Evaluation of Luciferase-based Reduction Neutralization Test for the Measurement of Neutralizing Antibody in DENV - infected Patient Sera

Pornsiri Somasa[1], Jiraphan Junjhon[2*], Peeraya Ekchariyawat[3], Chakrit Hirunpetcharat[4], Kriangsak Ruchusatsawat[5], Chunya Puttikhunt[6,7], Sutha Sangiambut[6,7], Nopporn Sittisombut[8]

Abstract

Virus reporter-based neutralization assays have been established to provide a feasible tools for the detection of neutralizing antibody against flavivirus. The luciferase-based reduction neutralization test (LRNT) was developed and evaluated previously using a set of well-characterized flavivirus and dengue virus (DENV)-2 specific monoclonal antibodies (MAbs). In this study, we further examined the performance of LRNT and its applicability in human serum comparatively to the reference method by employing various group of patient sera infected with DENV and other flavivirus. The specificity of LRNT was demonstrated by no neutralizing activity measurable at reciprocal dilution of 10 in all non-dengue infected sera. Among 10 Japanese encephalitis virus (JEV) infected sera, 4 serum samples showed no neutralizing activity against DENV-2, while 6 serum samples with previous exposed to DENV infection displayed degrees of neutralization. LRNT highlighted well agreement of neutralizing activity detected in all DENV-2 infected sera 0.994 (95% CI, 0.987 to 0.997%) and JEV-infected sera 0.996 (95% CI, 0.984 to 0.999%) compared to the reference method. Therefore, our study suggested that LRNT could be used as an alternative assay to detect DENV neutralizing antibody in suspected DENV-infected human serum.

Keywords: Antibody detection, DENV, Luciferase, Neutralization assay, Reporter virus

[1] Student of Infectious Diseases and Epidemiology, in Department of Microbiology, the Faculty of Public Health, Mahidol University, pornsiri.b@dmsc.mail.go.th

[2] Assistant Professor in Department of Microbiology, the Faculty of Public Health, Mahidol University, jiraphan.juh@mahidol.ac.th (* Corresponding author)

[3] Lecturer in Department of Microbiology, the Faculty of Public Health, Mahidol University, peeraya.ekc@mahidol.ac.th

[4] Associate Professor in Department of Microbiology, the Faculty of Public Health, Mahidol University, chakrit.hir@mahidol.ac.th

[5] Medical Technologist Senior Professional Level in Thai-National Institute of Health, Department of Medical Sciences, Ministry of Public Health, ruchusatsawat@yahoo.com

[6] Division of Dengue Hemorrhagic Fever Research and Siriraj Center of Research Excellence in Dengue and Emerging Pathogens, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand, chunyapk@biotec.or.th, ssangiambut@hotmail.com

[7] Molecular Biology of Dengue and Flaviviruses Research Team, Medical Molecular Biotechnology Research Group, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum-thani 12120,Thailand, ssangiambut@hotmail.com, chunyapk@biotec.or.th

[8] Professor in Department of Microbiology, the Faculty of Medicine, Chiang Mai University, nopporn.sittiso@cmu.ac.th

Introduction

Dengue is spread in many countries around the world, especially in tropical and sub-tropical areas. It is estimated that 390 million DENV infections occur annually in the world, of which 96 million cases were clinically apparent (Bhatt et al., 2013). Moreover, 3.6 billion people live in area at risk of DENV transmission (Bhatt et al., 2013). Neutralizing antibody response plays a major role against DENV infection (Rothman, 2011; Wahala & Silva, 2011). The neutralization occurs when antibodies bind to the virus, then prevent the infection of the susceptible host cells. The major target DENV neutralizing antibody is the envelope (E) protein. Generally, E protein consists of three beta-barrel domains included E domains I (EDI), II (EDII) and III (EDIII). The EDIII is a major target for neutralization by strongly neutralizing MAbs (Rothman, 2011).

