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**Original article**

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

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## Abstract

### *Background*

In April 2009, a novel influenza A (H1N1) virus emerged in North America and Mexico. In the face of a pandemic influenza, serology provides important public health data and is a valuable research tool. The labor intensiveness of the hemagglutination inhibition (HI) test is a major hindrance to its use on a large scale in routine. Recently, a commercial enzyme-linked immunosorbent assay (ELISA) (pandemic A (H1N1) IgG and IgA Genzyme Virotech®) has been developed. In addition, the need for surveillance led us to develop complement fixation test (CF) with 2009 A (H1N1) viral lysate. The following study was conducted to assess the antibody detection accuracy of this two tests in comparison with the HI test as “gold standard”.

### *Patients and methods*

Serum samples tested in this study were collected from 2 groups of subjects. The first group comprised 75 unvaccinated patients; the second group comprised 69 subjects receiving immunosuppressive therapy and vaccinated three weeks ago against 2009 A (H1N1). All were tested by the three techniques.

### *Results and conclusion*

Sero-prevalence was significantly higher in vaccinated than unvaccinated subjects ( $p = 0,032$ ). In vaccinated group, the ELISA IgA gave a sensitivity of 61% and a specificity of 78%, ELISA IgG gave the same value for specificity but 75% for sensitivity; the CF titre cut-off (80) gave a sensitivity of 15% and a specificity of 87%. The CF titer cut-off value that provided the highest sensitivity (71%) and specificity (78%) was 10. In unvaccinated patients, the ELISA IgG gave a sensitivity of 57% and 87% for specificity; the CF titre cut-off (80) gave a sensitivity of 70% and a specificity of 100%.; strong positive correlation ( $r^2=0.67$ ,  $p<0.0001$ ) was noted between CF and IH titers. CF results had good concordance with HI for general population screening. ELISA may be superior to CF for the detection of 2009 A (H1N1) antibodies among vaccinated patients.

## INTRODUCTION

In April 2009, a novel swine origin influenza A (H1N1) virus emerged in North America and Mexico [1]. The efficient transmission of this virus and the lack of immunity in most populations enabled it to rapidly spread across the world and necessitated the declaration of a pandemic by the World Health Organization [2]. Although reverse transcriptase PCR (RT-PCR) is the preferred diagnostic modality for influenza, it's extremely resource-intensive and false-negative RT-PCR results occur, especially if sampling was performed late in the illness or if the patient had received antiviral therapy [3]. Serology improves influenza diagnosis by capturing cases missed by RT-PCR [4]. Furthermore, influenza serology provides important public health data and is a valuable research tool. In particular, seroepidemiological studies can assess risk factors for infection and rates of transmission in defined populations with information on illness, demographics, and behavioral factors.

There are significant differences between 2009 A (H1N1) virus and seasonal influenza viruses both genetically and antigenically, the new virus was never detected in humans or animals before [5]. This has required clinical virology laboratories to adapt influenza detection assays to this new strain [6]. The hemagglutination inhibition test (HI) is reference technique for detection of influenza antibodies. However, the labor intensiveness of the HI test is a major hindrance to its use on a large scale in routine. Recently, a commercial enzyme-linked immunosorbent assay (ELISA) (pandemic A (H1N1) IgG and IgA Genzyme Virotech®) has been developed specifically for detecting antibody against 2009 A (H1N1) subtype. In addition, the need for surveillance led us to develop complement fixation test (CF) with 2009 A (H1N1) viral lysate that specifically recognizes pandemic H1N1 2009 influenza virus antibodies in human sera. The performance of serological methods like CF and ELISA for 2009 A (H1N1) strains has not been extensively validated. The following study was conducted to assess the antibody detection accuracy of these two tests in comparison with the HI test using a set of serum samples from two types of population: A (H1N1) vaccinated and unvaccinated.

### Patients and methods:

#### A. Subjects and survey design

Serum samples tested in this study were collected from 2 groups of subjects. The first group comprised 75 unvaccinated patients for 2009 A (H1N1). All patients, except those who had been vaccinated against 2009 A (H1N1), were eligible to participate during the survey period (between 29 November 2009 and 2 March 2010). The second group

comprised 69 subjects receiving immunosuppressive therapy and vaccinated three weeks ago against this strain; indeed, this population is more problematic in serology allowing a better evaluation of our techniques.

