Differential diagnosis of pandemic (H1N1) 2009 infection by detection of haemagglutinin with an enzyme-linked immunoassay

Q. Yuan[1], X.-D. Cheng[2], B.-C. Yang[1], Q.-B. Zheng[1], Y.-X. Chen[1], Q.-R. Chen[1], F. Zeng[1], R. Zhang[2], S.-X. Ge[1], X.-K. Hao[2], H. Chen[3], J. Zhang[1] and N.-S. Xia[1]

1) *National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Life Science, Xiamen University, Xiamen,* 2) *Centre of Clinical Laboratory Medicine of PLA, Xijing Hospital, Fourth Military Medical University, Xi'an and* 3) *State Key Laboratory for Emerging Infectious Diseases, University of Hong Kong, Hong Kong SAR, China*

Abstract

A sensitive and convenient immunoassay that can directly differentiate pandemic (H1N1) 2009 (pH1N1) virus from seasonal influenza virus can play an important role in the clinic. In the presented study, a double-sandwich ELISA (pH1N1 ELISA), based on two monoclonal antibodies against haemagglutinin (HA) of the pH1N1 virus, was developed. After laboratory determination of the sensitivity and specificity characteristics, the performance of this assay was evaluated in a cohort of 904 patients with influenza-like illness. All seven strains of pH1N1 virus tested were positive by pH1N1 ELISA, with an average lower detection limit of $10^{3.0 \pm 0.4}$ tissue culture infective dose $(TCID)_{50}$/mL (or 0.009 ± 0.005 HA titre). Cross-reaction of the assay with seasonal influenza virus and other common respiratory pathogens was rare. In pH1N1-infected patients, the sensitivity of the pH1N1 ELISA was 92.3% (84/91, 95% CI 84.8–96.9%), which is significantly higher than that of the BD Directigen EZ Flu A + B test (70.3%, $p < 0.01$). The specificity of pH1N1 ELISA in seasonal influenza A patients was 100.0% (171/171, 95% CI 97.9–100.0%), similar to that in non-influenza A patients (640/642, 99.7%, 95% CI 98.9–100.0%). The positive predictive value for pH1N1 ELISA was 97.7% and the negative predictive value was 99.1% in this study population with a pH1N1 prevalence of 10.1%. In conclusion, detection of HA of pH1N1 virus by immunoassay appears to be a convenient and reliable method for the differential diagnosis of pH1N1 from other respiratory pathogens, including seasonal influenza virus.

Keywords: Haemagglutinin, immunoassay, influenza A virus, influenza diagnosis, pandemic (H1N1) 2009 virus
Original Submission: 11 June 2010; **Revised Submission:** 26 September 2010; **Accepted:** 22 October 2010
Editor: J.-M. Pawlotsky
Article published online: 4 November 2010
Clin Microbiol Infect 2011; **17:** 1574–1580
10.1111/j.1469-0691.2010.03413.x

Corresponding author: N.-S. Xia, National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Life Science, Xiamen University, Siming Road 422, Xiamen 361005, China
E-mail: nsxia@xmu.edu.cn

Introduction

Over 214 countries had reported confirmed cases of pandemic (H1N1) 2009 (pH1N1) infection and at least 18 449 deaths were noted as of 6 August 2010 [1]. The spread of the virus highlights the importance of having convenient and reliable methods for diagnosis. Currently, RT-PCR is the mainstay for specific diagnosis of pH1N1 virus infection in the clinic, but its utility is questionable, because of the requirement for specialized equipment and long turn-around time. Hence, rapid influenza diagnosis tests (RIDTs) have been used on many occasions [2]. However, RIDTs cannot efficiently differentiate pH1N1 virus infection from seasonal influenza A virus infection, as they have poor sensitivity [2–5], with consequences for clinical management. Therefore, a sensitive and convenient immunoassay that can differentiate the pH1N1 virus from seasonal influenza virus is desirable. In this study, with haemagglutinin (HA) of pH1N1 virus as the detection target, a double-sandwich ELISA (pH1N1 ELISA) was developed and evaluated for its ability to differentiate pH1N1 virus from other respiratory pathogens, including seasonal influenza viruses.

Materials and Methods

Monoclonal antibodies used for pH1N1 ELISA

Two monoclonal antibodies (mAbs) (10B4 and 1E12) that recognize the cluster-specific epitopes in HA of pH1N1 virus were created by immunizing with a pH1N1 isolate

(A/California/04/2009(H1N1)) in mice. The specificities of the two mAbs were determined in a series of influenza viral isolates, using haemagglutination inhibition assays and cell-based microneutralization assays, performed as previously described [6,7] (Table 1). mAb 10B4 was then coated on the microplate, and mAb 1E12 was conjugated with horseradish peroxidase.

