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An optimized enzyme-linked lectin assay to measure influenza A virus neuraminidase inhibition antibody titers in human sera

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A B S T R A C T

Antibodies to neuraminidase (NA), the second most abundant surface protein on influenza virus, contribute toward protection against influenza. The traditional thiobarbituric acid (TBA) method to quantify NA inhibiting antibodies is cumbersome and not suitable for routine serology. An enzyme-linked lectin assay (ELLA) described by Lambre et al. (1990) is a practical alternative method for measuring NA inhibition (NI) titers. This report describes optimization of the ELLA for measuring NI titers in human sera against influenza A viruses, using H6N1 and H6N2 viruses as antigens. The optimized ELLA is subtype-specific and reproducible. While the titers measured by ELLA are somewhat greater than those measured by a miniaturized TBA method, seroconversion rates are the same, suggesting similarity in assay sensitivity under these optimized conditions. The ELLA described in this report provides a practical format for routine evaluation of human antibody responses to NA.

1. Introduction

Neuraminidase (NA) inhibiting antibodies are associated with protection against influenza (Murphy et al., 1972) and correlate with reduced viral shedding and disease symptoms in a human challenge study (Clements et al., 1986). These antibodies contribute to immunity by inhibiting release and spread of newly formed virus particles from infected cells (Compans et al., 1969). Despite the established importance of NA inhibiting (NI) antibodies, these titers are rarely measured in seroepidemiologic or vaccine studies because the traditional thiobarbituric acid (TBA) assay used to quantify these antibodies is impractical for large numbers of samples and employs hazardous chemicals. Alternative assays that have been developed include a miniaturization of the TBA method (Sandbulte et al., 2009) and an enzyme-linked lectin assay (ELLA) (Lambre et al., 1990). The read-out of each method is different – while both quantify products of enzyme activity, the TBA method measures the amount of free sialic acid, the soluble product of NA activity, whereas ELLA measures the amount of penultimate galactose that becomes available after the terminal sialic acid is cleaved from substrate. Despite this difference, the assay principals are the same, usually employing whole virus as a source of antigen and using fetuin, a highly glycosylated protein, as substrate.

Since HA-specific antibodies in human sera can block access of substrate to NA, it is essential to use viruses with a HA subtype that is not in circulation when performing either of these assays for human serology. The assay described in this manuscript uses reverse genetics-derived H6 reassortant viruses that contain the targeted NA (Sandbulte et al., 2011). This strategy follows the original approach to measure NA inhibition antibody titers in which H6 reassortant viruses generated by classical reassortment were used in the traditional TBA method (Kilbourne et al., 1968, 1990).

The substrate for NA, fetuin, is coated onto the surface of 96 well plates used in the ELLA. Virus is incubated in the wells in the presence or absence of serial serum dilutions. NA cleaves terminal sialic acid moieties from glycoprotein complexes, and therefore enzyme activity can be quantified by measuring the amount of galactose that is consequently at the terminus of each carbohydrate complex. This is accomplished using peroxidase-conjugated peanut-agglutinin (PNA), a lectin with specificity for terminal

Abbreviations: ELLA, enzyme-linked lectin assay; HA, hemagglutinin; HI, HA inhibition; NA, neuraminidase; NI, NA inhibiting/inhibition; PBS, phosphate-buffered saline; PNA, peanut agglutinin; TBA, thiobarbituric acid.

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galactose. Following incubation with PNA-peroxidase, a substrate for peroxidase is added, leading to a colorimetric change proportional to NA activity. The ELLA has recently been used to measure NI antibody titers in a number of studies (Cate et al., 2010; Couch et al., 2012, 2013; Fritz et al., 2012; Fries et al., 2013), but assay optimization and details of the assay procedure to measure titers against the NA of human seasonal viruses have not been reported. This report describes steps that are important for obtaining consistent results and provide data to support the use of ELLA in human serology.

2. Materials and methods

2.1. Viruses

Reassortant influenza viruses were generated by reverse genetics as described previously (Hoffmann et al., 2000; Sandbulte et al., 2011). These viruses contain the HA (H6) gene from A/turkey/Massachusetts/3740/1965, gene segments encoding internal proteins from A/Puerto Rico/8/1934 (PR/34), and one of the following NA gene segments: N1 of A/Texas/36/1991 (TX/91), A/New Caledonia/20/1999 (NC/99), A/Solomon Islands/3/2006 (SI/06), A/Brisbane/59/2007 (BR/07), A/California/07/2009 (CA/09); and N2 of A/Wisconsin/67/2005 (WI/05) or A/Uruguay/716/2007 (UR/07). The following wild type influenza B viruses were used: B/Florida/4/2006 (B/FL/06) as representative of the B/Yamagata lineage and B/Brisbane/60/2008 (B/BR/08) as representative of the B/Victoria lineage. Viruses were cultured in the allantoic cavity of 9–12 day old embryonated chicken eggs at 33 °C, harvested 72 h post-inoculation and stored in aliquots at −80 °C.

