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Short Report: High Throughput Quantitative Colorimetric Microneutralization Assay for the Confirmation and Differentiation of West Nile Virus and St. Louis Encephalitis Virus

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Abstract. An automated colorimetric micro-neutralization assay (CmNt) was developed for confirmation and differentiation of West Nile Virus (WNV)-positive human sera as a higher throughput alternative to the standard six-well plaque-reduction neutralization test (PRNT). CmNt was performed in high-capacity 96-well micro-titer plates and required 4–6 days to complete. Inhibition of infection was determined by reduced neutral red-dye retention and conveniently recorded by a colorimetric plate reader. Human sera previously confirmed by PRNT as either negative ($N = 52$), WNV positive ($N = 81$), or St. Louis encephalitis virus positive ($N = 12$) were tested by CmNt; interpreted results were virtually identical to PRNT with a reduced turnaround time and higher throughput. Additionally, a handful of dengue virus positive and negative specimens (four each) were tested by CmNt; interpreted results were identical to PRNT.

INTRODUCTION

Since the arrival of West Nile virus (WNV) in California in 2003, the California Department of Public Health, Viral and Rickettsial Disease Laboratory (VRDL) each year has screened up to 2,000 human sera by enzyme immunoassay for suspected arbovirus infection and confirmed up to 500 positive for WNV by plaque-reduction neutralization test (PRNT). Increased demand for PRNT motivated development of a higher throughput alternative—the colorimetric micro-neutralization assay (CmNt). In California, WNV has emerged amid a background of other potential arbovirus exposures both endemic, such as St. Louis and western equine encephalitis viruses (SLEV and WEEV), and imported, such as dengue (DENV) and chikungunya (ChikV) viruses. The plaque-reduction neutralization test (PRNT) remains the gold standard for serologic confirmation and differentiation of arbovirus infection or exposure.[1–3] However, PRNT is a low throughput assay, in part because of the six-well format and manual plaque counting; when confronted with ongoing, high demand for confirmatory testing, the per-run number of specimens that reasonably can be tested by PRNT quickly reaches its limit, resulting in delayed reporting. The VRDL experienced a surge of testing during the initial outbreak of WNV infections in 2004 and has seasonally since that time.

Other laboratories have developed higher throughput, micro-neutralization alternatives to PRNT.[4–9] One assay, a quantitative CmNt for testing antibodies to adenovirus, was considered most applicable for adaptation, offering the advantages of automated, colorimetric results in a microtiter format.[5] CmNt uses the same vital stain used in PRNT, neutral red, as an objective, quantifiable indicator of percent neutralization but obviates the need for an agarose overlay. In PRNT, neutral red highlights unstained viral plaques that are counted visually. In CmNt, the neutral red retained by remaining live cells is solublized and quantified by its optical density (OD) using a plate reader. The microtiter format increases the per plate capacity for specimens from 1 to 8, including two additional dilutions per specimen.

MATERIALS AND METHODS

PRNT. Positive and negative human sera were identified by PRNT at the VRDL as previously described.[10] Briefly, at 4 days before inoculation, Vero cells were cultured in six-well tissue-culture plates in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, sodium bicarbonate, penicillin, streptomycin, and glutamine. Serial dilutions of the test specimens were challenged with 100 plaque forming units (PFU) of virus, incubated for 1 hour at 37°C, and then adsorbed to confluent cell monolayers for an additional 1.5 hours at 37°C. After adsorption, a neutral red-containing agarose medium was overlaid in each well—a single overlay system for WNV and a double overlay system for SLEV. Plates were read at 4 days post-infection for WNV and 7–10 days post-infection for SLEV. Neutralization titer was the highest serum dilution showing > 80% reduction of plaques relative to a serum-free control. If a positive specimen failed to show at least a 4-fold difference in titer between the two viruses, it was interpreted as indeterminate.

Viruses and test sera. Sera were tested against the New York strain of WNV (35211 AAF; 9/23/99), the Ruis strain of SLEV (50-228T; 2/17/60) isolated from human brain tissue sent to the VRDL by a hospital in Fresno, CA, and the New Guinea C strain of DENV-2 (50-46T; 5/10/50); all were recently passaged in Vero cells. Test sera were from previously reported submissions maintained by the VRDL that were randomized and blinded before CmNt.

Cell culture. Stock cultures of Vero cells (African green-monkey kidney) were maintained in outgrowth medium consisting of 90% Eagle's minimal essential medium in Hanks' balanced salt solution and 10% fetal bovine serum buffered with 0.088% $NaHCO_3$ supplemented with 0.3% L-glutamine, 0.5% of 20,000 U/mL penicillin and streptomycin and 0.05 μg/mL amphotericin B. Cells were grown in 150-cm^2 flasks to a density of 4×10^7 cells/flask.