Currently, the laboratory diagnosis of DENV infection is primarily through the detection of DENV RNA or antibodies against DENV proteins (WHO, 2009; CDC, 2020). The detection of DENV RNA is highly sensitive and specific, but the viral genome can be detected in serum at 4–5 days onset of illness, limiting the utility of DENV RNA assays (WHO, 2009). Most DENV infections are diagnosed serologically using an enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition test (HI) (Gubler, 1998; Guzman & Kouri, 1996; Vorndam & Kuno, 1997; WHO, 2009). The greatest confidence in diagnosis may be gained by using the neutralization assays such as plaque reduction neutralization test (PRNT). The PRNT was firstly developed by Russel and Nisalak in 1967 (Russell, Nisalak, Sukhavachana & Vivona, 1967). This assay is considered the “gold standard” for measuring the neutralizing antibody against DENV infection. However, the detection of plaque number is restrictively for viruses that can generate plaque, thereby limiting the test for non-plaque producing virus strains.

A large-scale alternative assays which can be performed in 96-well plates, would be preferred to improve the performance of neutralizing antibody detection. Focus reduction neutralization test (FRNT) is employed to determine the neutralizing potential of antibodies. The sensitivity of the FRNT is sufficiently high for detecting seroconversion (Borisevich et al., 2008; Watanabe, Hirokawa, Kon, Tamura & Nishikawa, 2008). FRNT could determine the neutralization potential of human sera with broadly reactivity across the dengue serocomplex (Dejnirattisai et al., 2015). However, detection of neutralizing titer in specimen by FRNT can cause an operator-error prone manual readout. Therefore, this assay requires the specialized expertise.

Various forms of DENV reporters have been made to improve the detection system for neutralization assays. Our study carried out an alternative form of DENV reporter, luciferase-secreting single-round infectious particle reporter (SIP^{sLuc}) in detecting neutralizing antibody against DENV-2 (Junjhon et al., unpublished results). This test is relied on the detection of reduced amount of secreted Lucia luciferase derived by SIP^{sLuc} replication when it is neutralized by antibodies. The presence of Lucia

luciferase gene in the viral genome improves the reading of neutralizing antibody assay since the luciferase signal was detected by luminometer instead of doing enumeration of infectious virus by eyes.

Purpose

This study aimed to profile the neutralizing activity on acute and convalescent DENV-2 infected sera for further applied in dengue disease diagnosis and to expand the performance testing of LRNT in non-DENV and Japanese encephalitis virus (JEV) infected sera.

Research Methodology

Clinical samples. Human serum samples with previous diagnosed of non-dengue, dengue and JEV infections were used to test the performance of LRNT. All human serum samples were secondarily provided by the Arbovirus section, National Institute of Health of Thailand, Department of Medical Science, Ministry of Public Health, Thailand. Among 35 DENV-2 infected patient sera, 14 sets of pair serums (acute and convalescent serums) and 7 convalescent sera were used as dengue positive group. Thirty-five non-dengue and ten JEV infected sera were tested for the specificity of LRNT. Neutralizing activity detected from 35 positive DENV-2 and JEV infected sera were used to analyze the agreement between LRNT and FRNT.

The positive and negative dengue patient sera were previously diagnosed by three approaches: 1) virus isolation in C6/36 cell line 2) RT-PCR employing primers specific to each of the four DENV serotypes (Yenchitsomanus et al., 1996) and 3) in-house antibody capture ELISA assay (Innis et al., 1989) for the detection of anti-DENV IgM and anti-DENV IgG antibodies, respectively. The detection unit of antibody levels is expressed in binding index (BI) (Innis et al., 1989). The minimal detection unit of 40 and 100 for anti-dengue IgM and anti-dengue IgG, respectively was interpreted as positive. The serum sample positive from a minimum two of three approaches is diagnosed as DENV infection. The JEV-infected serum samples were previously confirmed by using JEV-IgM in-house ELISA.

