An Automated Microneutralization Plaque Assay to Measure Serum Antibodies against Influenza A Virus H1, H5 and H7 Subtypes

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ABSTRACT

As new vaccine technologies are developed to generate broadly cross-reactive antibodies by targeting conserved epitopes such as the stalk region of the HA, the M2 or the NA, the development and validation of new assays that measure protective antibodies will be necessary. The hemagglutination-inhibition (HI) antibody titer is currently the only universally accepted immune correlate of protection against influenza. HI titers of e1:40 are considered seroprotective; however, protection is a 50% reduction in the risk of influenza infection and not absolute. Therefore, it is necessary to consider whether better alternatives to measure protection exist or need to be developed. Virus neutralization (VN) assays may serve as a more direct measure of immune correlates of protection; however, a protective titer for neutralizing serum antibodies has not been established. In this study, a micro plaque reduction neutralization test (PRNT) was developed using wild type H1N1 and recombinant H5N1 and H7N7 influenza viruses. Hyperimmune serum from humans, ferrets and mice homologous to the test antigens was used to evaluate the sensitivity and specificity of the assay. The PRNT uses immunoperoxidase staining and an automated 96-well enzyme-linked immunospot (ELISPOT) reader to count foci and measure anti-influenza antibodies in MDCK cells accurately within 48 hours. PRNT titers are expressed in terms of conventional 50% PRNT end-point titers and compared to conventional VN for each subtype. The PRNT for human serum against pandemic 2009 H1N1 viruses revealed cross reactive antibody titers against the seasonal Brisbane/59 (H1N1) strain that was not detected by VN assay. The PRNT also revealed cross clade reactive antibodies for the H5N1 viruses not observed by the VN and was more sensitive for the detection of H7 antibodies than the VN assay. Overall, good agreement was observed between VN and the PRNT titers for all subtypes tested; however, the PRNT values were significantly higher than those of the VN test. The PRNT is a rapid and highly sensitive assay for the detection of influenza-neutralizing antibodies for multiple influenza subtypes. Using recombinant H5N1 and H7 influenza viruses, we are able to perform a rapid and sensitive neutralization assay for highly pathogenic influenza viruses and viruses with pandemic potential in a BSL-2 facility. Validation and evaluation of the PRNT as a measure of correlate of protection should be further explored.

BACKGROUND

Currently available seasonal influenza vaccines that target the surface molecule hemagglutinin (HA) are effective as long as there is an antigenic match between the vaccine strains and influenza viruses circulating or predicted to circulate. If the vaccine is a poor match or a pandemic strain emerges, current vaccines offer little protection. There is substantial interest to develop a universal influenza vaccine that would generate antibodies that would cross-react with all influenza A virus strains and subtypes and protect against antigenic variants and novel emerging influenza strains. As new antigenic targets are identified to generate universal vaccine candidates, it is imperative to have reliable methods to evaluate their immunogenicity. Various serologic tests are available to evaluate humoral responses against influenza; however, the hemagglutination inhibition (HI) assay is the only accepted measure of protection. The HI assay will identify the presence of HA antibodies but only measures the quantity of HA antibodies that prevent hemagglutination and not neutralizing capability. Therefore, to provide accurate and reliable data for immune correlates of cross-protection in vaccine studies, the successful development and validation of a rapid and highly sensitive influenza-specific neutralization assay for multiple influenza strains and subtypes is necessary.