Detection protocol of pH1N1 ELISA

A schematic diagram of the principle and manipulation of pH1N1 ELISA is shown in Fig. 1. For detection by pH1N1 ELISA, 50 μL of viral lysis buffer was added to the coated wells, and a 100-μL specimen aliquot was then added and mixed. After incubation for 60 min at 37°C, the plate was washed five times with a washing buffer. Then, 100 μL of 1E12–horseradish peroxidase solution was added to each well and incubated for 30 min at 37°C. After five washes, 100 μL of tetramethylbenzidine substrate solution was added and incubated at 37°C for 15 min, and the optical density $(OD)_{450/630\ nm}$ was measured with a microplate reader (Sunrise, Tecan, Switzerland) (Fig. 1). The final result was obtainable within 105 min. The cut-off value was set as mean + 4 standard deviations, equal to 2.1-fold of the mean value of two negative control wells or 0.105 if the mean negati value was <0.05. Bronchoalveolar lavage fluid, nasopharyngeal aspirate/nasopharyngeal swab (NPS), nasal swab/nasal wash, throat wash, oropharyngeal swab, cell culture supernatant and allantoic fluid specimens were available for the test without any pretreatment. However, the swab specimens need to be transported in viral transport medium (phosphate-buffered saline solution containing 100 U/mL kanamycin and 120 U/mL ampicillin) before testing.

TABLE 1. Haemagglutination inhibition (HI) and neutralization activities of monoclonal antibodies 10B4 and 1E12 against pandemic (H1N1) 2009 virus or seasonal influenza viruses

	1/HI titre		1/Neutralization titre	
Virus strain	**10B4**	**1E12**	**10B4**	**1E12**
2009 pandemic A/H1N1				
A/California/04/2009(H1N1)	6400	12 800	ND	ND
A/Xiamen/N583/2009(H1N1)	6400	12 800	12 800	12 800
A/Xiamen/N582/2009(H1N1)	12 800	12 800	12 800	12 800
Seasonal H1N1				
A/Xiamen/N66/2009(H1N1)	<10	<10	<10	<10
A/Xiamen/1172/2008(H1N1)	<10	<10	<10	<10
Seasonal H3N2				
A/Yancheng/N101/2009(H3N2)	<10	<10	<10	<10
A/Xiamen/1394/2008(H3N2)	<10	<10	<10	<10
Influenza B				
B/Yancheng/N105/2009	<10	<10	<10	<10
B/Xiamen/1325/2008	<10	<10	<10	<10

ND, no data.
Titre lower than 1 : 10 is considered to be negative.

Influenza viral isolates and other respiratory pathogens

Seven pH1N1 strains (Table 1), 78 influenza A virus strains (non-pH1N1), 20 influenza B virus strains and 59 strains of other common respiratory pathogens were used to determine the analytical sensitivity and cross-reactions of pH1N1 ELISA (Tables 2 and 3).

Clinical specimens

Clinical specimens from patients with influenza-like illness (ILI) were collected in Xi'an City, north-western China,

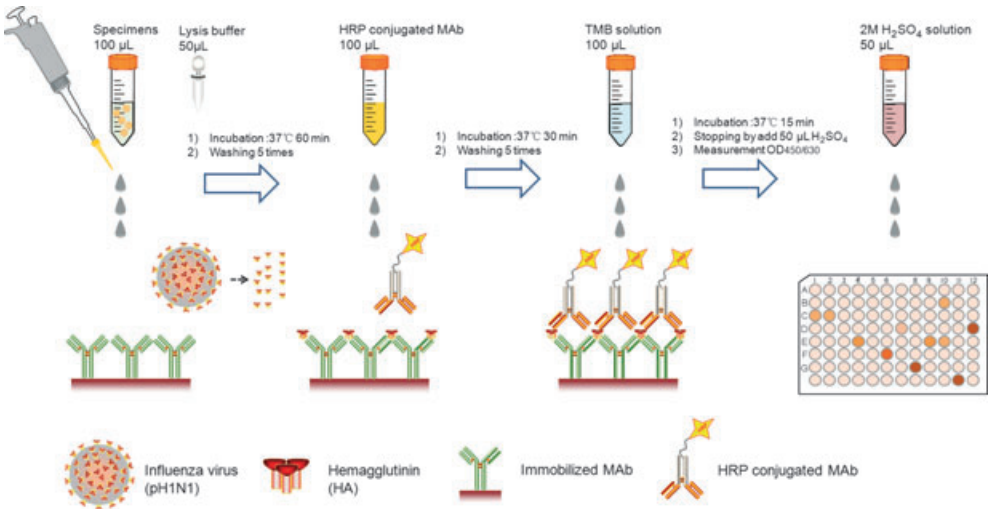

FIG. 1. A schematic diagram of the principle and manipulation of the double-sandwich ELISA for pandemic (H1N1) 2009 virus (pH1N1 ELISA). HA, haemagglutinin; HRP, horseradish peroxidase; mAb, monoclonal antibody; tetramethylbenzidine (TMB).