Cells. The African green monkey kidney (Vero) cell line (Yasumura & Kawakita, 1963) was used in the FRNT and LRNT assays. The cells were cultured in Minimum Essential Medium (MEM) (Gibco BRL, Waltham, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Waltham, USA), 2 mM L-glutamine (Gibco BRL, Waltham, USA), 100 units/ml penicillin-streptomycin (Gibco BRL, Waltham, USA) at 37°C in the presence of 5% CO_2. The Vero cells were propagated in T-25 or T-75 tissue culture flask (Corning Costar Corporation, Cambridge, MA, USA) at 37°C in the presence of 5% CO_2. Following a three-or four-day growth period with approximately 90-100% confluence of cell density, they were detached enzymatically by using 0.25% trypsin-EDTA solution (Gibco BRL, Thermo scientific, Waltham,

USA). The cell number of 4.5×10^5 and 1×10^6 cells were further used for passaging in T-25 and T-75 tissue culture flask, respectively.

The capsid (C)-expressing mosquito cell line and the mosquito (C6/36) cell were cultured in Leibovitz's (L-15) medium (Gibco BRL, Waltham, USA) supplemented with 10% (v/v) FBS (Gibco BRL, Waltham, USA), 2mM L-glutamine (Gibco BRL, Waltham, USA), 0.26% (v/v) tryptose phosphate broth (Sigma-Aldrich, St Louis, USA) and 100 units/ml penicillin-streptomycin (Gibco BRL, Waltham, USA) at 29°C. After four-day growth period, the cells were mechanically detached from the T-25 tissue culture flask by using a plastic cell scraper and resuspended in the L-15 medium containing 10% (v/v) FBS. About 1:20 of the cells were used for the passage in T-25 tissue culture flask.

Antibody. 4G2 is an anti-flavivirus E protein murine monoclonal antibody (MAb) with the IgG2a isotype (Henchal, Gentry, McCown & Brandt, 1982). The 4G2 binds to a conserved epitope on the E protein of the flavivirus family. It has been shown to recognize DENV, West Nile virus (WNV), JEV and Zika Virus (Henchal et al., 1982). It binds to the fusion loop at the extremity of EDII of E protein and prevents syncytia formation (Summers, Cohen, Ruiz, Hase & Eckels, 1989). This antibody was used in the focus immunoassay titration and FRNT. The anti-E antibody was kindly provided by Dr. Chunya Puttikhunt, Medical Biotechnology (BIOTEC), NSTDA.

The goat anti-mouse IgG H&L conjugated with alkaline phosphatase (AP) was purchased from a company (Abcam, Cambridge, UK). This antibody reacts specifically with mouse IgG and light chains common to other mouse immunoglobulins. The antibody was used in the focus immunoassay titration and FRNT.

Virus and DENV reporter. DENV-2, strain 16681 was kindly provided by Dr. Nopporn Sittisombut, Chiang Mai University. It was amplified in C6/36 cells to generate virus stock for all experiment conducted in this study. The 90% confluent cell monolayer of C6/36 cells were infected with the virus at a multiplicity of infection (MOI) 0.05 at 29 °C. Upon the presence of cell fusion at 6-8 days, the culture supernatant was harvested and subjected to centrifugation at 1,735×g for 10 min at 4°C. The clarified culture supernatant was distributed into small aliquots in the presence of 20% (v/v) FBS and kept at -80 °C.

Single-round infectious particle reporter (SIP^{sLuc}) of DENV-2, strain 16681 replicates for a single-cycle of infection within the host. It releases Lucia luciferase enzyme, providing a reporter function associated with DENV-2 replication. It was amplified in C-expressing C6/36 cells (Sangiambut et al., 2013) at 29°C. The culture supernatant containing infectious SIP^{sLuc} was harvested when cell fusion is observed and clarified by centrifugation at 1,735×g for 10 min at 4°C to remove cells debris. The clarified culture supernatant was distributed into small aliquots in the presence of 20% (v/v) FBS. The SIP^{sLuc} was subjected to detecting the infectious virus titer and luciferase activity by focus immunoassay titration and luciferase assay, respectively.

