibition assay. MN, microneutralization assay. CI, confidence interval. +, evidence of seroconversion. −, absence of seroconversion. $MN_{1:40}$ versus RT-PCR, kappa = 0.69; $MN_{1:20}$ versus RT-PCR, kappa = 0.64; $HAI_{1:40}$ versus RT-PCR, kappa = 0.54; $HAI_{1:20}$ versus RT-PCR, kappa = 0.60.

[b] Serological method sensitivity for detection of RT-PCR confirmed cases.

[c] Seroconversion was defined as an acute-phase serum titer of <1:10 with a convalescent titer of ≥1:40 or a significant increase (≥4-fold) in antibody titers between the two sera.

[d] Seroconversion was defined as an acute-phase serum titer of <1:10 with a convalescent titer of ≥1:20 or a significant increase (≥4-fold) in antibody titers between the two sera.

Statistical analyses included the calculation of sensitivity (95% confidence interval [95% CI]), kappa, and McNemar's test using SAS software (version 9.2). P values of ≤0.05 were considered significant.

Our serology substudy included 24 RT-PCR-confirmed index cases (median age, 15 years; range, 7 to 56 years) and 98 household contacts (median age, 30.5 years; range, 7 to 61 years), of which 34 also tested positive for pH1N1 by RT-PCR. Table 1 compares MN and HAI results. The strongest concordance between serological assays (kappa = 0.72) was achieved using convalescent-phase serum titer thresholds for seroconversion of 1:40 for MN ($MN_{1:40}$) and 1:20 for HAI ($HAI_{1:20}$). When comparing RT-PCR to serology (Table 2), the best concordance was with $MN_{1:40}$ (kappa = 0.69). Of the 9 samples positive by $HAI_{1:20}$ but negative by $HAI_{1:40}$, 6 (66%) were also positive by RT-PCR. In contrast, only 1 of 5 (20%) additional positive results by $MN_{1:20}$ compared to $MN_{1:40}$ was positive by RT-PCR. The sensitivity of $MN_{1:40}$ to detect seroconversion in RT-PCR-positive patients (83%; 95% CI, 70 to 91) was significantly higher ($P = 0.016$) than the sensitivity of $HAI_{1:20}$ (71%; 95% CI, 57 to 82).

Our study details the performance of MN and HAI compared with each other and with RT-PCR for the diagnosis of pH1N1 infection. $HAI_{1:20}$ had better concordance with MN and RT-PCR than $HAI_{1:40}$. The lower HAI threshold detected additional seroconversions, two-thirds of which were in RT-PCR-positive patients. Although HAI is a well-established method to estimate antibody titers against a particular influenza virus strain, results are dependent on the affinity of the hemagglutinin of the strain in question for the sialic acid receptors of the red blood cells used (13, 14). Therefore, careful validation of serological cutoffs is required when adapting HAI assays to a virus with a novel hemagglutinin, like that of pH1N1. Our pH1N1 HAI assay used TRBC; assays using other types of red blood cells might not demonstrate the same performance.

Our findings suggest that MN may be superior to HAI for detection of pH1N1 infection. $MN_{1:40}$ and $MN_{1:20}$ had good concordance with RT-PCR, whereas concordances of $HAI_{1:40}$ and $HAI_{1:20}$ with RT-PCR were only moderate. Moreover, the sensitivity of $MN_{1:40}$ for identifying seroconversion in RT-PCR-positive subjects was significantly greater than that of $HAI_{1:20}$. Although MN and HAI have demonstrated comparable sensitivities for assessing antibody responses to pH1N1 vaccines (2, 6), there are virtually no data comparing their performance for diagnosing pH1N1 infection. Cowling et al. observed a greater proportion of pH1N1 RT-PCR-positive patients with a ≥4-fold rise in titers by MN than HAI; however, no statistical comparison was reported (3). Beyond the aforementioned performance characteristics, it is important to also consider that, while both assays are time-consuming, MN is the more labor-intensive of the two and requires handling live virus.

Because of our lack of serological data in children <7 years old, and because we performed laboratory testing regardless of our subjects' symptoms, the spectrum of disease in our study may differ from those in other patient populations in whom MN and HAI might be used. This could affect the observed performance of the assays. For instance, Hung et al. have reported that being afebrile on presentation was associated with a poorer MN convalescent response (<1:40) among patients with RT-PCR-confirmed pH1N1 (8). Nevertheless, our study provides new data on serological diagnostic methods for pH1N1. MN results had good concordance with HAI and RT-PCR. Furthermore, MN may be superior to HAI for the diagnosis of pH1N1 infection.

This work was supported by the Fonds de la Recherche en Santé du Québec. G.B. is the holder of the Canada Research Chair on Emerging Viruses and Antiviral Resistance and is the Canadian Pandemic Team Leader on Antiviral Resistance and Evolution of Influenza Viruses.

We thank Patricia S. Fontela for her thoughtful review of the manuscript.

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