HHS Public Access

Author manuscript

J Virol Methods. Author manuscript; available in PMC 2022 March 28.

Published in final edited form as:

J Virol Methods. 2017 May ; 243: 61–67. doi:10.1016/j.jviromet.2017.01.008.

Evaluation of multiplex assay platforms for detection of influenza hemagglutinin subtype specific antibody responses

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Abstract

Influenza hemagglutination inhibition (HI) and virus microneutralization assays (MN) are widely used for seroprevalence studies. However, these assays have limited field portability and are difficult to fully automate for high throughput laboratory testing. To address these issues, three multiplex influenza subtype-specific antibody detection assays were developed using recombinant hemagglutinin antigens in combination with Chembio, Luminex®, and ForteBio® platforms. Assay sensitivity, specificity, and subtype cross-reactivity were evaluated using a panel of well characterized human sera. Compared to the traditional HI, assay sensitivity ranged from 87% to 92% and assay specificity in sera collected from unexposed persons ranged from 65% to 100% across the platforms. High assay specificity (86–100%) for A(H5N1) rHA was achieved for sera from exposed or unexposed to hetorosubtype influenza HAs. In contrast, assay specificity for A(H1N1)pdm09 rHA using sera collected from A/Vietnam/1204/2004 (H5N1) vaccinees in 2008 was low (22–30%) in all platforms. Although cross-reactivity against rHA subtype proteins was observed in each assay platform, the correct subtype specific responses were identified 78% to 94% of the time when paired samples were available for analysis. These results show that high throughput and portable multiplex assays that incorporate rHA can be used to identify influenza subtype specific infections.

Keywords

influenza; hemagglutinin; antibody; Chembio; Luminex®; antibody biosensor assay

1. Introduction

Influenza serology tests such as the hemagglutination inhibition (HI) and virus microneutralization (MN) assays are used for retrospective diagnosis and assessment

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of immune status. Both tests detect antibodies that inhibit the interaction of influenza hemagglutinin (HA) with receptors on red blood cells or cultured cells. The HA glycoprotein, originally named for its capability to agglutinate erythrocytes (Hirst, 1941), is a homotrimer with each monomer consisting of an ectodomain, transmembrane domain, and cytoplasmic tail (Verhoeyen et al., 1980). The precursor form of HA, HA0, is expressed as a single chain that is cleaved by host proteases to generate the native form of the protein. Native HA is comprised of HA1 and HA2 chains linked by a disulfide bond (Steinhauer, 1999).

Antibodies to acute viral infections appear in the blood as early as 10–14 days after infection (Katz, Hancock, and Xu, 2011; Miller et al., 2008) and can be maintained for decades after infection (Crotty et al., 2003). The continual antigenic drift and occasional antigenic shift of influenza viruses creates complex antibody profiles in individuals over a life time of repeated exposure to multiple influenza viruses and vaccines, impeding the resolution and interpretation of influenza subtype specific immunity.

The HI and MN assays are the most reliable and commonly used tests for serologic surveys and seroepidemiologic investigations (Katz et al., 2011; Laurie et al., 2013). Serosurveys evaluate a population's immune status to emerging influenza viruses. This is an important public health surveillance tool which can be used to identify susceptible populations and asymptomatic infections to determine virus transmission rates, and provide more accurate estimates of disease severity (Broberg, Nicoll, and Amato-Gauci, 2011; Laurie et al., 2013), which in turn can aid the risk assessment of viruses for their pandemic or epidemic potential. Public health serosurveillance studies often involve the collection of thousands of specimens, highlight the need for high-throughput platforms.

Whereas the HI assay can be performed with inactivated viruses provided by reference laboratories (such as, via https://www.internationalreagentresource.org/), the MN assay requires infectious viruses. Virus propagation needed for both assays may require heightened biological safety levels for emerging influenza viruses and limit assay portability. The development of sensitive and specific serologic assays that do not use whole virus, but do use noninfectious recombinant proteins, offers the potential for improved assay standardization and inter-laboratory reproducibility. At the same time, this change would also make the serology assays more suitable for remote locations, biological safety level 2 laboratories, and high-throughput applications.