Focus immunoassay titration. Infectious virus titer of DENV-2, strain 16681 and SIP^{sLuc} were determined using focus immunoassay titration in Vero cells. The 4×10^4 cells/well were plated onto 96-well culture plate (Corning Costar Corporation, Cabridge, USA). After 24 h seeded, cells were infected with 50 µl of 10-fold serially diluted virus in 450 µl of 2% (v/v) FBS-MEM at 37°C for 2 h. After that, 125 µl of 1.5% (v/v) carboxymethyl cellulose (CMC) in 2% (v/v) FBS-MEM was added onto cells to restrict the area of virus infection. At 48 h of infection at 37°C, the overlayer was removed by 5 times washing with 200 µl/well sterile PBS. The infected cells were then fixed and permeabilized with 3.7% (v/v) formaldehyde and 2% (v/v) triton X-100 consequently. To remove triton X-100 residue, the plate was washed with 200 µl/well of PBS for 5 times. The infected cells were probed with 50 µl of 4G2 in PBS at 37°C for 1 h. The plate was washed with 200 µl/well of PBS for 5 times to remove unbound antibody. The 50 µl of 1:2,500 diluted AP conjugated goat anti-mouse IgG H&L (Abcam, Cambridge, UK) in blocking buffer (0.25% (v/v) Tween 20, 10% (v/v) FBS in PBS) was reacted with E-4G2 complex within the cells at 37 °C for 1 h. The plate was washed with 200 µl/well of PBS for 5 times, followed by sterile water. Finally, the infected cells were detected by adding 50 µl/well of AP chromogenic substrates containing 5-bromo-4-chloro-3'-indoly phosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) in AP buffer (100 mM Tris-HCl pH 9.5, 100 mM Nacl, and 5 mM $MgCl_2$) at room temperature in the dark for 15-20 min. The cells were rinsed with sterile water to stop the reaction. The infected cells were observed as the dark purple spot. DENV-2, strain 16681 as represented a group of infected cells as the focus. A single infected cell was represent the focus of SIP^{sLuc}. The infectious virus titer was expressed as focus forming unit (FFU) per ml.

Luciferase activity assay. Lucia luciferase enzyme catalyzes the oxidation of coelenterazine substrate to yield coelenteramide and light signal to be detected. The amount of Lucia luciferase was detected by using QUANTI-Luc detection kit (Invivogen, San Diego, USA). An equal volume of 50 µl of 1:2 diluted of substrate was mixed with the SIP^{sLuc} infected culture supernatant in 96 well-opti plate. Then, the luciferase signal was promptly detected by the VICTOR ™ X series Multilabel plate reader.

Neutralization assays. Neutralization assays were performed with human sera. Briefly, the $4x10^4$ cells/well of Vero cells were seeded with 10% (v/v) FBS containing MEM medium in 96-well culture plate and incubated at 37°C for 24 h. The human sera were heat-inactivated at 56°C for 30 min and 30 µl of 4-fold serial dilutions of sera from 1:10 to 1: 10,485,760 in 2% (v/v) FBS-MEM were incubated with an equal volume of DENV-2, strain 16681 or SIP^{sLuc} containing 100 FFU at 37°C. After 1 h of incubation, 50 µl of antibody-virus mixture was added onto Vero cells monolayer in 96-well culture plate and incubated at 37 °C for 2 h. For FRNT, 125 µl of 1.5% CMC in 2% (v/v) FBS containing MEM was then added onto each well. After incubation at 37 °C for 48 h. FRNT, the plate was stained by focus immunoassay. The serum

dilution resulting in 50% reduction of infectious titer referred to no serum condition was interpreted as $FRNT_{50}$

For LRNT, the complex was removed and cells were 5 times washed with sterile 1x PBS to eliminate luciferase residue before replenishing with 125 µl of 2% FBS-MEM. The culture supernatant was harvested for the measurement of luciferase activity after 48 hr of infection at 37 °C. The antibody dilution resulting in 50% reduction of relative light unit (RLU) value referred to the negative control is defined as $LRNT_{50.}$

Statistical analyses. The measurement agreement of test between FRNT and LRNT were tested by using the Intraclass Correlation Coefficient (ICC) (Koo & Li, 2016). The values greater than 0.90 indicate excellent reliability.