#### B. Laboratory testing

Assays were performed in the WHO Collaborating Centre for Reference on Influenza in Lyon (France).

**Hemagglutination assay:** Details on the HI assays have been published elsewhere [7]. Viral antigen used was A/California/7/2009 virus (National Institute for Medical Research, UK), which was propagated in 10 to 11 day-old embryonated chicken eggs. Hemagglutination assay (HA) was performed in order to measure the amount of hemagglutinin antigen present in the test virus suspension prior to running HI assay. One HA unit of the test virus was defined as the highest virus dilution that displayed complete hemagglutinating activity. Four HA units of the antigen of 2009 A (H1N1) virus were used. After centrifuging, sera were obtained, aliquoted, and then frozen at -80°C. Non specific inhibitors were removed from serum by overnight treatment with receptor-destroying enzyme (RDE) of 4 volumes at 37°C for 18 h, and then incubated at 56°C for 60 min. The serum was then absorbed with 50% chicken erythrocytes of equal volume to serum. Eight titrations (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280) were prepared for each serum sample to test for specific antibody against 2009 A (H1N1) virus by HI assay. Serums were incubated with 50 µl of the test antigen for 60 minutes at room temperature. Thereafter, the reaction wells were added with 50 µl of 0.5% chicken erythrocyte suspension and further incubated for 60 minutes at room temperature. All experiments included negative and positive controls, and a serum agglutinating activity control. The HI titre was calculated as the reciprocal of the highest dilution of serum that was able to inhibit the hemagglutination of the chicken erythrocytes induced by the influenza virus. The 1:40 titre was regarded as seropositive, i.e. the participant was immune against this virus [8]. The ih assays took approximately 25 ours for completion.

**Complement Fixation:** Some weeks after the emergence of 2009 A (H1N1) influenza virus, we developed complement fixation test in our laboratory. The strain of the first case identified (A/California/7/2009) was obtained from MDCK cell culture supernatant medium. Viral lysate was prepared by purification and conserved at -80°C [9]. Then, we determined by titration the optimal concentration of CF reagents *ie* antigen, complement and hemolytic system [10]. CF was performed as previously described [11] with Siemens® CF reagents. Briefly, the first step was the thermal

inactivation of patient's serum to inactivate endogenous complement which may disturb the test calibration. Patient serum was diluted from 1:10 to 1:640 and combined with antigen. Guinea pigs complement was added and incubated for 60 minutes at 37°C.

The positive control serum was prepared from blood of ferret immunized with 2009 A (H1N1) strain. Negative serum control, antigen and complement control were tested. Sensitized sheep red blood cells (RBC) are added and incubated at 37°C for 25 minutes. When antibody to the antigen is present, complement is bound and the RBCs settle out and form a pellet on the bottom of the well. When antibody is absent, unbound complement lyses the RBCs and no pellet is formed. A CF result was considered positive for both IgG and IgM antibodies at titers of 1:80 or more. The CF assays took approximately 5 hours for completion.

**ELISA:** The specimens were tested for IgG and IgA by using VIROTECH ELISA® test kits (Genzyme Virotech, Germany) for the influenza virus 2009 A (H1N1) according to the manufacturer's recommendations. Optical density values were then converted into sample-to-positive (S/P) ratios using a formula provided by the manufacturer. Samples with S/P ratio equal to or greater than 11 were considered to be positive for antibody against 2009 A (H1N1). The specific viral antigens coating the ELISA plates are related with A/California/7/2009 hemagglutinin antigen. The IgG and IgA assays took approximately 2 and 4 hours for completion, respectively.

### C. Statistical analysis

The database was maintained in Microsoft Excel (version 2003) and analysed using Epi Info statistical package (version 3.5.3). Frequencies were calculated for categorical variables. The percent sensitivity and specificity, the negative and positive predictive value for CF and ELISA were determined by comparing to IH. IH was used as the "gold standard". Linear regression models were performed to estimate the correlation between serum antibody titers measured by CF vs HI and by ELISA vs HI assays. *P* values of less than 0.05 were considered significant.