2.2. Serum samples

The following animal sera were used: ferret antisera against NC/99, UR/07, B/FL/06 and B/BR/08 generated by infecting ferrets with the respective wild-type influenza viruses, and cotton rat sera from naïve as well as PR/34 (H1N1)-immune animals. Ferret and cotton rat inoculations were performed following federal guidelines under a protocol approved by the institutional Animal Care and Use Committee. Pooled rabbit antisera (Capralogics, Hardwick, MA, USA) collected before and after immunization with purified NA were also used in this study. The NA was purified from WI/05 by cellulose acetate electrophoresis (Sultana et al., 2011). De-identified human sera were obtained from a clinical vaccine study in which groups of young, healthy adults were immunized with either a live or inactivated trivalent seasonal influenza vaccine. The study was approved by the Institutional Research Involving Human Subjects Committee. Antibody and cellular immune responses were measured before and 4 weeks after vaccination; these results were reported previously (Eichelberger et al., 2011). Unless otherwise noted, antisera were heat-inactivated at 56 °C for 45 min before conducting assays.

2.3. ELLA procedure

The principles of the ELLA described by Lambre et al. (1990) and Cate et al. (2010) were followed to optimize and validate the method. The standard operating procedure (SOP) for this method is included in this manuscript as supplementary information. Fetuin (Sigma, St. Louis, MO, USA) was diluted to 25 μg/ml in 0.1 M phosphate buffered saline (PBS) and 100 μl added to each well to coat high-binding 96-well plates (Nalge Nunc, Rochester, NY, USA). Plates were stored at 4 °C and used 24 h to 2 months after coating. To determine the amount of antigen (virus) to use in ELLA, serial dilutions of the targeted H6 reassortant virus were prepared in Dulbecco's PBS (pH 7.4)–0.9 mM $CaCl_2$–0.5 mM $MgCl_2$ containing 1% bovine serum albumin (BSA) and 0.5% Tween and then dispensed (50 μl/well) into fetuin-coated plates containing an equal volume of PBS. The plates were incubated for 16–18 h at 37 °C, then washed 6 times with PBS–0.05% Tween 20 (PBST) before adding 100 μl peanut agglutinin (PNA) conjugated to horse-radish peroxidase (HRPO, Sigma). PNA–HRPO was used at the highest dilution that gave the maximum signal when titrated on fully digested fetuin. Plates were incubated at room temperature for 2 h and washed 3 times with PBST before adding o-phenylenediamine dihydrochloride (OPD, Sigma) to the plate. The color reaction was stopped after 10 min by the addition of 1 N H_2SO_4. The plates were read at 490 nm for 0.1 s using a Victor V 96-well plate reader (PerkinElmer, Waltham, MA, USA). The dilution of virus (antigen) that resulted in 90–95% maximum signal was elected for use in serology.

To measure the NI titers, each serum sample was heat treated (56 °C for 45 min) and then diluted serially in PBS–BSA. Fifty microliters of each dilution was added to duplicate wells of a fetuin-coated plate. An equal volume (50 μl) of the selected virus dilution was added to all serum-containing wells in addition to at least 4 wells containing diluent without serum that served as a positive (virus only) control. At least 4 wells were retained as a background control (PBS only). The plates were incubated for 16–18 h at 37 °C. As described for the virus titration, the plates were washed and PNA–HRPO was added to all wells. After a 2 h incubation period, the plates were washed and peroxidase substrate (OPD) was added. The color reaction was stopped after 10 min and absorbance read. The mean absorbance of the background (A_{bkg}) was subtracted from the test wells and positive control (A_{pos}) wells. The percent NA activity was calculated by dividing the mean absorbance of duplicate test wells (A_{test}) by the mean absorbance of virus only wells and multiplied by 100, i.e. $(A_{test} - A_{bkg})/(A_{pos} - A_{bkg}) \times 100$. To determine percent NA inhibition, the percent activity was subtracted from 100. The NI titers were defined as the reciprocal of the last dilution that resulted in at least 50% inhibition. An alternative way to report results is to calculate the titers of replicate wells independently and then report the geometric mean of the duplicates as the 50% end-point NI titer. In some instances the exact 50% inhibition (IC_{50}) was determined by 4 parameter logistics regression analysis (GraphPad Prism software). An assay was considered valid if the background absorbance was less than 10% of the virus only control, control sera had a similar NI titer to the median established in previous assays ($\leq$2-fold difference), and the raw A_{490} values of the duplicates did not vary more than 20%.

2.4. Miniaturized TBA method

The miniaturized TBA method was followed as described previously (Sandbulte et al., 2009).

2.5. Statistical analysis

Microsoft Office Excel was used to calculate standard deviations (SDs) of NI titers, percent coefficient of variation (%CV) for repeat assays and Pearson's correlation coefficient. Bland–Altman analysis (Bland and Altman, 1999) was performed using GraphPad Prism to assess the agreement between ELLA and TBA results.