TABLE 2. Lower detection limits of pandemic (H1N1) 2009 ELISA on pandemic (H1N1) 2009 virus cultures

Virus[a]	Lower detection limit	
	$TCID_{50}$/mL (in log_{10})	HA titre[b]
A/Xiamen/N465/2009(H1N1)	2.9	0.008
A/Xiamen/N582/2009(H1N1)	2.8	0.008
A/Xiamen/N584/2009(H1N1)	2.9	0.004
A/Xi'an/A29/2009(H1N1)	3.4	0.004
A/Xi'an/A35/2009(H1N1)	2.4	0.016
A/Xi'an/A36/2009(H1N1)	3.6	0.008
A/HK/41 9521/2009(H1N1)	NA	0.008
A/CA/04/2009(H1N1)	NA	0.016
Average LDL(mean ± SD)	3.0 ± 0.4	0.009 ± 0.005

CA, California; HA, haemagglutinin; HK, Hong Kong; LDL, lower detection limit; NA, not applicable; SD, standard deviation; TCID, tissue culture infective dose.
[a]A/HK/41 9521/2009(H1N1) and A/CA/04/2009(H1N1) were inactivated viral cultures of allantoic fluids, and others were live viral cultures of MDCK cell supernatants.
[b]The HA titre is the reciprocal of the highest dilution of virus with complete haemagglutination.

TABLE 3. Tested influenza viral cultures

Influenza A	Tested HA titre	Subtype or strain (no.)	No. tested	No. positive
H1	16–1024	H1N9 (1)[a]; H1N1 (24)[b]	25	1
H2	256	A/DK/Shantou/992/2000(H2N8)	1	0
H3	16–1024	H3N3(2)[c]; H3N2(25)[d]; H3N8(1)[e]	28	0
H4	256	A/DK/Siberia/378/2001(H4N6)	1	0
H5	16–1024	H5N1(13)[f]	13	0
H6	256	A/TEAL/Hongkong/W312/1997(H6N1)	1	0
H7	256	A/DK/C/A47/1947(H7)	1	0
H8	256	H8N4(2)[g]	2	0
H9	256	A/Qa/Hongkong/G1/1997(H9N2)	1	0
H10	256	A/DK/Shantou/1796/2001(H10N4)	1	0
H11	256	H11N3(1)[h], H11N8(1)[i]	2	0
H12	256	A/DK/Hongkong/838/1980(H12N5)	1	0
H13	256	A/Gull/Maryland/704/1977(H13N5)	1	0

HA, haemagglutinin.
[a]H1N9: A/WDK/Shantou/520/2000.
[b]H1N1 viral strains included: A/DK/Shantou/1734/2003, A/Shantou/104/2005, A/Shantou/517/2005, A/NewCaledonia/20/1999, A/Xiamen/N66/2009, A/Xiamen/1172/2008, A/Xiamen/116/2006, A/Xiamen/3141/2006, A/Xiamen/149/2006, A/Xiamen/98/2006, A/Xiamen/3123/2006(H1N1), A/Xiamen/1168/2006, A/Xiamen/12/2006, A/Xiamen/N49/2009, A/Xiamen/1247/2008(H1N1), A/Xiamen/1169/2008, A/Xiamen/1175/2008, A/Xiamen/1170/2008, A/Xiamen/1355/2008(H1N1), A/Xiamen/1393/2008, A/Xiamen/1180/2008, A/Xiamen/1152/2008, A/Xiamen/3126/2006(H1N1), A/Xiamen/16/2006.
[c]H3N3 viral strains included: A/DK/Shantou/708/2000, A/DK/Shantou/1283/2001.
[d]H3N2 viral strains included: A/Shantou/602/2005/, A/Shantou/177/2005, A/SW/Hongkong/1311/2001, A/Shantou/798/2005, A/Shantou/820/2007, A/Yancheng/N101/2009, A/Xiamen/1394/2008, A/Xiamen/042/2007, A/Shantou/602/2005, A/Xiamen/025/2007, A/Xiamen/044/2007, A/Xiamen/067/2007, A/Xiamen/030/2007, A/Xiamen/040/2007, A/Xiamen/1124/2007, A/Xiamen/170/2007, A/Shantou/820/2007, A/Xiamen/1129/2007, A/Xiamen/012/2007, A/Xiamen/023/2007, A/Xiamen/074/2006, A/Xiamen/1380/2006(H3N2), A/Xiamen/1013/2006, A/Xiamen/014/2007, A/Xiamen/028/2007.
[e]H3N8: A/EQ/Jinlin/1989.
[f]H5N1 viral strains included: A/Ck/Hongkong/Yu22/2002, A/Qa/Gangxi/575/2005, A/DK/Vietnam/S654/2005, A/CK/Indonesia/2A/2004, A/Dk/Hunan/1265/2005, A/Shenzhen/406H/2006, A/CPHeron/Hongkong/18/2005, A/DK/Fujian/897/2005, A/MDk/Jiangxi/2295/2005, A/Vietnam/1194/2004, A/Ck/Shanxi/CV042/2006, A/CK/Vietnam/568/2005, A/Indonesia/542/2006.
[g]H8N4: A/TURKEY/Ontario/6118/1968, A/TURKEY/Ontario/6118/1968.
[h]H11N3: A/DK/Shantou/4253/2003.
[i]H11N8: A/DK/Shantou/834/2001.