In their current form, the HI and MN require technical expertise, limiting their use to experienced laboratories with access to virus. Reagent preparation, liquid handling, and data collection are time consuming components of testing. Alternative serologic assays, such as enzyme-linked immunosorbent assay (ELISA), have been attempted to improve the functionality of serologic assays. Nonetheless, for influenza A virus, ELISA has lacked subtype specificity (Burlington et al., 1985) and multiplexing capabilities (Watson et al., 2009). The multiplex protein microarray was developed to investigate antibody profiling by Koopmans and colleagues, the bivariate model of microarray using recombinant hemagglutinins (rHAs) from A(H1N1)pdm09 and A/1918(H1N1) influenza viruses was used to achieve high sensitivity and specificity (Boni et al., 2013; Freidl et al., 2014;

Freidl et al., 2016; Huijskens et al., 2013; te Beest et al., 2014). More recently, Wang et al developed mPLEX-Flu assay in which MagPix platform using full length HAs from multiple strains of influenza A and B viruses, investigated antibody profile and antigenic profile of HA (Wang et al., 2015). Our previous study indicated that assay sensitivity and specificity for one novel subtype HA maybe lower in persons exposed to other novel subtype HA than those unexposed to any novel subtype HA (Li et al., 2014) Despite these recent advances, assay sensitivity and specificity, using a panel of well-characterized sera from recently exposed and unexposed persons, has not been fully investigated in this context.

The 2009 A(H1N1) pandemic and A(H7N9) outbreaks highlight the public health need for rapid and reliable assays to detect influenza subtype-specific antibody responses (Boni et al., 2013; Broberg et al., 2011; Laurie et al., 2013). New technologies and assays are urgently needed to improve assay speed, portability, throughput, sensitivity, specificity, and reproducibility. To address these issues, we evaluated both rapid lateral flow and high throughput laboratory platforms in combination with a set of rHAs and a panel of well-characterized human serum specimens collected from persons exposed or unexposed to specific subtype influenza antigens. Results from these studies demonstrate the utility and caveats of using these platforms to assess influenza subtype-specific antibody responses following influenza virus infection, potentially when novel subtype influenza virus emerges.

2. Material and Methods

2.1. Human sera.

To evaluate test platforms, 21 paired acute (S1, 1–7 days post symptom onset) and convalescent (S2, 15–52 days post symptom onset) sera were collected from A(H1N1)pdm09 (pH1) virus-infected persons during the first wave of the 2009 A(H1N1) pandemic (April to July, 2009) (Table 1). All patients showed seroconversion (a 4-fold or greater rise in antibody titer in convalescent compared with acute phase sera with convalescent sera titers ≥ 40) by MN and/or by HI (Table 1). The collection of these sera was a part of the CDC U.S. Public Health Emergency Response to the pandemic that did not require CDC Institutional Review Board (IRB) review. In addition, 15 paired pre- (S1, day 0) and post-vaccination (S2, day 28 or 56) sera were collected from U.S. residents enrolled in 2009 who received one dose of 15 μg pH1 monovalent, non-adjuvanted, split vaccine and 23 U.S. residents who received 2 doses of 90 μg A/Vietnam/1203/2004 (H5N1) (H5) monovalent, non-adjuvanted, split vaccine in 2008 (Table 1). S1 sera represent either pre-vaccination or acute sera, S2 sera represent either post-vaccination or convalescent sera (Table 1 and Table 2). Subjects that received pH1 or H5 vaccines showed a 4-fold or greater rise in either turkey red blood cell (RBC) or horse RBC HI titer, respectively (Table 1). IRB approval was granted for the collection of H5N1 vaccination specimens by the Centers for Disease Control and Prevention (CDC; ClinicalTrials.gov identifier: NCT00417560) and for the pH1 vaccination sera by Emory University (IRB# 22371). Forty paired sera were collected from U.S. residents who received 2009–10 trivalent Northern Hemisphere inactivated vaccines and showed serum conversion to either A/Brisbane/59/2007 (H1N1) (sH1) and/or A/Brisbane/10/2007 (H3N2) (H3) (Table 1, data not shown). All serum panels were collected from healthy individuals aged 1–79 years of age (Table 1). The

serum samples used in determination of sensitivity, specificity for unexposed or exposed to heterosubtypic influenza antigen were described in Table 2.