3. Results

3.1. Assay optimization

The published ELLA method (Lambre et al., 1990) was optimized for routine analysis of human sera. This assay uses reassortant viruses with a mismatched HA as antigen (source of NA enzyme) to avoid non-specific inhibition by H1 and H3-specific antibodies in human sera. The H6 reassortant viruses containing the targeted

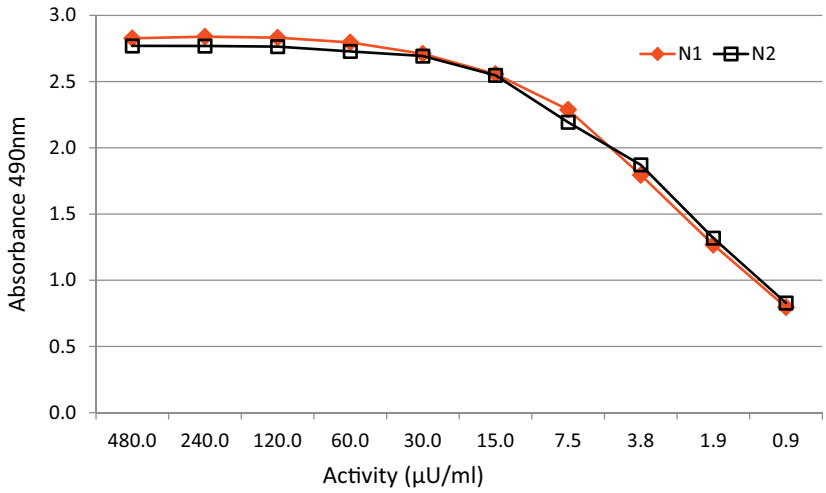

Fig. 1. Examples of H6N1 and H6N2 virus titrations. Serial dilutions of $H6N1_{NC/99}$ (N1 shown in red filled symbols) and $H6N2_{WI/05}$ (N2 shown in black open symbols) were incubated for 18 h in fetuin-coated plates and the reactivity with PNA determined as described in Section 2. Average absorbance of 2 wells is plotted against enzyme activity of N1 and N2 antigens. The enzyme activity of each antigen stock was determined previously using bacterial neuraminidase as the standard.

NA were generated by reverse genetics as described previously (Hoffmann et al., 2000; Sandbulte et al., 2009). Fetuin was used as substrate in the assay.

The assay requires the use of a defined amount of antigen (enzyme). Titration of different batches of H6N1 and H6N2 viruses on fetuin-coated plates resulted in expected sigmoidal regression curves, with maximum signal at $A_{490\,nm}$ ~3.0 and background <0.2. Bacterial NA purified from *Vibrio cholera* (Sigma) resulted in a similar titration curve. The linear range of absorbance values corresponded to enzyme activity units of 1–15 μU NA/ml (Fig. 1). Three different human sera and normal rabbit serum were titrated at each virus concentration, showing dependence of NI titer on the amount of antigen, with low titers at high virus concentrations and high titers when low amounts of antigen were added to the wells (Table 1). A minimal difference in NI titer was recorded when dilutions of virus that resulted in 50–90% of the maximum signal were used. This corresponded to the amount of virus within the linear region of the virus titration curve and resulted in a signal 5–10 times greater than background. Under optimized conditions this corresponded to absorbance signals (A_{490nm}) of 1.5–2.5. To exploit the full range of the assay, the dilution of virus that was at the top of the linear range (90–95% of maximal signal) was elected for routine serology because this amount of virus resulted in greatest signal:noise ratio and allowed maximum assay range (16-fold increases in titer could be measured). In the examples shown in Table 1, the amounts of virus selected for serology were dilutions of 1:640 for CA/09 and 1:60 for UR/07.

Since human sera usually contain NA-specific antibodies, initial experiments used sera from naïve animals to establish conditions that minimize the effect of non-specific inhibitors. Naive cotton rat serum tested in ELLA against different dilutions of PR/34 showed considerable inhibition of NA activity when the recommended amount of virus was used (Fig. 2). Freeze-thawing (F/T) of the serum sample did not reduce the non-specific inhibition significantly. However, heat-treatment at 56 °C for 45–60 min was sufficient to reduce non-specific inhibition so that titers of naïve serum against either H6N1 or H6N2 reassortant viruses were <5. Non-specific inhibition of NA activity is dependent on HA subtype and therefore needs to be considered when assays use antigens that contain HA subtypes other than H6. In instances when heat-inactivation is insufficient to eliminate inhibitors, serum samples can be treated with receptor destroying enzyme (RDE) prior to heat treatment (results not shown).

Following heat-treatment, 2-fold dilutions of serum samples were added to duplicate wells of a washed, fetuin-coated plate. An equal volume of virus (antigen) was then added. Initial experiments included an incubation step of serum and virus in a 'dilution' plate. This resulted in inconsistent results, most likely reflecting adherence of virus to the dilution plate wells. The assay was therefore simplified to add serum dilutions and virus directly to the fetuin-coated plate.