where the first confirmed pH1N1 patient, a university student, was reported on 26 June 2009. After a 2-month summer vacation ending at the end of August, a pH1N1 outbreak in Xian occurred in a university, such that, by 4 September, over 50 students were hospitalized. As the pandemic progressed, 920 patients reported fever, cough or other symptoms of ILI for the period from 24 to 27 September at Xijing Hospital (Xian, China). Given these circumstances, permission to conduct a laboratory-based study was obtained from the institutional review board of Xijing Hospital. From this cohort, 904 (98.3%), patients were recruited into the study. NPS specimens from these patients were carefully collected by using sterile polyester, according to a standard method [8], and were transported to the microbiology laboratory in 1.5 mL of viral transport medium. The time of sampling was <48 h post-onset for each patient.

Influenza diagnostic tests

The specimens of ILI were first tested by CDC real-time RT-PCR (rRT-PCR). For the test, viral RNA was extracted from 200-μL specimens by use of the Biomek NX Laboratory Automation Workstation (Beckman Coulters, Brea, CA, USA), and was then determined by the CDC recommended protocol [9]. The subtypes of influenza A virus were determined by DNA sequencing and phylogenetic analysis. For the sequencing analysis, a 281-nucleotide fragment (nucleotides 84–370 in the NP gene of influenza A virus) was obtained by nested PCR. The first round of PCR was performed with an outer primer set of NPF1 (5′-AGC AAA AGC AGG GTA GAT AA-3′) and PyR533 (5′-AGT GTT GAA CCT TGC ATT AGA GAG-3′) for 30 cycles. The second round was performed with an inner primer set of PyF41 (5′-GAT CAT ATG AAC AAA TGG AGA CTG-3′) and PyR375 (5′-AAC TCT CCT TAT TTC TTC TTT GTC-3′). The PCR products were sequenced on an ABI Prism 3130X automatic genetic analyzer (Applied BioSystems, Foster City, CA, USA). The tree was constructed by the neighbour-joining method, with MEGA software, version 4.0. After these tests, all specimens were stored at −80°C. Two months later, all specimens were taken out and blindly tested by pH1N1 ELISA and BD Directigen EZ Flu A + B (RIDT; Becton, Shannon, County Clare, Ireland).

Statistical analysis

The sensitivity, specificity, positive predictive value and negative predictive value were determined with the CDC rRT-PCR result as the reference standard. The unadjusted chi-square test was used for categorical independent variables. Estimation of the 95% CI was performed with exact binomial methods. Calculations were conducted with SPSS statistical software, version 16.0 (SPSS, Chicago, IL, USA).

Results

The lower detection limit of pHINI ELISA for viral isolates

Seven pHINI viral isolates were two-fold serially diluted and tested by pHINI ELISA. The lower detection limit was determined as $10^{3.0 \pm 0.4}$ tissue culture infective dose $(TCID)_{50}$/mL (or 0.009 ± 0.005 HA titer) (Table 2).

Evaluating the cross-reactions of pHINI ELISA

Seventy-eight strains of influenza A virus (non-pHINI, H1–H13 subtypes), 20 strains of influenza B virus and 15 types of other common respiratory pathogen were tested by pHINI ELISA; all were negative except for one bird strain of influenza A/H1N9 (A/WDK/Shantou/520/2000), for which the viral titre was higher than 16 HA (approximately 1800-fold higher than the lower detection limit of the assay) (Tables 3 and 4).