2.2. Chembio Dual Path Platform.

The Chembio Platform (Chembio Diagnostic Systems, NY) was used to construct a portable rapid influenza antibody test containing the full length ectodomain recombinant HAs with histidine tag from A/California/4/2009 (H1N1pdm09, pH1), cat# FR-180; A/Brisbane/59/2007 (H1N1, sH1), cat# FR-65; A/Brisbane/10/2007 (H3N2, H3), cat# FR-61; A/Vietnam/1203/2004 (H5N1, H5), cat# FR-39; A/shorebird/DE/68/2004 (H13N9, H13), cat# FR-73 were obtained through the Influenza Reagent Resource (CDC Influenza Reagent Resource, Atlanta, Georgia). Positive and negative control antigens included a recombinant protein A and H13 rHA, respectively. Each rHA and the protein A were sprayed onto a nitrocellulose strip mounted in a 95 mm × 50 mm plastic cassette (Fig. 1). The test utilized a filter pad on the vertical sample path to remove red blood cells when testing whole blood and a pad impregnated with dried colloidal gold conjugated protein A on the lateral buffer path to detect antibody bound to immobilized rHA antigens (Fig. 1). To perform the assay, 10 μl of serum or plasma was added to 250 μl of sample diluent and 55 μl of the diluted sample was then added to the sample port. After incubation at room temperature for 5 minutes, five drops of running buffer were added to the buffer port (Fig. 1). The colloidal gold-protein A/anti HA antibody/rHA or colloidal gold-protein A/human IgG/protein A was formed for testing antigen or protein A control, respectively. The test was read after 15 minutes of incubation at room temperature using a Chembio Rapid Influenza Immunity Test Reader. The threshold values were determined to achieve optimal sensitivity for post-vaccination or convalescent serum samples (S2) and specificity for serum samples collected from unexposed (S1) or exposed (S2) to heterosubtype influenza for all three platforms in this study (Table 2 and Table 3). The threshold values for determining a positive result were established to achieve the combination of at least 87% (20/23) sensitivity for post-vaccination or convalescent serum samples (S2) and at least 65% (15/23) specificity for S1 sera from unexposed persons in Chembio DPP kits (Table 3).

2.3. Antibody biosensor assay.

The biolayer interferometry technology platform from ForteBio®, Inc (Menlo Park, CA) was used to develop a laboratory assay for detection of influenza HA antibody. An Octet Red instrument was used for all measurements. Biosensors were prepared by complexing chemically biotinylated rHAs (H13, H5, pH1, sH1, and H3) to streptavidin coated biosensors following the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). All steps were performed at 30°C. Briefly, the biosensor tips were pre-wet with sample diluent and washing buffer to establish a test baseline. Biosensors were then read by a dip and read method: Biosensors were dipped in serum sample for 120 seconds, washing buffer for 30 seconds, and sample diluent for 30 seconds, and colloidal gold protein A for 60 seconds. Binding rates, measured in nm/s, for the last step were analyzed by ForteBio® version 6.5 software. The threshold values for determining a positive result were established to achieve the combination of at least 87% sensitivity for post-vaccination or convalescent serum samples (S2) and at least 65% specificity for S1 sera from unexposed persons (Table 3).

2.4. Multiplexed fluorescence microsphere immunoassay.

Six distinct LumAvidin® avidin-coupled polystyrene microspheres (Luminex®, Austin, Texas) were conjugated to 5 trimeric rHAs (H13, H5, pH1, sH1, and H3) obtained from International Reagent Resource and a protein A control. The rHA and protein A were biotinylated using a Sulfo-NHS-LC biotinylation kit (Pierce Biotechnology, Rockford IL) at a molar coupling ratio of 1:5 (protein:Sulfo-NHS-LC-biotin) and then bound to the LumAvidin microspheres at a concentration sufficient to saturate all of the available avidin binding sites (>6.70×10^5 biotin binding sites per microsphere). One hundred microliters of assay buffer (1× phosphate buffer saline (PBS) containing 1% (w/v) bovine serum albumin (BSA), 0.05% (v/v) Tween-20, 0.5 M NaCl, 0.05% (w/v) sodium azide) was added to the appropriate wells of the 1.2 μm 96 well filter plate (Millipore, Billerica, MA) to pre-wet the membrane and then aspirated from each well using a vacuum manifold apparatus. Fifty microliters of microspheres containing two thousand microspheres from each of the six regions were added to each well of the filter plate (12,000 microspheres/well total). Fifty microliters of the appropriate diluted serum samples were then transferred into corresponding wells of the 96-well filter plate in duplicate and incubated in the dark at room temperature for 20 minutes on an orbital shaker at 600 rpm (VWR, Radnor, PA). The filter plate was washed with 100 μl of assay buffer two times followed by a 20 minute incubation with 100 μl of protein A-phycoerythrin conjugate (protein A-RPE) on an orbital shaker at 600 rpm (VWR, Radnor, PA) in the dark. The plate was washed three times with 100 μl of read buffer (1× PBS with 0.05% (v/v) Tween-20, 1% (w/v) BSA, and 0.05% (w/v) sodium azide) followed by the incubation in read buffer at room temperature for 3 minutes on an orbital shaker at 600 rpm to suspend the beads. The plate was read in the Luminex® 200 system, the median fluorescence intensity (MFI) for 100 microspheres for each of the six regions was recorded at each dilution point and results were analyzed with xPONENT 3.0 software. The threshold values for determining a positive result were established to achieve the combination of at least 87% sensitivity for post-vaccination or convalescent (S2) serum samples and at least 65% specificity for S1 sera from unexposed persons (Table 3).