Our initial experiments hoped to reduce assay time by optimizing conditions to allow short virus and substrate incubation times. However, the overall signal obtained after short incubations was low (data not shown) and therefore to preserve assay robustness, the assay was optimized for overnight (16–18 h) incubation of virus/serum on the fetuin-coated plate. After this incubation, the lowest dilution of PNA-HRPO that resulted in maximum signal was added and the plates held at room temperature for 2 h. OPD was then added as substrate to all wells. OPD was used because it is used for other assays in our laboratory, however, it can be replaced by 3,3′,5,5′-tetramethylbenzidine (TMB).

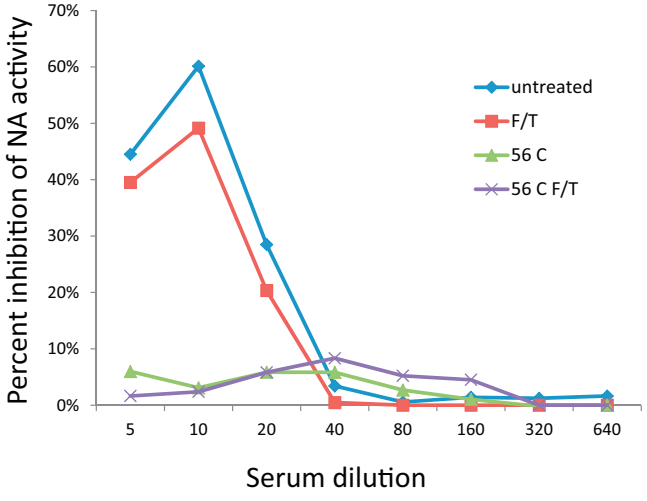

Fig. 2. Heat treatment of sera is required to reduce non-specific inhibition of NA activity. Serum from naïve cotton rats was either not treated, heated at 56 °C for 60 min, frozen and thawed 3 times (F/T), or heat-treated in addition to 3 freeze–thaws.

Table 1
NA inhibition titers of serum samples incubated with different amounts of antigen.

Serum sample	Dilution of H6N1$_{CA/09}$ (percent of maximum signal)							
	160 (100)	320 (98)	640 (89)	1280 (80)	2560 (46)	5120 (30)	10,240 (18)	20,480 (11)
1	<10	<10	<10	<10	<10	<10	10	10
2	40	40	80	160	160	160	320	320
3	320	640	1280	2560	2560	5120	5120	5120
4	160	320	640	640	1280	2560	5120	5120
	Dilution of H6N1$_{UR/07}$ (percent of maximum signal)							
	20 (100)	40 (99)	80 (82)	160 (67)	320 (43)	640 (23)	1280 (15)	2560 (8)
1	10	<10	<10	<10	10	10	20	20
2	20	40	40	80	80	80	160	160
3	80	160	320	320	640	640	2560	2560
4	40	80	80	160	320	320	640	640

3.2. Assay specificity

The specificity of ELLA was examined with monospecific sera. These included antisera from ferrets previously infected with NC/99 (H1N1), UR/07 (H3N2), B/FL/06 and B/BR/08 and antiserum from rabbits immunized with NA purified from WI/05 (H3N2). NI titers were measured against H6N1$_{NC/99}$, H6N2$_{WI/05}$, and H6N2$_{UR/07}$, B/FL/06 and B/BR/08. Ferret anti-NC/99 inhibited enzyme activity of H6N1$_{NC/99}$ by a 64-fold higher titer than H6N2 viruses (Table 2). Similarly, rabbit antiserum specific for WI/05, had a 32-fold higher inhibition titer against H6N2$_{WI/05}$ than H6N1$_{NC/99}$. Control sera did not inhibit enzyme activity of H6N1$_{NC/99}$ or H6N2$_{WI/05}$. Ferret anti-B/FL/06 (B/Yamagata lineage) and anti-B/BR/08 (B/Victoria lineage) antisera did not react with the NA of influenza A viruses demonstrating specificity for the B antigens. Since H6 reassortants cannot be generated with the NA of B strains, the antibody titers measured against B viruses may not accurately quantify NA-specific antibodies because HA-specific antibodies may hinder access of substrate to NA's active site and thereby contribute to the reduced signal; nevertheless, the NI titer of the B/FL/06-specific serum was greater against the homologous virus than the heterologous B/Victoria-lineage and vice versa, suggesting that these NAs are indeed antigenically distinct. Overall, these results demonstrate that virus-specific antisera show specificity for the homologous NA, although a small degree of cross-reactivity with a different subtype is observed when sera have very high homologous NI titers.