## RESULTS

### A. Vaccinated group

Our study included 69 vaccinated patients with immunosuppressive therapy (median age, 39.4 years; range, 5 to 56 years, Sex Ratio F/H=1.46), which sera were tested by ELISA, CF and IH assays for 2009 A (H1N1). Table 1 indicates the results of different tests. Of the subjects, 42 (61%) were seropositive by ELISA IgG, 42 (61%) with ELISA IgA, 10 (14.5%) with CF and 51 (74%) with IH test. Subjects aged 21-35 years recorded the highest seroprevalence rate

(51%,  $p < 0.05$ ). Forty five (65%) patients had IH titre superior than 1:160. HI-titres 1:160 are more frequent in younger age groups old than in individuals > 35 years ( $p = 0.01$ ).

Table 1: Details of the positive and negative antibody response among different sex and age-groups in vaccinated subjects.

Vaccinated patients	IgG		IgA		CF		HAI		
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	
Male	34	21	20	13	28	5	23	29	12
Female	35	21	7	22	6	5	36	22	6
<b>Age (years)</b>									
0-20	30	9	21	9	21	2	28	22	8
21-35	33	27	6	27	6	6	27	23	10
> 35	6	6	0	6	0	2	4	6	0

(pos: positive, neg: negative, CF: Complement fixation, HAI: Hemagglutination inhibition)

The ELISA IgA gave a sensitivity of 61% (46-74, IC:95%) and a specificity of 78% (52-93, IC:95%), ELISA IgG gave the same value for specificity but 75% (60-85, IC:95%) for sensitivity. The CF titre cut-off (80) gave a sensitivity of 15% (6-27, IC:95%) and a specificity of 87% (52-93, IC:95%) (Table 2).

Table 2: Serology assay sensitivity and specificity summary

	Vaccinated patients (95% CI)				Unvaccinated patients (95% CI)			
	Se	Sp	PPV	NPV	Se	Sp	PPV	NPV
IgA (%)	61 (46-74)	78 (52-93)	89 (73-96)	42 (24-59)	35 (20-52)	94 (82-99)	87 (59-98)	60 (46-72)
IgG (%)	75 (60-85)	78 (52-93)	52 (31-71)	91 (77-97)	57 (39-72)	87 (71-95)	81 (60-93)	68 (52-80)
IgA+G (%)	86 (73-94)	78 (52-93)	92 (80-97)	67 (43-85)	65 (47-79)	85 (68-93)	80 (61-92)	71 (55-83)
CF (%)	15 (6-27)	87 (52-93)	67 (34-90)	24 (13-36)	70 (53-84)	100 (90-100)	100 (86-100)	78 (63-88)

Se: Sensibilité ; Sp : Spécificité. VPP: positive predictive value, VPN: negative predictive value, CI: confidence interval, CF : Complement fixation

The CF titer cut-off value that provided the highest sensitivity (71%) and specificity (78%) was 10. We next assessed whether a combination of IgA and IgG titers could maximize sensitivity and specificity. Combining resulted in a sensitivity of 86% (73-94, IC95%) and a specificity of 87% (52-93, IC95%) for all ages. Weak positive correlation (Spearman's rank correlation,  $r^2 < 0.5$ ,  $p < 0.0001$ ) was noted between ELISA IgA, IgG, CF and IH titers.



### B. Unvaccinated group

Our study included 75 unvaccinated patients (median age, 42.3 years; range, 19 to 65 years, Sex Ratio F/H=1.08). Table 3 indicates the results of different tests. Of the subjects, 26 (34.6%) were seropositive by ELISA IgG, 15 (20%) with ELISA IgA, 31 (41.3%) with CF and 37 (49.3%) with IH test. Subjects aged 19-30 years recorded the highest seroprevalence rate (65.3%,  $p < 0.05$ ).

Table 3: Details of the positive and negative antibody response among different sex and age-groups in unvaccinated subjects.