2.5. Statistics.

Statistical analyses were performed using GraphPad Prism 5 software. Fisher exact tests were used to compare statistical differences, p values of less than 0.05 were considered statistically significant.

3. Results

A total of 99 paired (total 198) human serum samples were tested in this study, appropriate serum numbers were used to assess the sensitivity and specificity of Chembio, Luminex®, and ForteBio® assays (Table 1 and Table 2) for reactivity against H5, pH1, and H13 HA antigens. The reactivity against sH1 and H3 antigens were not further analyzed here. The sensitivities of different assay platforms ranged from 87% to 92% and the specificities in sera collected from persons unexposed to heterosubtype HAs under evaluation ranged between 65% and 100% (p>0.05, Table 3).

The specificities in sera collected from persons exposed to other subtype influenza were also evaluated. S2 sera collected from H5N1 vaccine study held in 2008 showed only 22–30% specificity to pH1 rHA in all three platforms, that was significantly lower than that in unexposed persons ($p<0.05$, Table 3). Interestingly, S2 sera collected from persons infected with pH1- infected, or vaccinated with pH1 or the 2009–10 Northern Hemisphere trivalent (TIV) showed similar specificities for H5 rHA ($p>0.05$, Table 3). Overall, the performance of each platform was similar ($p>0.05$, Table 3), only difference between assay platform was observed for H5 specificity in exposed persons between Chembio (99%) /Luminex (100%) and ForteBio (86%) ($p<0.05$, Table 3).

These results demonstrate that each of the platforms can detect cross-reactive antibody responses to other subtype HAs and highlight the difficulty of assaying influenza subtype specific antibody responses in the presence of pre-existing or subtype cross reactive antibodies induced by previous exposure to different homologous and heterologous subtypes of influenza.

Because of the diverse and complex exposure history to influenza virus(es) and/or vaccine(s) in most individuals, the use of well-timed pre- and post-exposure paired sera is essential for the accurate detection of antibody responses indicative of recent infection or vaccination; a post-exposure serum (S2) HI or MN titer that is four fold higher than the baseline (S1) serum HI or MN titer indicates that a vaccine has induced a positive antibody response (Katz et al., 2011). In the context of our assay, we observed significant S2/S1 fold rises for multiple test antigens (Table 5 and data not shown). We further analyzed the relationship between S2/S1 fold rise values and assay sensitivity for the Chembio and Luminex® platforms using paired human serum samples from each serum source (Table 1). The fold rise threshold values for the ForteBio® platform were not calculated because the binding rates produced in this assay were not easily converted to fold rise data. To achieve reliable fold rise results, any result from a pre-vaccination or acute sample that was below 15% of the assay threshold value in Table 3, was arbitrarily adjusted to 15% of the threshold values. When testing paired antisera and setting the threshold for a positive result at $S2/S1 \geq 2$ in this study, the assay sensitivity to all rHA ranged from 72% to 96% for both Chembio and Luminex® platforms. In contrast, when a fold rise threshold of ≥ 3 or ≥ 4 was used, lower test sensitivity and higher specificity were achieved (Table 4 and data not shown). Altogether, these data indicate that a S2/S1fold rise cut off of 2 yields acceptable test sensitivity when paired antisera specimens are available for study.

Seven or 5 paired sera showed more than 2 fold rise against more than one rHA including heterologous novel subtype HA in DPP or Luminex, respectively (Table 5 and data not shown). We next evaluated the sensitivity of assays using the highest fold rise thresholds for paired sera from H5 vaccinated persons and pH1 infected or vaccinated persons. As shown in Table 3, some subtype cross-reactivity was detected in S2 sera, particularly for H5 vaccine sera and this was also observed in 7 (in DPP) or 5 (in Luminex) out of 59 paired serum specimens when the highest fold rise thresholds were used to interpret results (Table 5). Eighteen out of 23 serum samples from H5 vaccine recipients elicited the highest fold rise value for H5 rHA in the Chembio assay (78%) and 21 out of 23 in the Luminex® assay (91%) ($p>0.05$, Table 5). Similarly, 34 out of 36 and 33 out of 36 serum samples

from pH1 infected persons and vaccine recipients showed the highest fold rise to pH1 rHA in Chembio (94%) and in Luminex® (92%) assays, respectively ($p>0.05$, Table 5). We found that the test antigen that yielded the highest S2/S1 fold rise value likely indicated the correct HA subtype specific antibody response when the signal fold rises in multiple rHAs were observed. These results imply that use of paired sera can improve the accuracy of the Chembio and Luminex® assays when considering the highest fold rise result.