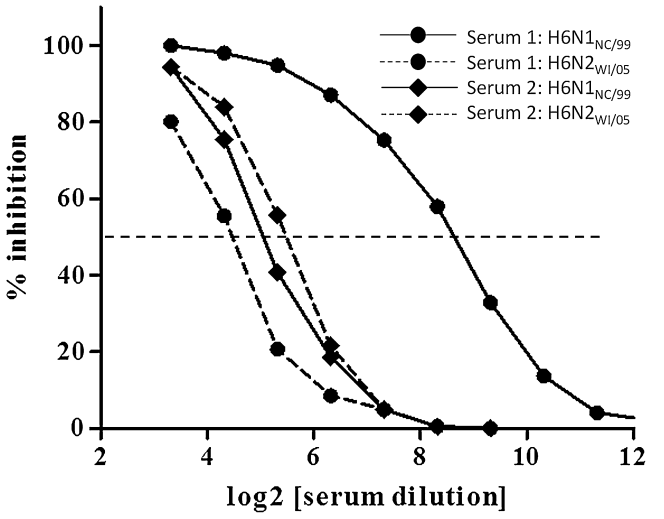

Fig. 3. Titration of sera against H6N1$_{NC/99}$ (solid lines) and H6N2$_{WI/05}$ (dashed lines) viruses. Percent enzyme inhibition for serial dilutions of serum 1 (filled circle) and serum 2 (filled diamond) is shown on each graph with the dashed horizontal line indicating 50% inhibition. The NA inhibition titers of sera 1 and 2 against the NA of NC/99 were 320 and 20 respectively; the NA inhibition titers of sera 1 and 2 against the NA of WI/05 were 20 and 40 respectively.

3.3. Assay linearity

NA inhibition titration curves were established for human serum samples having a range of titers against N1$_{NC/99}$ and N2$_{WI/05}$. This was accomplished by adding serial dilutions of serum samples to a fetuin-coated plate in duplicate wells, followed by addition of standardized amounts of H6N1$_{NC/99}$ and H6N2$_{WI/05}$ antigens. After incubation overnight, the assay was completed as outlined in the materials and methods section (the detailed method is provided as supplementary information). Absorbance increased as the amount of serum decreased, indicating the presence of increasing amounts of active NA. Percent inhibition was proportional to the amount of serum and followed a sigmoidal curve. Examples of human serum titrations are shown in Fig. 3; one serum has a relatively high titer against the N1 antigen but lower titer against the N2 antigen whereas the second serum has similar titers against N1 and N2 antigens. To assess the linearity of the assay, NI antibody

Table 2
Specificity of ELLA.

NA antigen[a]	NI titer of antiserum against					
	None	H1N1	H3N2		B	
		NC/99	WI/05[b]	UR/07	B/FL/06	B/BR/08
NC/99 (N1)	<10	1280	80	<10	<10	<10
WI/05 (N2)	<10	10	2560	80	<10	<10
UR/07 (N2)	10	20	2560	160	<10	<10
B/FL/06	<10	<10	40	<10	640	40
B/BR/08	<10	<10	<10	<10	40	160

[a] The N1 and N2 antigens were in the form of H6N1 and H6N2 reassortant viruses, respectively. Wild type B viruses were used in this assay.
[b] Antisera against NC/99, UR/07, B/FL/06 and B/BR/08 were generated by infection of ferrets. The anti-WI/05 serum was from rabbits immunized with purified NA purified mixed with an equal volume of Freund's complete adjuvant. The purification and mixing may have resulted in denaturation of some of the antigen.

Table 3
Assay precision: titration of samples within the same and different plates. Four replicates of each titration were conducted on each plate.

Plate	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	Titer ± SD[a]	%CV	Titer ± SD	%CV	Titer ± SD	%CV	Titer ± SD	%CV	Titer ± SD	%CV
Antigen 1: H6N1$_{NC/99}$										
1	<2.3	0	5.3 ± 0	0	6.3 ± 0	0	7.3 ± 0	0	6.3 ± 0	0
2	<2.3	0	6.1 ± 0.5	8.2	6.3 ± 0	0	7.3 ± 0	0	6.3 ± 0	0
3	<2.3	0	5.6 ± 0.5	8.9	6.3 ± 0	0	7.3 ± 0	0	6.3 ± 0	0
All	<2.3	0	5.6 ± 0.5	8.7	6.3 ± 0	0	7.3 ± 0	0	6.3 ± 0	0
Antigen 2: H6N2$_{WI/05}$										
1	<2.3	0	12.3 ± 0	0	4.3 ± 0	0	4.3 ± 0	0	5.7 ± 0.5	8.9
2	<2.3	0	12.3 ± 0	0	4.3 ± 0	0	4.3 ± 0	0	5.3 ± 0	0
3	<2.3	0	12.3 ± 0	0	4.3 ± 0	0	4.3 ± 0	0	6.3 ± 0	0
All	<2.3	0	12.3 ± 0	0	4.3 ± 0	0	4.3 ± 0	0	5.7 ± 0.5	8.7

[a] The titer is shown as log 2.

titers of different dilutions of 4 different samples with known titers were measured. The results demonstrated that the IC_{50} calculated for each dilution varied <10% from the assigned value (results not shown) and supported relative accuracy of the method for measuring NI antibody titers over a range of 5–2560.