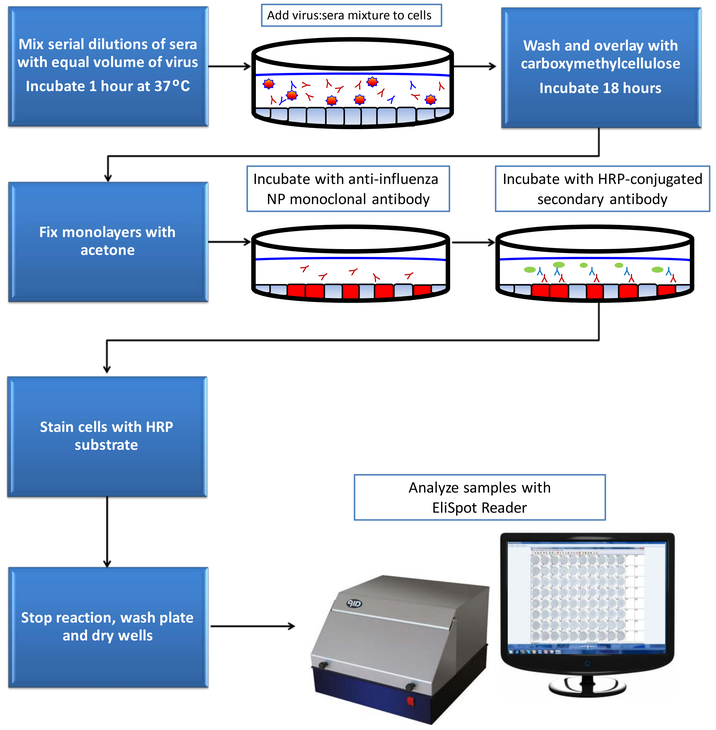
METHODS

Viruses and Cells: The H1N1 viruses: A/Brisbane/59/2007, A/California/07/2009 (H1N1pdm virus) and the recombinant H5N1 viruses: A/Viet Nam/1203/2004, A/Indonesia/05/2005, A/goose/Guiyang/337/2006, A/Japanese White Eye/Hong Kong/1038/2006 and H7N7 virus A/Netherlands/219/03 were obtained from the World Health Organization influenza-collaborating laboratories. MDCK cells were obtained from the American Type Culture Collection (Manassas, VA).

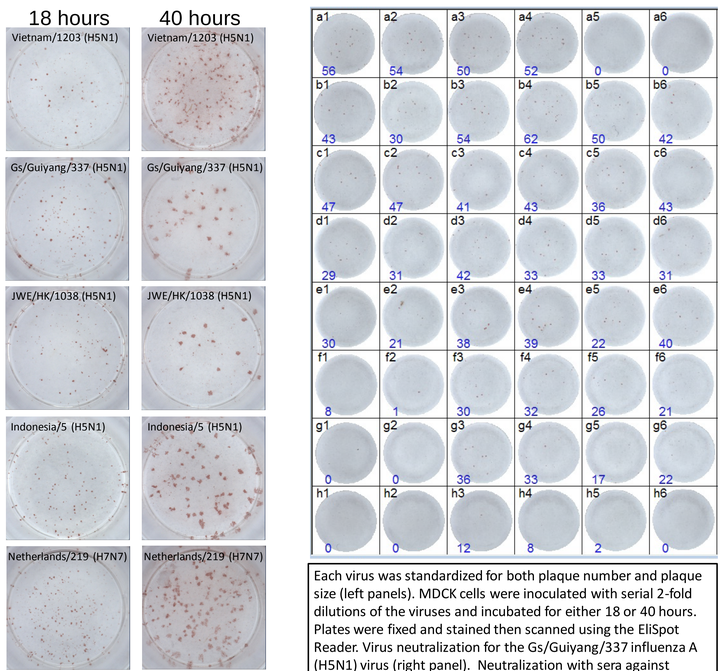
Serological testing: Hyperimmune sera to H1, H5 and H7 viruses were obtained from the World Health Organization influenza-collaborating laboratories. MDCK cells were obtained from the American Type Culture Collection. Hemagglutination inhibition (HI) titers were determined with receptor destroying enzyme (RDE) treated sera, diluted 1:10 in PBS and tested by the HI assay. Virus-neutralizing antibody titers were determined in MDCK cells. 2-fold serial dilutions of heat-inactivated serum were incubated with 100 $TCID_{50}$ of virus for 1 h at 37˚C. The mixture was then added to MDCK cells and incubated for 72 h at 37˚C in 5% CO_2. After 72 h, HA activity of the supernatant was assessed by the HA assay with 0.5% packed chicken red blood cells (H5 and H7) and guinea pig red blood cells (H1). Neutralizing titers were expressed as the reciprocal of the serum dilution that inhibited 50% of the HA activity of 100 $TCID_{50}$ of virus.

Plaque Reduction Neutralization Test: Serial 2-fold dilutions of heat-inactivated sera from 1:100 to 1:12,800 were mixed with an equal volume of virus, 50-100 focus forming units (FFU), and incubated for 60 min at 37˚C. After incubation, the virus:sera mixture was added to MDCK cells and incubated for 1 h. The cells were washed with PBS and overlaid with 1.0% carboxymethylcellulose and incubated for 18 h. The monolayers were fixed with acetone and prepared for staining. Wells were then incubated with anti-influenza monoclonal antibody against nucleoprotein (NP), followed by a horseradish peroxidase-conjugated secondary antibody. Cells were then stained with the insoluble HRP substrate, 3-amino-9- ethylcarbazole (AEC). The reaction was stopped, wells washed and allowed to dry. The plates were scanned using the AID EliSpot Reader image analyzer (AID Diagnos tika, Strassberg, Germany) and evaluated using the AID AID ELISpot Reader Software V5.0.