4. Discussion

Here we evaluated one portable lateral flow and two high-throughput platforms to detect influenza HA subtype specific antibody responses using purified rHA proteins. For all platforms, we measured the performance times, ease of use, and reagent stability for up to 1 year (data not shown). The sensitivity for these assays ranged from 87% to 92%, the assay specificity ranged from 65% to 100% for unexposed individuals and 22% to 100% for exposed individuals (Table 3). We did not observe significant differences in test sensitivity and specificity among the three test platforms except Fortebio which showed lower H5 specificity in exposed persons compared to Chembio or Luminex ($p<0.05$, Table 3). However, qualitative differences between the different test platforms make them more or less suitable for seroepidemiologic investigations or serosurveys.

The advantages of the Chembio portable lateral flow platform include its ease of use, time to result, and portability. The DPP kit was designed to provide results in a non-laboratory setting in less than 25 minutes with blood obtained from a single finger prick. This was not further investigated in the study. The ForteBio® laboratory-based platform analyzed 96 samples for five rHAs and the positive control in approximately four hours. The ForteBio® Octet RED96 has an eight-well simultaneous read-out, so that up to eight individual specimens can be tested against each rHA in parallel. Another benefit of the ForteBio® device is that it is capable of measuring antibody and antigen association and dissociation kinetics in addition to estimation of endpoint values, although this function was not evaluated in this study. The newer Octet QK384 platform could improve the assay throughput, though we did not evaluate the newer equipment in this study.

The Luminex® platform is a versatile and cost-effective technology platform for development of multiple analyte profiles (Binnicker, Jespersen, and Rollins, 2011; Martins, 2002; Watson et al., 2009). This platform has been used to investigate the antibody responses to many viral, bacterial, and parasitic infections (Binnicker et al., 2011; Firnhaber et al., 2011; Lammie et al., 2012; Martins et al., 2002; Pickering et al., 2002). Watson et al used the Luminex® platform to detect antibody responses to avian influenza A virus NP, M1, and NS1 proteins for avian influenza surveillance in poultry (Watson et al., 2009). Antibody profile following 2012 seasonal trivalent influenza vaccination was investigated by mPLEX-Flu assay (Luminex®) (Wang et al., 2015). In our hands, the Luminex® prototype offered significant benefits for laboratory-based high throughput serologic testing. The Luminex® assay analyzed 96 samples against five rHAs and the positive control in approximately two hours. The Luminex® required fewer reagents, had high accuracy, high reproducibility, less aliquot errors, limited staff time, required small sample volumes, continuous reading, and wide linear range (Binnicker et al., 2011; Martins, 2002; Wang

et al., 2015). Another advantage of the Luminex® technology is the ability to perform analysis of up to 100 different bead regions in a single well. Therefore, it is feasible for the Luminex® platform to include all known influenza HA subtypes in a single test, allowing for future expansion of this assay to rapidly detect antibody responses to emerging influenza A viruses. These attributes are important for influenza outbreak investigations in which a potentially large number of serological samples must be tested and analyzed quickly (Koopmans et al., 2012).

Previous influenza infection or vaccination generates a complex influenza HA-specific antibody profile in humans that varies greatly among individuals (Hancock et al., 2009). Therefore, demonstration of a rising titer in paired S1 and S2 serum specimens is necessary to determine if an individual has recently been infected with a circulating influenza virus or has mounted a response to influenza vaccine. For novel influenza virus subtypes, a single convalescent phase serum specimen may be sufficient to determine a recent exposure to influenza, if there is a documented low level of age-specific, subtype cross-reactive antibody present in the population (Veguilla et al., 2011). Nevertheless, the ability to reliably identify influenza subtype specific responses, even when some level of subtype cross-reactive antibody exists in the population, remains a highly desirable feature of next generation influenza serologic tests. As such, the use of paired sera will always improve the specificity of serologic testing, including novel HA subtypes.