3.4. Assay precision

Repeatability of the assay was determined by measuring geometric mean NA inhibition titers (GMT) of 5 sera. One operator performed the assays in which each sample was tested 8 times on 3 separate 96-well plates. The results obtained for titrations within the same plate were highly reproducible (CV <10%). Plate-to-plate variability was also minimal (Table 3). In some instances (e.g. sample 2 tested against H6N1 virus), the percent inhibition calculated for a particular dilution was close to 50% inhibition in all tests but because the titer is defined as the reciprocal of the last dilution that resulted in at least 50% inhibition, a lower titer was assigned even when percent inhibition was 49%. As a result there were some instances when a 2-fold difference was reported for replicate serum titrations even though the assay variability was minimal (the average relative standard deviation calculated at approximately 50% percent inhibition for assays against either N1 or N2 antigens was 7%). This was the largest difference observed for sample 2 shown in Table 3 (H6N1$_{NC/99}$ antigen), even when assays were run on different days (total of 24 titrations). The titers for other samples shown in Table 3 were the same in all 24 tests, reflecting the unambiguous assignment of titer when percent inhibition was well below 50% (e.g. sample 1 against either the N1 or N2 antigen) and when the percent inhibition was clearly greater than 50% at the assigned titer (e.g. sample 2 against the N2 antigen). Therefore, these results show that when all samples are considered for repeatability, a 4-fold difference in 50% end point titer is statistically significant ($p \leq 0.05$). It is likely that smaller differences in titers reported as IC_{50} or geometric means from repeat assays would be considered significant.

Intermediate precision of the assay was also assessed, with 2 technicians running the same samples. Both operators were experienced and trained in the same standard operating procedure. Comparable results were obtained for each sample, with measurement of no more than a 2-fold difference in titer.

Changes to the source of fetuin, storage conditions of fetuin (4 °C instead of −20 °C), use of coated plates stored for different lengths of time at 4 °C and use of different plate readers were evaluated for assay robustness. The changes were acceptable because the modifications did not result in NI titers that differed from the titers obtained with the written SOP by more than 2-fold. As described earlier, the amount of antigen (virus) in the assay was critical and therefore the dilution of virus used for the purpose of serology was defined as the dilution that results in 90–95% of maximum signal (Table 1). Although assay sensitivity may be increased by using less antigen (allowing elucidation of smaller differences in antigenic structure), an amount of antigen that gives a signal ≥10-fold background and within the linear range of the virus titration resulted in reproducible sample titers.

3.5. Comparability between ELLA and mini-TBA methods

Comparison of NI antibody titers of ferret antisera measured by the mini-TBA and ELLA against a number of N1 (NA of TX/91, NC/99, SI/06 and BR/07) and N2 (NA of AI/68, WU/95, SY/97, PA/99, WY/03, NY/04, UR/07) antigens demonstrated excellent correlation between NI titers, although the absolute titer was somewhat greater when measured by ELLA (Fig. 4A and B; Fig. S1A, B). This study also compared absolute titers and response rates measured by mini-TBA and ELLA methods of 32 serum pairs reported previously for a clinical study comparing responses to live, attenuated and inactivated split trivalent influenza vaccines (Hassantoufighi et al., 2010). As for the ferret sera and reported by Fritz et al. (2012), the human antibody titers measured by mini-TBA and ELLA methods showed some correlation (Fig. 4C and D), however NI titers generated by ELLA were often greater than the titers measured by the mini-TBA method (Fig. S1C, D). Bland–Altman analysis showed better agreement of ELLA and mini-TBA NI antibody titers for ferret sera than human sera (Fig. S1), with the mini-TBA assay generating lower NI antibody titers against both H6N1 and H6N2 antigens for the majority of human sera tested. The bias (±SD) for agreement between TBA and ELLA titers against the H6N1 antigen was −2.2 (±15.7) for ferret sera but −45.0 (±19.3) for human sera; and against the H6N2 antigen was −2.8 (±30.2) for ferret sera but −22.1 (±24.2) for human sera.

There was similar sensitivity in determining seroconversion by ELLA and mini-TBA methods. For paired samples taken before vaccination and 4 weeks later, increases in NI titers were evident in a similar proportion of volunteers; of 32 volunteers, the mini-TBA method identified 12 volunteers with increased serum NA inhibition titers against the N1 component (NC/99) and 13 volunteers with increased NA inhibition titers against the N2 component (WI/05) after vaccination (Hassantoufighi et al., 2010). The ELLA identified a similar number of volunteers that had increased NI titers following vaccination: the 12 volunteers identified by mini-TBA as well as two additional subjects had increased NI titers against the N1 component (14 seroconversions when considering a 2-fold increase as significant); 12 of the 13 volunteers who had increased NI titers identified by mini-TBA against the N2 component were identified by ELLA in addition to 2 volunteers who did not have increased titers measured in the mini-TBA assay (Table 4).

Table 4
Assay sensitivity: NA inhibition titers against A/WI/05 (H3N2) following seasonal influenza vaccination of healthy adults.