Results

Neutralizing activity of DENV-2 infected sera

Thirty-five clinical samples from patients with DENV-2 infection were used to measure the performance of LRNT along with FRNT. These sera neutralized DENV-2, 16681 and SIP^{sLuc} with a similar range of $FRNT_{50}$ (Table 1). Most of DENV-2 infected sera displayed high neutralizing titers in a range from 1:14,066 to 1:73,570 (Table 1). ICC analysis showed strong agreement between $FRNT_{50}$ and $LRNT_{50}$ in all sera tested.

Table 1. NT_{50} titers of DENV-2 infected serum samples assayed by FRNT and LRNT

Serum samples	$FRNT_{50}$ titer	$LRNT_{50}$ titer
P1/L08-168A	2794	3565
P2/L08-168C	22134	26844
P3/L08-199A	14854	10372
P4/L08-199C	285952	227194
P5/L08-230A	602	605
P6/L08-230C	7829	9500
P7/L08-267C	187050	154345
P8/L08-322C	45236	54311
P9/L08-345C	21257	24166
P10/L08-372C	58977	48279
P11/L13-003C	155291	104356
P12/L13-056C	21137	23393

Serum samples	$FRNT_{50}$ titer	$LRNT_{50}$ titer
P13/L13-147C	73570	83335
P28/R15-229A	17	13
P29/R15-229C	51045	38014
P34/R15-342A	3645	1735
P35/R15-342C	7582	5969
P44/L15-203A	189	336
P45/L15-203C	73120	61461
P57/R17-002A	41239	33547
P58/R17-002C	585119	574689
P61/R17-056A	25270	19256
P62/R17-056C	45888	37470
P64/R17-068A	14066	9835
P65/R17-068C	192797	139746
P66/R17-073A	1056	748
P67/R17-073C	38798	30945
P68/R17-077A	390	546
P69/R17-077C	16011	19232
P70/R17-081A	3653	4396
P71/R17-081C	16932	17493
P72/R17-093A	144	190
P73/R17-093C	103457	128125
P74/R17-095A	<10	<10
P75/R17-095C	42523	43178

A=Acute serum, C=Convalescent serum

Agreement of DENV-2 infected sera on the ICC analysis is 0.994 (95% CI, 0.987 to 0.997%), indicating excellent reliability of LRNT.

In DENV-2 infected human sera, there were 4 groups of neutralizing activity titers detected (Figure 1). The first group had no neutralizing activity (Figure 1A). The second group displayed low neutralizing activity with $FRNT_{50}$ and $LRNT_{50}$ values of 1:2,794 and 1:3,565, respectively (Figure 1B). The third group showed moderate neutralizing activity with $FRNT_{50}$ and $LRNT_{50}$ values of 1:22,134 and 1:26,844, respectively (Figure

1C). The fourth group demonstrated high neutralizing activity with $FRNT_{50}$ and $LRNT_{50}$ values of 1:73,210 and 1:61,461, respectively (Figure 1D).

Correlation of neutralizing titers in acute and convalescent sera

The increase of NT_{50} titers at ≥4-fold in convalescent samples from acute samples has been used to indicate active DENV infection. In this study, 14 acute and convalescent of DENV-2 infected sera were demonstrated for studying correlation of neutralizing titers by FRNT and LRNT. There was similar neutralizing activity obtained by both FRNT and LRNT assays. Among 14 convalescent sera, 12 convalescent sera displayed ≥4-fold rising of $FRNT_{50}$ and $LRNT_{50}$, while only two samples showed 2 to 3-fold rising of $FRNT_{50}$ and $LRNT_{50}$ (Figure 2).