When the assay specificity was analyzed in sera collected from individuals with known exposure to heterosubtype influenza, we observed a lower specificity (22–30%) for pH1 in samples collected from H5-vaccinated persons in 2008 compared to unexposed persons (65–74%) in all three platforms ($p<0.05$, Table 3). Interestingly, pH1N1 virus infection or vaccination, or receipt of 2009–10 northern hemisphere TIV, did not result in a significant lower specificity to H5 rHA in Chembio (99%) and Luminex (100%) platforms ($p>0.05$, Table 3), though lower specificity to H5 was observed in Fortebio (86%) ($p<0.05$, Table 3). The signal fold rises in multiple rHAs were observed in Chembio DPP and Luminex assays (Table 5 and data not shown), fortunately, the highest fold rises are related to correct subtype exposures in most cases (Table 5). This phenomenon is not readily observed when testing antisera with the HI assay. Whereas the HI assay measures the presence of antibodies that inhibit HA binding to sialic acid moieties, prototype platforms evaluated herein measure total antibodies bound to all exposed epitopes of HA proteins. Similar results have been observed in previous study, children with primary H1N1 or H3N2 influenza virus infections developed antibodies to H8 HA by ELISA, but not by HI (Burlington et al., 1985). In the 18 HA subtypes identified so far (Shaw and Palese, 2013; Tong et al., 2012; Tong et al., 2013), the HA1 domain contains more subtype and strain specific epitopes than the HA2 domain (Krystal et al., 1982). Thus, antibody binding to the HA2 domain in our assays may lead to greater subtype cross-reactivity than observed in a typical HI assay. The reduced subtype cross-reactivity was observed in ELISA and protein microarray when globular head domain rHA was used (Boni et al., 2013; Freidl et al., 2014; Huijskens et al., 2013; Li et al., 2014; te Beest et al., 2014), therefore, Chembio and Luminex® platforms using globular head domain rHA are currently under development. To overcome the interference of cross-reactive antibodies detected in these binding antibody assays (Table 3, Table5 and data not shown) (Burlington et al., 1985; Freidl et al., 2016; Li et al., 2014; Wang et al.,

2015), we are investigating the serum adsorption with seasonal rHA to remove cross-reactive antibodies in these platforms.

In summary, we have evaluated three cell-free, virus-free portable lateral flow or high-throughput assays to detect influenza HA subtype specific antibody responses in human sera. This study demonstrated a “proof of concept” phase for both screening and diagnostic test development. The Chembio assay may be useful for identifying specimens in the field that warrant more detailed analysis in the laboratory. The ForteBio® and Luminex® platform offer multiplexed high throughput systems that can simultaneously detect antibodies against multiple subtypes under identical assay conditions. The multiplex platforms will provide critical information when analyzing antibody responses in serum samples collected from the areas where several subtype influenza viruses including novel subtype(s) co-circulate. Additional strategies, such as the use of recombinant globular head domain HA as antigen or depletion of subtype cross-reactive antibodies by serum adsorption, may improve assay subtype specificity for novel subtypes, particularly when paired serum specimens are not available. In their current form, these multiplex assays will be useful tools for pre-screening specimens, prior to triage testing with more conventional subtype-specific methods such as HI and MN assays.

Acknowledgments

This work was supported by BARDA under contract between CDC and Battelle Memorial Institute. Recombinant HAs with Histidine Tag were obtained through the International Reagent Resource (https://www.internationalreagentresource.org/). We thank Heather Tatum, Leilani Thomas, and Peter Browning for managing the serum samples.

Trade names are only used for identification and they are not endorsed by the Public Health Service or by the U.S. Department of Health and Human Services. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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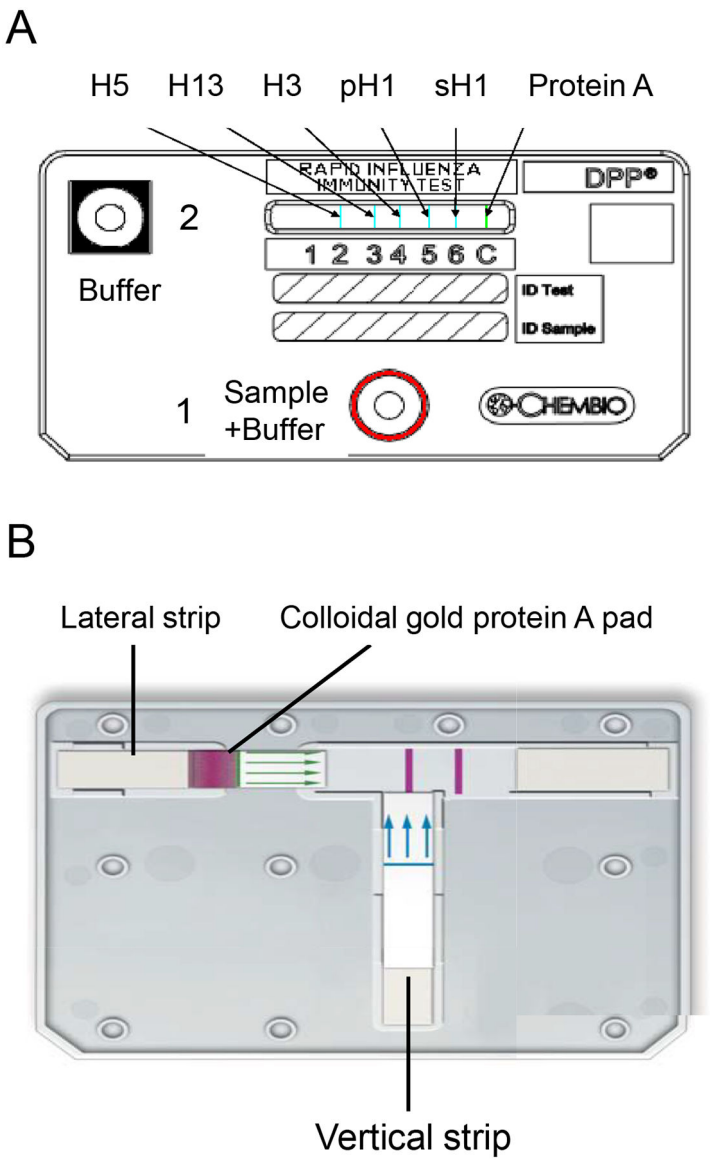