Neutral red. The optimal concentration of neutral red used in CmNt was determined to be 0.0132%; higher concentrations exceeded the linear portion of the OD curve generated by the spectrophotometer. Cell control values were maintained in the OD range of 1.400–1.900, and virus control values were

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TABLE 1
Randomly selected and abbreviated WNV versus SLEV reciprocal neutralization titers—10 from each category plus one indeterminate result

Specimen	PRNT			CmNt		
	WNV	SLEV	Interpretation	WMN	SLEV	Interpretation
1	< 20	< 20	Neg	< 20	< 20	Neg
2	< 20	< 20	Neg	< 20	< 20	Neg
3	< 20	< 20	Neg	< 20	< 20	Neg
4	< 20	< 20	Neg	< 20	< 20	Neg
5	< 20	< 20	Neg	< 20	< 20	Neg
6	< 20	< 20	Neg	< 20	< 20	Neg
7	< 20	< 20	Neg	< 20	< 20	Neg
8	< 20	< 20	Neg	< 20	< 20	Neg
9	< 20	< 20	Neg	< 20	< 20	Neg
10	< 20	< 20	Neg	< 20	< 20	Neg
11	20	320	SLEV	20	320	SLEV
12	40	≥ 640	SLEV	20	640	SLEV
13	< 20	≥ 640	SLEV	< 20	640	SLEV
14	< 20	≥ 640	SLEV	< 20	80	SLEV
15	20	80	SLEV	< 20	320	SLEV
16	80	≥ 640	SLEV	40	320	SLEV
17	40	320	SLEV	40	320	SLEV
18	< 20	320	SLEV	< 20	320	SLEV
19	40	160	SLEV	40	320	SLEV
20	80	320	SLEV	20	160	SLEV
21	320	< 20	WNV	80	< 20	WNV
22	320	< 20	WNV	160	< 20	WNV
23	≥ 640	< 20	WNV	160	< 20	WNV
24	≥ 640	< 20	WNV	160	< 20	WNV
25	≥ 640	< 20	WNV	160	< 20	WNV
26	≥ 640	< 20	WNV	320	< 20	WNV
27	320	< 20	WNV	160	< 20	WNV
28	≥ 640	< 20	WNV	160	< 20	WNV
29	320	< 20	WNV	80	< 20	WNV
30	80	< 20	WNV	80	< 20	WNV
31	160	< 20	WNV	40	20	Indeterminate

between 0.300 and 0.600 to maintain a difference of 0.800–1.000 between cell control values and virus control values.

Control era. Positive control sera were from previously tested specimens exhibiting high PRNT titers to either WNV or SLEV. Negative control sera tested negative against both viruses.

CmNt. The diluent for serum and virus dilutions was 0.75% bovine albumin-phosphate buffered saline (BA-PBS) supplemented with 100 U/mL penicillin and streptomycin and 0.05 µg/mL amphotericin B. The same viral preparations that were titrated for use in the PRNT to produce 100 PFU/0.1 mL were used in the CmNt with a one-half log increase in titer that was determined empirically to yield an interpreted result comparable with PRNT. Sera were diluted 1:20 and heat-inactivated at 56°C for 30 minutes; 50 µL of sera were plated starting at 1:20 with 2-fold serial dilutions in duplicate in a microtiter plate. Diluted virus was added at 50 µL per well to all wells except cell control wells. The plates were then agitated on a plate shaker at 200 rpm for 10 minutes. After mixing, the plates were incubated at 37°C and 5% CO_2 for 1 hour. Vero cells were trypsinized, and 40,000 cells/well were added to prepared cell culture media consisting of 95% Eagle's minimal essential medium in Earle's balanced salt solution and 5% fetal bovine serum buffered with 0.176% $NaHCO_3$ supplemented with 0.03% L-glutamine, 100 U/mL of penicillin and streptomycin, and 0.5 µg/mL of amphotericin B; 150 µL of this cell suspension was added to all wells, including virus and cell controls. The plates were then incubated at 37°C and 5% CO_2 until 2–3+ cytopathic effect (CPE; scored visually on a scale from 1+ to 4+) was observed in the virus control wells (4 days for WNV and 5–7 days for SLEV).