Volunteer #	Mini-TBA			ELLA		
	Pre	Post	Fold increase	Pre	Post	Fold increase
1	10	10		20	20	
3	40	40		40	40	
4	10	10		20	20	
5	40	20		20	20	
6	5	10	2	10	20	2
7	10	20	2	20	40	2
8	5	20	4	10	40	4
9	<5	<5		<10	<10	
10	<5	5	2	<10	10	2
11	10	10		10	20	2
12	20	40	2	10	40	4
13	80	80		40	40	
14	5	5		20	20	
15	20	20		20	20	
17	10	5		20	20	
18	10	20	2	10	20	2
19	<5	5	2	<10	10	2
20	10	10		40	40	
21	20	20		40	80	2
22	10	10		20	20	
23	10	10		40	20	
24	10	10		40	40	
25	10	10		20	20	
26	40	40		40	40	
28	10	20	2	20	40	2
29	10	20	2	20	40	2
30	10	40	4	20	80	4
31	10	10		40	40	
32	20	40	2	40	80	2
33	10	20	2	20	40	2
34	40	80	2	80	80	
35	80	40		40	20	

4. Discussion

NA inhibiting antibodies correlate with reduction in influenza disease (Murphy et al., 1972) and therefore serology that routinely includes measurement of NI antibody titers is warranted. In the past, this was difficult to accomplish with the standard TBA method, but development of a miniaturized TBA method (Sandbulte et al., 2009) and use of the enzyme-linked lectin assay (Lambre et al., 1990) have resulted in more practical platforms that have allowed NI titers to be measured in a number of studies. This report describes steps that are important for obtaining consistent results and demonstrates reproducibility of the ELLA method. An SOP that can be used by investigators interested in performing this assay routinely is provided as supplementary information.

This study is not the first to use ELLA to measure human serum NI antibody titers – others have adapted the method published by Lambre et al. (1990) and measured responses to NA in clinical studies that demonstrate a number of important points. Couch et al. (2012) demonstrated that current influenza vaccines contain immunogenic amounts of NA and that NI antibody seroconversion rates are greater when vaccine dose is increased (Cate et al., 2010). Fritz et al. (2012) compared the TBA and ELLA methods in a study measuring NI titers after vaccination with a cell grown inactivated whole virus H5N1 vaccine, demonstrating increased sensitivity of ELLA compared to the TBA method and showing excellent increases in NI titer following a single dose of vaccine. NI titers were not increased following a boost with this vaccine, possibly reflecting competition between HA and NA antigens when they are presented on the same whole virus (Johansson and Kilbourne, 1993). Since antigenic competition can be circumvented by immunizing with dissociated HA and NA antigens in mice (Johansson and Kilbourne, 1996), it would be of interest to determine whether NI titers are boosted following vaccination with split, inactivated influenza vaccines. NI titers measured after vaccination with VLPs suggest the requirement for dissociated HA and NA antigens may not be applicable to all vaccine types – responses to both HA and NA were boosted following receipt of a second dose of adjuvanted H7N9 VLPs (Fries et al., 2013). Interestingly, significant increases in NI but not HAI titers were evident after the first dose of both unadjuvanted and adjuvanted VLPs in this clinical study, suggesting that the N9 component of the VLP vaccine is more immunogenic than H7 or that the NI assay has greater sensitivity than the HAI assay.

Although NI titers measured by ELLA correlate with the titers measured in the mini-TBA assay, the values measured by ELLA are often higher than measured by the more traditional assay. This difference was more noticeable for titration of human sera than ferret sera. It is not known whether the difference in assay bias reflects the relatively low NI antibody titers of samples used in the human analysis or whether it is due to qualitative differences in antibodies or the presence of additional non-specific inhibitors in human sera. Further studies to identify the factors that contribute to this difference will be helpful in understanding how best to interpret results and may lead to assay improvements.

As for other serologic assays, non-specific inhibitors present in serum samples need to be removed to avoid inaccurate measurement of antibody titers. The non-specific inhibition observed in the ELLA when animal (mouse and cotton rat) sera were tested against H6N1 and H6N2 viruses was removed by heat-treatment (56 °C for 45 min), indicating the presence of thermolabile β-inhibitors. β-inhibitors are Ca^{2+}-dependent (C-type) lectins that bind to mannose-rich glycans on glycoproteins (Anders et al., 1990). Surfactant protein D is an example of a β-inhibitor in the lung; both infectivity and NA activity are inhibited in the presence of this inhibitor (White et al., 2005), indicating that SP-D has capacity to bind to glycans on HA and NA, blocking their functional activity. Conglutinin is a β-inhibitor present in human serum