TABLE 4. Tested non-influenza respiratory pathogens

Pathogen	Test dosage	No. tested	No. positive
Enterovirus	$10^{5.5–7.0}$ $TCID_{50}$/mL	21	0
Adenovirus	$10^{6.0}$ $TCID_{50}$/mL	1	0
Measles virus	$10^{6.0}$ $TCID_{50}$/mL	1	0
Parainfluenza virus	$10^{6.0}$ $TCID_{50}$/mL	1	0
Respiratory syncytial virus	$10^{6.0}$ $TCID_{50}$/mL	1	0
Coronavirus	$10^{5.0–6.0}$ $TCID_{50}$/mL	5	0
Bordetella pertussis	$\geq 10^7$ CFU/mL	1	0
Legionella pneumophila	$\geq 10^7$ CFU/mL	1	0
Streptococcus pneumoniae	$\geq 10^7$ CFU/mL	1	0
Candida albicans	$\geq 10^7$ CFU/mL	1	0
Mycobacterium tuberculosis	$\geq 10^7$ CFU/mL	1	0
Diphtheria bacillus	$\geq 10^7$ CFU/mL	1	0
Haemophilus influenzae	$\geq 10^7$ CFU/mL	1	0
Neisseria gonorrhoeae	$\geq 10^7$ CFU/mL	1	0
Mycoplasma pneumoniae	$\geq 10^7$ CCU/mL	1	0

CCU, colour changing units; $TCID_{50}$, tissue culture infective dose.

Reproducibility of pHINI ELISA

Intra-assay reproducibility was evaluated from 20 measurements of four pHINI-positive specimens. The mean OD values determined by pHINI ELISA of the specimens were 2.334 ($10^{4.4}$ $TCID_{50}$/mL), 0.996 ($10^{4.0}$ $TCID_{50}$/mL), 0.598 ($10^{3.8}$ $TCID_{50}$/mL) and 0.378 ($10^{3.6}$ $TCID_{50}$/mL), and the coefficients of variation were 4.0%, 4.3%, 4.5% and 4.1%, respectively. Inter-assay reproducibility was evaluated from 12 assays (six baths; two assays were randomly selected from each bath) with the same specimens, and the coefficients of variation were 5.8%, 4.5%, 5.3% and 2.9%, respectively.

Descriptions of clinical specimens

Among the 904 ILI patients, with ages ranging from 6 months to 86 years, CDC rRT-PCR and sequencing analysis (shown in Supporting information in Fig. S1) revealed that 91 (10.1%) patients were infected with pHINI virus, 171 (18.9%) patients were infected with seasonal influenza A virus, including five seasonal H1, 152 seasonal H3 and 14 untyped cases (determined by phylogenetic analysis and shown in Fig. S1), and the remaining 642 (71.0%) patients were not infected with influenza A virus. The demographic characteristics of pHINI patients and seasonal influenza A patients in this cohort are described in Table 5. In this period, seasonal influenza A virus infection (18.9%, 95% CI 16.4–21.6), rather than pHINI infection (10.1%, 95% CI 8.2–12.2) was predominant ($p < 0.01$) in ILI patients. The incidence of pHINI virus infection was higher in the age groups 6–10 years (9.7%, 95% CI 6.4–14.0, $p < 0.05$), 11–15 years

TABLE 5. Positive ratio of pandemic (HINI) 2009 (pHINI) ELISA based on age/sex group among influenza A patients

		2009 Pandemic HINI		Seasonal influenza A	
Variable	No. (%)	No. (%)	pHINI ELISA No. positive (%)	No. (%)	pHINI ELISA No. positive (%)
Total	904	91 (10.1)	84 (92.3)	171 (18.9)	0
Age (years)					
0–5	140 (15.5)	3 (2.1)	3 (100)	27 (19.3)	0
6–10	257 (28.4)	25 (9.7)[a]	23 (92.0)	66 (25.7)	0
11–15	259 (28.7)	53 (20.5)[b]	48 (90.6)	33 (12.7)	0
16–20	63 (7.0)	6 (9.5)[a]	6 (100)	9 (14.3)	0
21–30	96 (10.6)	3 (3.1)	3 (100)	19 (19.8)	0
31–40	38 (4.2)	1 (2.6)	1 (100)	6 (15.8)	0
>40	51 (5.6)	0 (0)	0	11 (21.6)	0
p-value	NA	<0.01	0.90	0.34	NA
Sex					
Female	408 (45.1)	39 (9.6)	34 (87.2)	84 (20.6)	0
Male	496 (58.2)	52 (10.5)	49 (94.2)	87 (17.5)	0
p-value	NA	0.73	0.49	0.28	NA

NA, no application.
Two non-influenza A cases (2/642) were positive in pHINI ELISA and were considered to be 'false-positive'. The two patients were both female; one was 8 years old and the other was 15 years old.
[a]$p < 0.05$, [b]$p < 0.01$.