Unvaccinated patients	IgG		IgA		CF		HAI		
	pos	neg	pos	neg	pos	neg	pos	neg	
Male	36	12	27	7	32	15	24	18	21
Female	39	14	22	8	28	16	20	19	17
Age									
19-30	46	17	29	9	12	17	12	6	9
31-45	16	4	12	4	25	6	12	17	14
46-55	7	5	4	1	12	4	13	8	11
>55	6	0	4	1	11	4	7	6	4

(pos: positive, neg: negative, CF: Complement fixation, HAI: Hemagglutination inhibition)

Twenty tree (71.8%) patients had IH titre superior than 1:80. The ELISA IgA gave a sensitivity of 35% (20-52, IC95%) and a specificity of 94% (82-99, IC95%); ELISA IgG gave a sensitivity of 57% (39-72, IC95%) and 87% (71-95, IC95%) for specificity. The CF titre cut-off (80) gave a sensitivity of 70% (53-84, IC95%) and a specificity of 100% (90-100, IC95%) (Table 2). The CF titer cut-off value that provided the highest sensitivity (78%) and specificity (88%) was 10. Combining IgA and IgG titers resulted in a sensitivity of 65% (47-79, IC95%) and a specificity of 85 (68-93, IC95%). Strong positive correlation ( $r^2=0.67$ ,  $p < 0.0001$ ) was noted between CF and IH titers, but not with ELISA IgG and IgA (Figure 1).

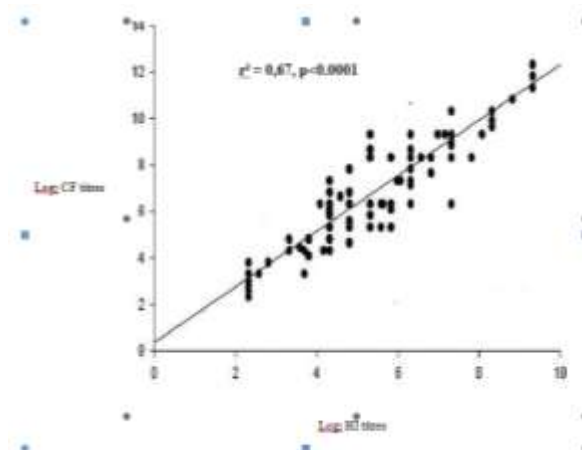


Figure 1: Linear regression and correlation between  $\log_2$  Hemagglutination inhibition (HI) titers and  $\log_2$  Complement fixation (CF) titers.

Our tree serology test (IgG, IgA and CF) were significantly more specific in unvaccinated subjects than vaccinated with immunosuppressive therapy (91% vs 59%,  $P < 0.001$ ) but not more sensitive. Sub-typing RT-PCR for seasonal influenza strains was performed for patients (4) with a positive result in CF test but negative in IH for pandemic strain, results confirmed that it was seasonal A(H1N1) strain, these correspond to cross-reactivity between the two strains concerning our developed CF test.

### DISCUSSION

Recent advances in technology have added to the number of diagnostic tools used for the detection of influenza infections. These include culture, immunoassay antigen staining, electron microscopy, RT-PCR and serologic testing. However, non serologic tests can be problematic owing to cost, lengthy turnaround times and the required expertise and specialized equipment. Serologic testing can be used diagnostically if acute and convalescent samples are obtained. He can provide useful information about recent or past viral infections within a community at a reasonable cost and in a short turnaround time [12].

In the face of a pandemic influenza, the application of the typical HI assay is limited due to some disadvantages. The labor intensiveness of the HI test is a major hindrance to its use on a large scale in routine. Recently, a commercial ELISA (pandemic A (H1N1) IgG and IgA Genzyme Virotech®) has been developed specifically for detecting antibody against 2009 A (H1N1) subtype. In addition, the need for surveillance led us to develop CF test with 2009 A (H1N1) viral lysate that specifically recognizes

pandemic H1N1 2009 influenza antibodies in human sera. In this study, we compare new CF and ELISA for 2009 A (H1N1) strain with the IH assay as “gold standard” serology test.