After 2–3+ CPE had developed in the virus control and correlated with the virus back titration, the plates were aspirated in a biological safety cabinet (BSC). Excess fluid was blotted onto plastic-backed absorbent paper, and 100 µL of warmed neutral red dye (prepared as a 1:25 dilution of a 0.33% stock in test diluent) was added to all wells of the plate. Plates were

TABLE 2
WNV versus SLEV neutralization results summary

		PRNT				
		WNV pos.	SLEV pos.	Neg.	Indeterminate	Total
CmNT	WNV pos.	80	0	0 (FP)	0	80
	SLEV pos.	0	12	0 (FP)	0	12
	Neg.	0 (FN)	0 (FN)	59 (TN)	0	59
	Indeterminate	1 (FN*)	0	0	0	1
	Total	81 (TP)	12 (TP)	59 (TN)	0	152

*Antibody was detected, but there was a less than 4-fold difference in titer between WNV and SLEV CmNt results.
pos. = positive; neg. = negative; TN = true negative; TP = true positive; FN = false negative; FP = false positive.

TABLE 3
Dengue neutralization comparison (reciprocal neutralization titers)—DENV negative specimens were positive for either WNV (3) or SLEV (1)

	PRNT				CmNt			
Specimen	WNV	SLEV	DEN	Interpretation	WNV	SLEV	DEN	Interpretation
1	> 640	< 20	20	WMV	640	80	20	WNV
2	80	< 20	20	WNV	320	80	< 20	WNV
3	160	< 20	20	WNV	160	40	< 20	WNV
4	< 20	320	40	SLEV	80	640	40	SLEV
5	160	20	1,280	DENV	320	80	2,560	DENV
6	320	80	1,280	DENV	320	320	1,280	DENV
7	160	< 20	5,120	DENV	320	80	2,560	DENV
8	80	20	5,120	DENV	640	160	2,560	DENV

incubated for 75 minutes at 37°C and 5% CO_2. The plates were then washed two times each with PBS (in the BSC) with a final aspiration cycle. Residual PBS in the plates was blotted onto plastic-backed absorbent paper immediately after washing to avoid inaccuracies; 100 μL of acid alcohol (50% ethanol and 1% acetic acid in water) was added to all wells. Plates were removed from the BSC and placed on a plate shaker at 200 rpm for 30 minutes. Plates were read on a Powerwave XS spectrophotometer at $\lambda = 540$ nm using KC Junior software (Bio-Tek Instruments, Winooski, VT).

The endpoint of viral neutralization was the highest dilution of serum resulting in a 50% or greater reduction of neutral red absorbance.[11] Each 96-well plate had a row of eight cell control wells and a row of eight virus control wells. The endpoint OD value for the plate was determined using the mean cell control (CC) OD value and the mean virus control (VC) OD value in the following calculation: 50% reduction of viral infectivity = 0.5 (CC–VC) + VC.

Test specimens were run in duplicate, and the mean value of each pair of dilutions was calculated. If a positive specimen failed to show at least a 4-fold difference in titer between the two viruses, it was interpreted as indeterminate.

RESULTS AND DISCUSSION

An abbreviated list of WNV versus SLEV data is presented in Table 1 (randomly selected by computer), and the entire data set is summarized using a contingency table (Table 2). The total number of specimens that completed testing in parallel (PRNT and CmNt) was 152. There were no CmNt false positives; all 52 PRNT-negative specimens were also negative by CmNt. Of the 93 specimens that were PRNT positive (either WNV or SLEV), one was indeterminate by CmNt—a WNV-positive specimen that scored only a 2-fold greater titer against WNV compared with SLEV in CmNt. This discordant specimen was treated as a false negative for statistical purposes.

The goal of the test strategy is to differentiate the viral infections based on a 4-fold or greater difference in neutralizing antibody titer, regardless of absolute titer values. Therefore, a higher challenge dose could be used in the CmNt (despite resulting in some lower absolute titers), allowing for the convenience of same-day inoculation of both viruses on a Monday with WNV termination on Friday of the same week. The one indeterminate WNV specimen result by CmNt (specimen 31) shows the slight limitation of this strategy; however, a WNV titer for this specimen was detected, and the indeterminate result would not be overlooked as a false negative.

An additional eight sera, including four DENV positives, were tested against WNV, SLEV, and DENV in CmNt (Table 3). CmNt interpretations correlated with PRNT and thus, differentiated DENV from WNV and SLEV.

Interpreted WNV/SLEV results show that CmNt is comparable with the PRNT in terms of sensitivity (99%) and specificity (100%); however, in a situation where large numbers of specimens need to be tested and reported quickly, the CmNt offers several time-saving and qualitative advantages over PRNT. The 96-well format allows for eight specimens per plate with eight dilutions each; the six-well PRNT format typically tests one specimen per plate with only six dilutions each. Incubation time for SLEV is reduced to 5–7 days in CmNt versus 7–10 days in PRNT. Cells are added to test plates on the test day; PRNT requires seeding of cells 4 days before the test day. Finally, percent neutralization is determined by OD using a plate reader, which removes subjectivity from the testing and allows for faster data acquisition.

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