(22.6%, 95% CI 15.7–21.9, p <0.01) and 16–20 years (9.5%, 95% CI 3.6–19.6, p <0.05), whereas the incidence of seasonal influenza A virus infection was consistent among all age groups (p 0.34).

Diagnostic performance of pHINI ELISA

All 904 specimens from patients with ILL were tested by pHINI ELISA and BD Directigen EZ Flu A + B. The OD value distribution by pHINI ELISA in 904 patients is shown in Fig. S2. Among 91 specimens of pHINI patients, pHINI ELISA yielded 84 positive results, and its sensitivity was 92.3% (95% CI 84.8–96.9). The sensitivity of the test was not statistically different among patients of different ages and sex (p 0.49) (Table 5), and was significantly higher than that of BD Directigen EZ Flu A + B (70.3%, 95% CI 59.8–79.5, p <0.01; Table 6). The signal/cut-off value of pHINI ELISA was negatively correlated with the cycle threshold value of CDC rRT-PCR (n = 91, R^2=0.789; Fig. 2). This showed that the signal/cut-off value of the assay correlated well with viral load. The specificities of pHINI ELISA were 100.0% (171/171, 95% CI 97.9–100.0%) and 99.8% (640/642, 95% CI 99.1–99.9) in seasonal influenza A patients and in non-pHINI ILI patients, similar (p >0.05) to that of BD Directigen EZ Flu A + B (99.8%, 95% CI 99.1–99.9) (Table 6).

For pHINI ELISA in this cohort with a pHINI prevalence of 10.1%, the negative predictive value was 97.7%, higher than that of BD Directigen EZ Flu A + B (89.5%, p <0.01), and the positive predictive value was 99.1%, similar to that of the latter (99.5%, p 0.19).

Discussion

The HA antigen of pHINI virus is genetically and serologically different from those of other annual seasonal influenza A viruses [7,10,11]. Hence, the use of immunoassays based on the pHINI virus cluster-specific antigenic determinants of HA could aid in the differentiation of pHINI virus infections from other infections. The concept has been demonstrated in this study and, to our knowledge, for the first time.

The sensitivity of the pHINI virus assay (92.3%, 95% CI 84.8–96.9) was significantly higher than that of BD Directigen EZ Flu A + B in nasopharyngeal specimens. The high sensitivity of the assay may be attributed to the high affinity of the mAbs used and/or enzyme-induced signal amplification. The assay also showed excellent specificity among seasonal influenza A patients (100%, 95% CI 96.4–100%) as well as among non-influenza A patients (99.7%, 95% CI 98.9–99.9%). Because of the low prevalence of seasonal H1 virus during the study period (0.6%, 95% CI 0.2–1.3%), only five seasonal H1-positive specimens were tested

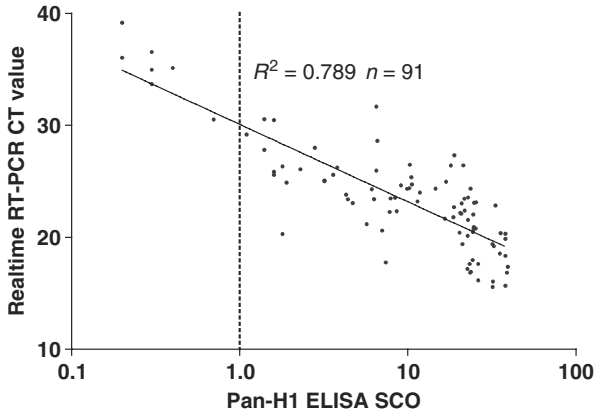

FIG. 2. Correlation between the signal/cut-off (S/CO) value of pandemic (HINI) 2009 ELISA and cycle threshold (C_T) value of the CDC real-time RT-PCR in 91 pandemic HINI patients. Broken lines indicate the lower detection of the Pan-H1 ELISA.