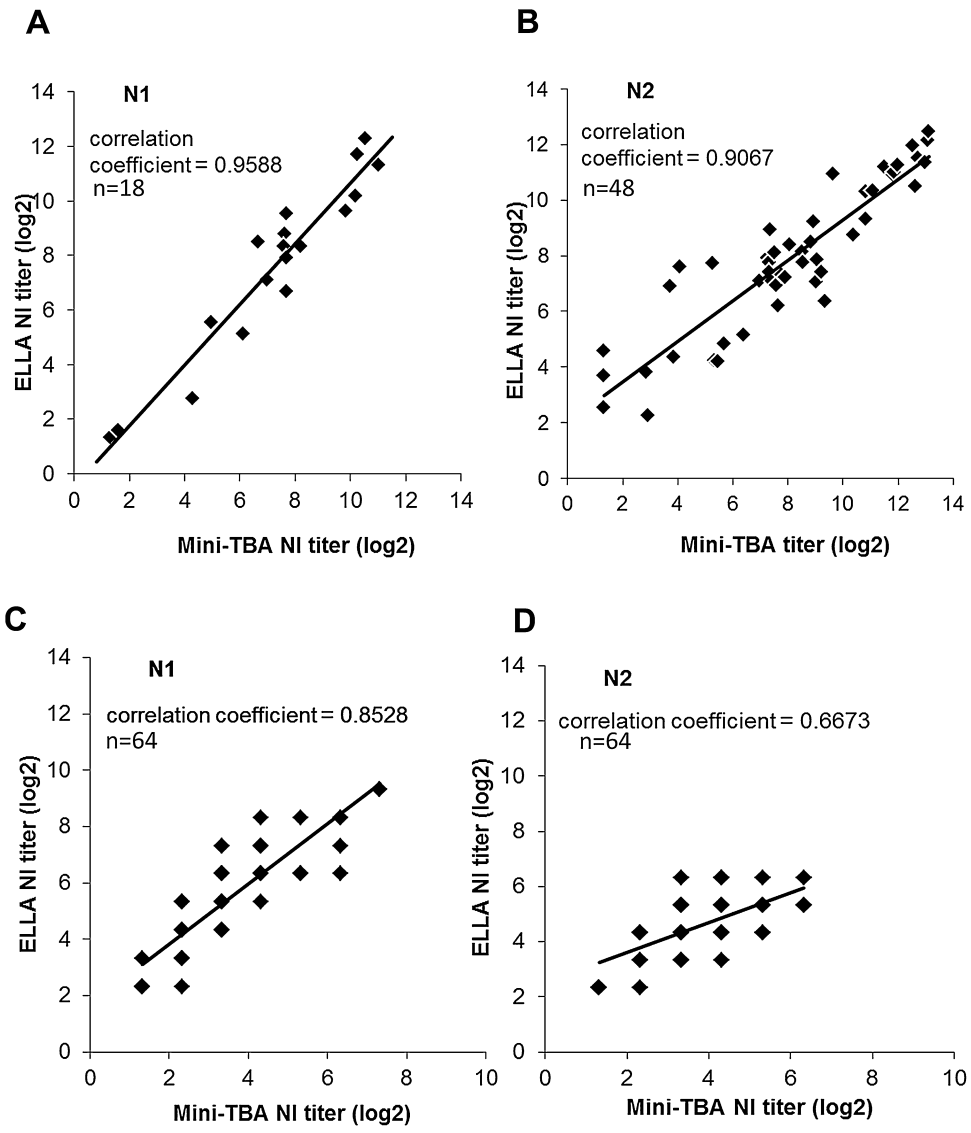

Fig. 4. Correlation between NI titers measured by TBA and ELLA. Titers are shown for ferret (A, B) and human (C, D) sera tested against H6N1 (A, C) and H6N2 (B, D) antigens. The number of titers compared (*n*) and Pearson's correlation coefficient are indicated on each figure.

(Hartshorn et al., 1993) that may contribute to non-specific inhibition of NA activity in the ELLA. Other non-specific inhibitors (α and γ-class) of influenza HA have been described, particularly in relation to H3N2 virus hemagglutination and infectivity (Rogers et al., 1983; Matrosovich et al., 1998; Job et al., 2013), however, these do not appear to result in non-specific inhibition of NA activity in assays that use H6 reassortant viruses as antigen.

Various forms of NA have been used in ELLAs – purified recombinant NA (Fritz et al., 2012), reassortant H6N1 and H6N2 viruses (this report), detergent split wild-type virus (Cate et al., 2010) and virus like particles (Couch et al., 2013; Fries et al., 2013). Although it is advantageous to use reassortant whole viruses that contain NA in the "natural" form in ELLA, it has not been possible to generate reassortant viruses expressing the NA of influenza B viruses and mismatched HAs. Purified recombinant NA protein or VLPs are therefore needed to measure antibody responses to the NA of influenza B viruses. Regardless of antigen source, all assays show a correlation between TBA and ELLA titers (our data as well as that reported by Fritz et al. (2012)), with NI antibody titers measured by ELLA somewhat greater than measured by the TBA method.

The results of assays that examine repeatability show that a 4-fold rise in NA inhibition titer is indicative of seroconversion. However, when consecutive serum samples are run on the same plate and the raw data examined to verify that differences in titer are not ambiguous (as may be the case when percent inhibition is close to 50%), assay variability may be less, allowing a 2-fold increase in NI titer to be used as evidence of a NA-specific antibody response. Seroconversion rates that are based on 2-fold differences in NI titer should be verified by performing repeat assays.

In conclusion, the steps taken to optimize and validate the ELLA for measuring NA inhibiting antibody titers in human sera are described in this report. The data show that this very practical assay is subtype specific, and yields reproducible results. Since routine measurement of NI antibody titers is now possible, the immunogenicity of NA in influenza vaccines can be examined more routinely and NI antibody titers that correlate with protection from clinical disease can be identified.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.09.003.

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