Of the vaccinated with immunosuppressive therapy subjects, 61% were seropositive by ELISA IgG, 61% with ELISA IgA, 14,5% with CF and 74% with IH test. Of the unvaccinated patients, 34.6% were seropositive by ELISA IgG, 20% with ELISA IgA, 41.3% with CF and 49.3% with IH test. The mucosal IgG levels correlate well with the respective serum levels, indicating passive diffusion from the systemic compartment, whereas IgA is produced locally [13]. Sero-prevalence was significantly higher in vaccinated than unvaccinated subjects ( $p = 0,032$ ). Indeed, the use of pandemic vaccine for patients with immunological disorders was recommended and increases the rates of A (H1N1) antibody. Under the influence of drugs, one third of this group not developed protective antibody. Although well tolerated, the sero-protection rate following pH1N1 vaccination is lower than that would be expected. Evaluation of pandemic H1N1 (2009) influenza vaccine described the seroprotective rate post-vaccination for solid tumors, was 50% compared to 27% for hematological malignancy [14]. The ELISA IgA in vaccinees gave a sensitivity of 61% and a specificity of 78%, ELISA IgG gave the same value for specificity but 75% for sensitivity. Combining IgA and IgG titers resulted in a sensitivity of 86% and a specificity of 87% with 92% as Positive Predictive Value (PPV). These results suggest that seropositivity criteria based on a combination of serological titers can provide maximal sensitivity and specificity for the detection of 2009 H1N1 virus-specific antibody. The CF titre cut-off (80) gave a sensitivity of 15% and a specificity of 87%. The ELISA with hemagglutinin antigen was more sensitive than CF in this group. Our results were similar to previous observations based on similar outcomes [15, 16]; the specificity of the ELISA methods depends greatly on the specific antigens used. CF assays give false-negative antibody response results for the majority of vaccinees group of Harry and al. study [15], CF detects heterologous antibodies and antibodies to type-specific nucleoproteins [17] that would disappear more rapidly [13]. In addition, they are probably not synthesized in large quantities after vaccination (particles of hemagglutinin antigen) and particularly among immuno-compromised patients. For all this reasons, weak positive correlation ( $r^2 < 0.5$ ,  $p < 0.0001$ ) was noted between ELISA, CF and IH titers in this population. The CF titer cut-off value that provided the highest sensitivity (71%) and specificity (78%) was 10. We suggest that 1:80 titer threshold may

underestimate the numbers of seropositive antibody sera for A (H1N1). More studies are needed to careful validation of serological cut-off value of CF test for immuno-compromised patients.

In unvaccinated patients, only sero-prevalence obtained by CF and IH tests was close to what was reported by others authors in French population (60%) [18]. The ELISA IgG gave a sensitivity of 57% and 87% for specificity. The CF titre cut-off (80) gave a sensitivity of 70% and a specificity of 100% with 92% as PPV. The IgG ELISA, lacked specificity and sensibility in this group. Rates and affinity of the antibodies detected by CF test in based population are certainly higher than among immuno-compromised patients. In addition, the use of viral lysate with multitude of antigen and possibility to detect all antibodies isotypes can explain why the sero-prevalence was significantly higher by CF compared with ELISA test ( $p < 0.01$ ). On the other hand, cross reactivity with others influenza subtype and auto-antibodies can contribute to some false positives observed in ELISA test [19]. Strong positive correlation ( $r^2 = 0.67$ ,  $p < 0.0001$ ) was noted between CF and IH titers. Consequently, the protocol presented here would facilitate the generation of reliable results during epidemiological surveys of the immune status against the pandemic influenza strain. Subjects aged less than 35 years recorded the highest sero-prevalence rate. This was reported by many publications during the pandemic phase [16,18]. Young people are more likely to gather in large groups, or attend venues where there are likely to be large numbers of people and contacts, e.g. schools, universities, concerts or sports events. In addition, the response of the immune system of the elderly patients to antigen stimulation is limited. Cross reactions were detected among 5 subjects, reflecting on the one hand the presence of commons antigens between seasonal and pandemic strain and the other, the low incidence of these reactions for our CF test.

## CONCLUSION

In summary, our study provides data on new serological diagnostic methods for 2009 A (H1N1). CF results had good concordance with HI for based population. ELISA may be superior to CF for the detection of 2009 A (H1N1) antibodies among vaccinated patients. CF assay should not be used to assess the antibody response to influenza virus vaccination [15]. These two assays could serve as a useful tool for the continued surveillance of the immune status of different risk groups, and could help to establish an effective vaccination policy and discern which groups should be vaccinated first, the ELISA technology for immuno-compromised and

vaccinated patients, CF test for general population screening.

#### ACKNOWLEDGMENT

No conflict of interests is declared.

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