TABLE 6. Performance of different assays in patients with influenza like illness

	pHINI ELISA		BD Directigen EZ Flu A + B	
	Positive	Negative	Positive	Negative
Pandemic HINI (n = 91)[a]	84	7	64	27
Seasonal influenza A (n = 171)[a]	0	171	123	48
Non-influenza A (n = 642)[a]	2	640	1	641
Sensitivity 1 (95% CI)	92.3 (84.8–96.9)		70.3 (59.8–79.5)	
Sensitivity 2 (95% CI)	NA		71.9 (64.5–78.5)	
Specificity 1 (95% CI)	100 (96.4–100)		NA	
Specificity 2 (95% CI)	99.7 (98.9–99.9)		99.8 (99.1–99.9)	
Predicted value	For 2009 pandemic HINI		For total influenza A	
PPV, % (95% CI)	99.1 (98.2–99.7)		99.5 (97.1–99.9)	
NPV, % (95% CI)	97.7 (91.9–99.7)		89.5 (87.1–91.7)	

NA, not applicable; NPV, negative predictive value; pHINI, pandemic (HINI) 2009; PPV, positive predictive value. Sensitivity 1: sensitivity in pandemic HINI patients. Sensitivity 2: sensitivity in seasonal influenza A patients. Specificity 1: specificity in seasonal influenza A patients. Specificity 2: specificity in non-influenza A patients.
[a]Data are no. positive/no. negative.

(all were negative). However, 78 strains of influenza A viral cultures (including 23 strains of H1N1 of human origin) and 20 strains of influenza B viral cultures were tested, with negative results being obtained by the assay even at much higher titres (over 1000-fold higher than the lower detection limit of the assay). This strongly suggests the assay does not cross-react with other influenza viruses (Table 3). Interestingly, a strain of A/H1N9 (A/WDK/Shantou/520/2000) virus of bird origin tested positive at a titre of over 16 HA (approximately 1800-fold higher than the lower detection limit of the assay), and this may indicate a potential association between the former and pH1N1 virus.

Pandemic (H1N1) 2009 virus infection is responsible for serious lung damage [12], especially in pregnant women [13], young children and people with chronic diseases [14]. Regarding antiviral therapy, pH1N1 virus is sensitive to oseltamivir but seasonal influenza virus is usually resistant [15]. This argues for rapid diagnosis as part of the assessment of patients with ILI who present to the clinic. An accurate diagnosis will result in better medical care [16]. Accurate diagnosis of pH1N1 virus infection depends on several aspects: (i) quality of the specimen—nasopharyngeal specimens are more appropriate for detection (nasopharyngeal aspirate or NPS), especially for antigen testing [17,18]; (ii) the time when the specimen was collected in relation to the onset of symptoms—a previous study showed that the decrease in viral concentration was correlated with time elapsed from symptom onset [19]; and (iii) the sensitivity and specificity of the tests used—previous studies demonstrated that most of the current RIDTs had sensitivities ranging from 11% to 88% [2,20], although they were very convenient for one-step operation. Hence, specimens that are negative by RIDT should undergo further testing with more sensitive assays. RT-PCR is the most sensitive method for the diagnosis of pH1N1 virus infection. However, it is expensive and time-consuming. In an influenza pandemic, laboratories may have to process a large number of specimens in a short period (e.g. over 200 specimens per day in this study). In this study, even with the use of a high-throughput automation workstation (96 channels) for RNA extraction and PCR, detection of 200 specimens by RT-PCR would take a minimum of 6 h. Thus, a reliable ELISA for the detection pH1N1 virus infection will enhance the effectiveness of disease control in the following ways: (i) it has a shorter turn-around time; (ii) it has lower costs, making it practicable for developing countries; and (iii) it can be a suitable tool for surveillance in a large population.

Because of the overlapping of the dominant antigenic determinant regions with cell receptor binding sites in HA of influenza virus, the possibility of the occurrence of significant antigenic variation in HA increases with time. Hence, it is necessary to closely monitor the recognition abilities of the mAbs using in pandemic-specific HA assays, and update the mAbs when appropriate.

Although there is a significant level of herd immunity against pH1N1 virus and despite the fact that vaccine is now available [7,21–23], there is still a substantial at-risk population. On the other hand, pH1N1 virus is evolving, and there is a possibility of more virulent strains emerging. Hence, a sensitive and convenient assay, as presented, for the direct detection pH1N1 virus has potentially important public healthy applications. However, the performance of this assay should be investigated in more specimens from other geographical areas.

Acknowledgements

We gratefully acknowledge A. Yeo for editorial assistance in writing this article. We especially thank M. H. Ng (NIDVD, Xiamen) for critical suggestions with regard to this work.

Financial Support

This work was supported by grants from Key Special Subjects of Infectious Diseases (2008ZX10004-006), the Key Project of Science & Technology of Fujian Province (2008Y0059) and the National High Technology Research and Development Program (2010AA022801).