Fig. 1.
Design of the Chembio Dual Path Platform cassette. A. External View. B. Internal View.

Table 1.

Human serum panels used in this study

Serum source	Age range (yrs)	S1[a]			S2[b]		
		n	HI GMT (95% CI)[g]	MN GMT (95% CI)[g]	n	HI GMT (95% CI)[g]	MN GMT (95% CI)[g]
H5N1 vaccinees[c]	18–65	23	6 (5–7)	12 (10–14)	23	118 (88–159)	122 (86–173)
A(H1N1)pdm09 infected persons[d]	4–79	21	7 (5–9)	NT[h]	21	91 (67–124)	NT
pH1N1 vaccinees[e]	18–65	15	13 (8–21)	NT	15	176 (111–277)	NT
TIV vaccinees[f]	1–3, 22–83	40	NS[i]	NS	40	NS	NS
Total number of specimens		99			99		

[a] Pre-vaccination sera (S1) from vaccine studies or acute sera (S1) from A(H1N1)pdm09 virus infected persons were collected from US residents.

[b] Post-vaccination sera (S2) or Convalescent (S2) showed HI ≥ 40, MN ≥ 80, or 4-fold or greater rise in antibody titer, all sera collected from US residents

[c] Sera were collected from split A/Vietnam/1203/2004 (H5N1) vaccine study

[d] Sera were collected from pH1N1 infection persons

[e] Sera were collected from split A/California/07/09 (pH1N1) vaccine study

[f] Sera were collected from vaccinees received trivalent 2009–10 Northern Hemisphere inactivated vaccines, 30 and 40 serum samples showed serum conversion to A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 (H3N2) in HI and MN, respectively.

[g] HI or MN geometrical mean titers against exposed influenza virus strains and 95% confidential interval were shown

[h] NT: not done

[h] NS: not shown

Table 2.

Serum panels used to determine the sensitivity and specificity for either unexposed or exposed to heterologous subtype influenza viruses

rHA	S2 sera for sensitivity[a]				S1 sera for specificity (unexposed persons)[b]				S2 sera for specificity (exposed persons)[c]			
	Total	Serum resources			Total	Serum resources			Total	Serum resource(s		
		H5N1	pH1N1 cases	pH1N1 vaccine		H5N1	pH1N1 vaccine	TIV		H5N1	pH1N1 cases	pH1N1 vaccine	TIV
H5	23	23	N/A	N/A	78[d]	23	15	40	76[e]	N/A	21	15	40
pH1	36	N/A[f]	21	15	23[g]	23	N/A	N/A	23[h]	23	N/A	N/A	N/A
H13	N/A	N/A	N/A	N/A	78[i]	23	15	40	99[j]	23	21	15	40

[a] The sensitivity was determined by using convalescent or post-vaccination sera (S2) listed in Table 1 that showed HI ≥ 40, MN ≥ 80, or 4-fold or greater rise in antibody titer

[b] The specificity was determined by using pre-vaccination sera (S1) from persons unexposed to heterologous subtype influenza viruses

[c] The specificity was determined by using S2 sera listed in Table 1 that showed HI ≥ 40, MN ≥ 80, or 4-fold or greater rise in antibody titer to heterologous subtype influenza viruses

[d] S1 sera (pre-vaccination) collected from persons that would have received pH1N1, TIV, or A/Vietnam/1203/2004 (H5N1) split vaccine (unexposed to A/Vietnam/1203/2004 (H5N1))

[e] S2 sera (convalescent and post-vaccination) collected from persons vaccinated with pH1N1 and TIV split vaccine (unexposed to A/Vietnam/1203/2004 (H5N1))