ASSAY WORKFLOW



VIRUS STANDARDIZATION AND PLAQUE REDUCTION NEUTRALIZATION TEST



Each virus was standardized for both plaque number and plaque size (left panels). MDCK cells were inoculated with serial 2-fold dilutions of the viruses and incubated for either 18 or 40 hours. Plates were fixed and stained then scanned using the EliSpot Reader. Virus neutralization for the Gs/Guiyang/337 influenza A (H5N1) virus (right panel). Neutralization with sera against VN/1203 and Indo/05 are shown.

SEROLOGICAL ASSAY RESULTS

Table 1. Comparison of H1 antibody titers measured by the plaque reduction neutralization test, virus neutralization and HI assays

Influenza Viruses[a]		H1N1 specific sera[b]				
		h-Cali/09-A	h-Cali/09-B	f-Brisbane/07	f-Cali/09	m-PR8
A/Brisbane/59/07 (H1N1)	PRNT	400	<100	>12,800	<100	NT
	VN	<100	<100	6,400	<100	NT
	HI	80	20	1,280	<20	NT
A/California/04/07 (H1N1)	PRNT	800	800	400	>12,800	NT
	VN	1,600	200	100	1,600	NT
	HI	160	20	<20	<20	NT
A/Puerto Rico/8/1934 (H1N1)	PRNT	800	400	100	<100	>12,800
	VN	<100	<100	<100	<100	800
	HI	<20	<20	<20	<20	640

[a]All test viruses are wildtype

[b]Serum sources: h=human serum; f=ferret serum; m=mouse serum; NT= Not tested

Table 2. Comparison of H5 antibody titers measured by the plaque reduction neutralization test, virus neutralization and HI assays

Influenza Viruses[a]		Clade 1	2.1	2.1	2.2	2.3.4	4
		VN/1203	h-INDO/05	f-INDO/05	Egypt/N03072	JWE/HK/1038	gs/Guiyang
A/Viet Nam/1203/04 (H5N1)	PRNT	1,600	<100	200	<100	<100	200
	VN	400	<100	<100	<100	<100	<100
	HI	40	<20	<20	<20	<20	<20
A/goose/Guiyang/337/06 (H5N1)	PRNT	800	100	400	<100	<100	1,600
	VN	100	<100	<100	<100	<100	400
	HI	40	<20	<20	<20	<20	80
A/JWE/Hong Kong/1038/06 (H5N1)	PRNT	800	400	800	200	3,200	100
	VN	<100	<100	100	<100	200	<100
	HI	20	<20	<20	<20	40	<20
A/Indonesia/05/05 (H5N1)	PRNT[b]	-	-	-	-	-	-
	VN	<100	200	800	<100	<100	<100
	HI	NT	NT	NT	NT	NT	NT

[a]All test viruses were recombinant (6+2) viruses on the PR8 backbone (6) with the HA and NA (2) derived from the respective virus

[b]Virus was standardized but PRNT was not successful; NT= Not tested

Table 3. Comparison of H7 antibody titers measured by the plaque reduction neutralization test, virus neutralization and HI assays

		Netherlands/New York[a]	Netherlands/219[b]
A/Netherlands/219/03 (H7N7)	PRNT	>12,800	400
	VN	800	200
	HI	320	80

[a]Ferret antisera to Recombinant H7 HA from influenza A/New York/107/2003 (H7N2) and influenza A/Netherlands/219/2003 (H7N7) viruses

[b]Ferret antisera to influenza A/Netherlands/219/2003 (H7N7) (6+2) virus

RESULTS AND CONCLUSIONS

- The PRNT revealed the presence of neutralizing antibodies against heterologous viruses not detected by the HI assay (blue boxes). HI and neutralization titers against homologous viruses are shown in red.
- As shown in Table 1, serum h-Cali/09-B had a HI titer of 20 against the heterologous Brisbane/59 (H1N1) but did not have detectable neutralization titers with either neutralization assay. The PR8 virus was neutralized by sera against Brisbane/59 and California/09.
- As shown in Table 2, in the absence of a detectable HI titer, serum specific for Indonesia/05 was found to have cross clade neutralizing antibodies against the clade 1, clade 2.3.4 and clade 4 test antigens by the PRNT assay (blue boxes). In addition, serum specific for Egypt/N03072 was found to neutralize the clade 2.3.4 test antigen.
- As shown in Table 3, the PRNT was more sensitive then the HI or standard neutralization against the H7N7 test antigen.

In conclusion, the PRNT assay is a sensitive assay for detecting antibodies against multiple influenza A subtypes and is suitable for high-throughput evaluation of immunogenicity. Neutralization titers were detected in the absence of a HI titer; however, the immune correlate of protection for neutralization has not been established and must be further explored.