Transparency Declaration

All authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. A phylogenetic tree constructed on the partial nucleoprotein gene sequences of influenza A virus.

Figure S2. The distribution of reactivity(OD value) by pH1N1 ELISA in all 904 patients with ILI.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. World Health Organization. *Pandemic (h1n1) 2009—update 112*. Available at: //http://www.who.int/csr/don/2010_08_06/en/index.html (last accessed 6 August 2010).
2. Vasoo S, Stevens J, Singh K. Rapid antigen tests for diagnosis of pandemic (swine) influenza A/H1N1. *Clin Infect Dis* 2009; 49: 1090–1093.
3. Centers for Disease Control and Prevention. Performance of rapid influenza diagnostic tests during two school outbreaks of 2009 pandemic influenza A (H1N1) virus infection—Connecticut, 2009. *MMWR* 2009; 58: 1029–1032.
4. Uyeki T. Diagnostic testing for 2009 pandemic influenza A (H1N1) virus infection in hospitalized patients. *N Engl J Med* 2009; 361: e114.
5. Faix DJ, Sherman SS, Waterman SH. Rapid-test sensitivity for novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 2009; 361: 728–729.
6. Kyriakis CS, Olsen CW, Carman S *et al.* Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs, Europe. *Emerg Infect Dis* 2010; 16: 96–99.
7. Hancock K, Veguilla V, Lu X *et al.* Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med* 2009; 361: 1945–1952.
8. Baden LR, Drazen JM, Kritek PA, Curfman GD, Morrissey S, Campion EW. H1N1 influenza A disease—information for health professionals. *N Engl J Med* 2009; 360: 2666–2667.
9. World Health Organization. *CDC protocol of realtime RTPCR for swine influenza A (H1N1)*. Geneva. Available at: http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf (last accessed 28 April 2009).
10. Garten RJ, Davis CT, Russell CA *et al.* Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 2009; 325: 197–201.
11. Smith GJ, Vijaykrishna D, Bahl J *et al.* Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 2009; 459: 1122–1125.
12. Hui DS, Lee N, Chan PK. Clinical management of pandemic (H1N1) infection. *Chest* 2009; 137: 916–925.
13. Louie JK, Acosta M, Jamieson DJ, Honein MA. Severe 2009 H1N1 influenza in pregnant and postpartum women in California. *N Engl J Med* 2010; 362: 27–35.
14. Nicoll A, Coulombier D. Europe's initial experience with pandemic (H1N1) 2009—mitigation and delaying policies and practices. *Euro Surveill* 2009; 14: 19279.
15. Hall RJ, Peacey MP, Ralston JC *et al.* Pandemic influenza A(H1N1)v viruses currently circulating in New Zealand are sensitive to oseltamivir. *Euro Surveill* 2009; 14: 19282.
16. Bonner AB, Monroe KW, Talley LI, Klasner AE, Kimberlin DW. Impact of the rapid diagnosis of influenza on physician decision-making and patient management in the pediatric emergency department: results of a randomized, prospective, controlled trial. *Pediatrics* 2003; 112: 363–367.
17. Loens K, Van Heirstraeten L, Malhotra-Kumar S, Goossens H, Ieven M. Optimal sampling sites and methods for detection of pathogens possibly causing community-acquired lower respiratory tract infections. *J Clin Microbiol* 2009; 47: 21–31.
18. Agoritsas K, Mack K, Bonsu BK, Goodman D, Salamon D, Marcon MJ. Evaluation of the Quidel Quickvue test for detection of influenza A and B viruses in the pediatric emergency medicine setting by use of three specimen collection methods. *J Clin Microbiol* 2006; 44: 2638–2641.
19. Lee N, Chan PK, Hui DS *et al.* Viral loads and duration of viral shedding in adult patients hospitalized with influenza. *J Infect Dis* 2009; 200: 492–500.
20. Chen Y, Xu F, Gui X *et al.* A rapid test for the detection of influenza A virus including pandemic influenza A/H1N1 2009. *J Virol Methods* 2010; 167: 100–102.
21. Miller E, Hoschler K, Hardelid P, Stanford E, Andrews N, Zambon M. Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study. *Lancet* 2010; 375: 1100–1108.
22. Liang XF, Wang HQ, Wang JZ *et al.* Safety and immunogenicity of 2009 pandemic influenza A H1N1 vaccines in China: a multicentre, double-blind, randomised, placebo-controlled trial. *Lancet* 2010; 375: 56–66.
23. Zhu FC, Wang H, Fang HH *et al.* A novel influenza A (H1N1) vaccine in various age groups. *N Engl J Med* 2009; 361: 2414–2423.