[f] N/A: not applicable

[g] S1 sera (pre-vaccination) collected from persons who received A/Vietnam/1203/2004 (H5N1) split vaccine in 2008 (unexposed to A(H1N1)pdm09); pH1N1 and TIV vaccine studies were performed after A(H1N1)pdm09 pandemic

[h] S2 sera (post-vaccination) collected from persons who received A/Vietnam/1203/2004 (H5N1) split vaccine in 2008 (unexposed to A(H1N1)pdm09)

[i] S1 sera (pre-vaccination) collected from persons who received A/Vietnam/1203/2004 (H5N1), pH1N1, or TIV split vaccine (unexposed to A/shorebird/DE/68/2004 (H13N9))

[j] S2 sera (convalescent and post-vaccination) collected from persons vaccinated with H5N1, pH1N1, and TIV split vaccine or pH1N1 virus (unexposed to A/shorebird/DE/68/2004 (H13N9))

Table 3.

Test sensitivity and specificity for three recombinant influenza hemagglutinin antigens using three testing platforms

rHA	Chembio[a]			Luminex[b]			ForteBio[c]		
	Sensitivity[d]	Specificity[e]		Sensitivity	Specificity		Sensitivity	Specificity	
		Sera from unexposed persons (S1)	Sera from persons exposed to heterologous subtype (S2)		Sera from unexposed persons (S1)	Sera from persons exposed to heterologous subtype (S2)		Sera from unexposed persons (S1)	Sera from persons exposed to heterologous subtype (S2)
H5	87% (20/23)	95% (74/78)	**99% (75/76)** [f]	87% (20/23)	100% (78/78)[g]	100% (76/76)	91% (21/23)	94% (73/78)[g]	**86% (65/76)** [f]
pH1	89% (32/36)	**65% (15/23)** [h]	**30% (7/23)** [h]	89% (32/36)	**74% (17/23)** [i]	**30% (7/23)** [i]	92% (33/36)	**65% (15/23)** [j]	**22% (5/23)** [j]
H13	N/A[k]	99% (77/78)	93% (92/99)	N/A	100% (78/78)	93% (92/99)	N/A	99% (77/78)	98% (97/99)

[a] Chembio cut-off values, H5: 165, pH1: 155, H13: 105

[b] Luminex cut-off values, H5: 2300, pH1: 2650, H13: 1130

[c] ForteBio cut-off values, H5: 0.4, pH1: 0.4, H13: 0.4

[d] The sensitivity were determined by using convalescent (S2) or post-vaccination sera (S2) listed in Table 1 that showed HI ≥ 40, MN ≥ 80, or 4-fold or greater rise in antibody titer

[e] The specificity were determined by using either sera from unexposed persons (S1) or sera (S2) from convalescent or post-vaccination sera listed in Table 1 that showed HI ≥ 40, MN ≥ 80, or 4-fold or greater rise in antibody titer to heterologous subtype influenza viruses

[f] $p<0.05$

[g] $p>0.05$

[h-j] $p<0.05$

[k] N/A: not applicable

The percentages that showed significant difference were highlighted

Table 4.

When testing paired serum samples, the assay sensitivity is dependent on fold rise threshold[a]

Fold rise threshold	Chembio		Luminex	
	H5[b]	pH1[c]	H5	pH1
2	96% (22/23)[d]	86% (31/36)[e]	87% (20/23)[d]	72% (26/36)[e]
3	87% (20/23)[f]	56% (20/36)[g]	65% (15/23)[f]	53% (19/36)[g]
4	57% (13/23)[h]	50% (18/36)[i]	57% (13/23)[h]	28% (10/36)[i]

[a]To achieve reliable fold rise values, all low S1 value s were set to 15% of the cut-off value in Table 3.

[b]The sera collected from persons who were vaccinated with split A/Vietnam/1203/2004 (H5N1) vaccine were described in Table 1.

[c]The sera were collected from pH1N1 infected and split A/California/07/09 (pH1N1) vaccinated persons were described in Table 1.

[d-i]$p>0.05$

Table 5.

When using paired serum specimens, greater assay sensitivity was achieved by choosing the result with the highest fold rise[a]

Positive sera	Chembio			Luminex		
	H13	H5	pH1	H13	H5	pH1
H5	17% (4/23)	**78% (18/23)** [b]	4% (1/23)	9% (2/23)	**91% (21/23)** [b]	0% (0/23)
pH1	0% (0/36)	6% (2/36)	**94% (34/36)** [c]	3% (1/36)	6% (2/36)	**92% (33/36)** [c]

[a]To achieve reliable fold rise values, all low S1 value s were set to 15% of the cut-off value in Table 3.

b-c $p>0.05$

The highest fold rise to exposed antigen is shown in bold numbers