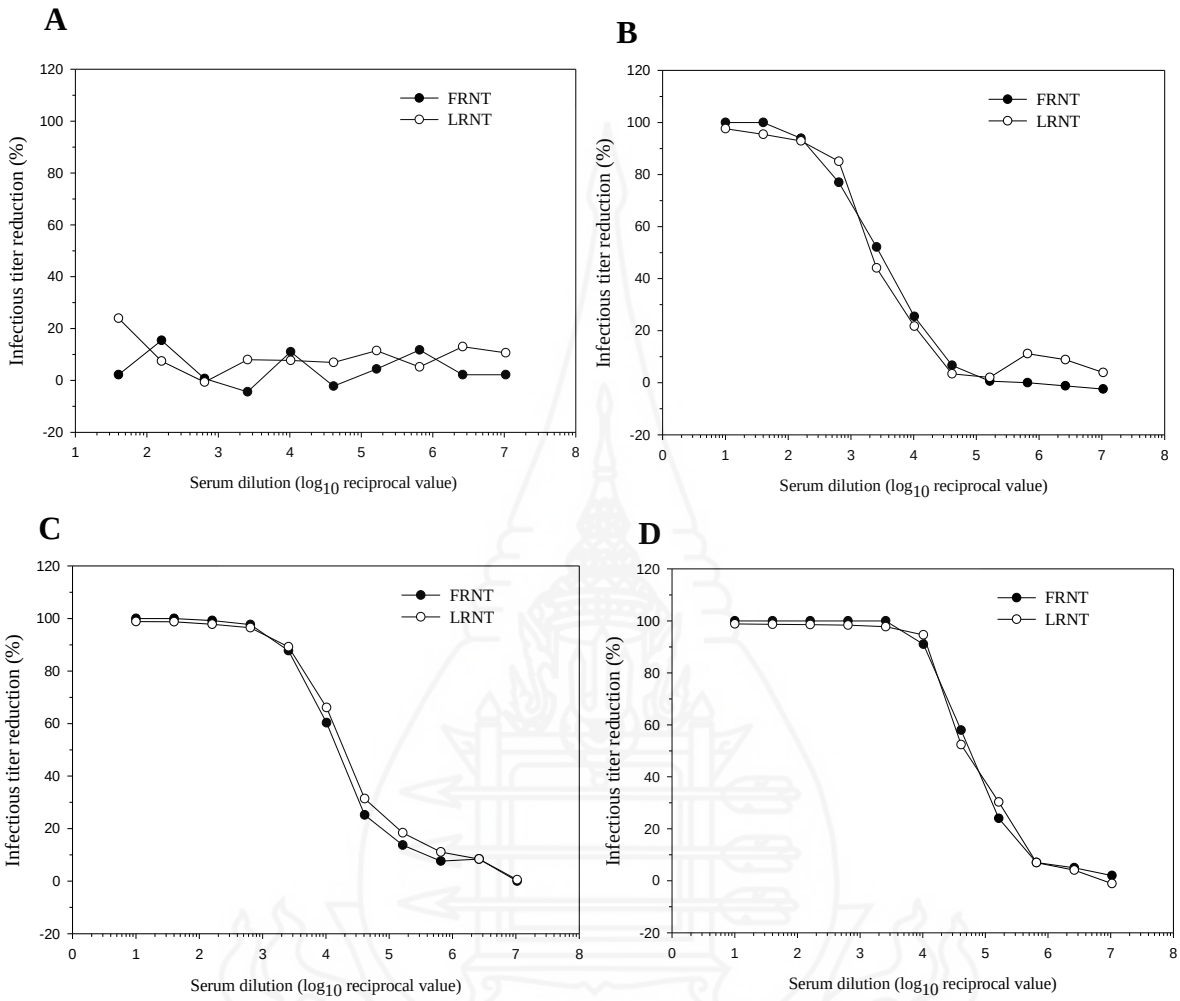

Figure 1. Neutralizing antibody assays of human sera infected with DENV-2. DENV-2 infected sera were reacted with DENV-2, strain 16681 or virus reporter at 37°C. After 48 hours of infection in Vero cells, focus-based detection method (closed circle) and luciferase-based detection method (opened circle) were employed to determine neutralizing activity of the sera. A) non neutralizing antibody was detected in patient sera. Dose-dependent neutralization patterns were observed by lower (B), moderate (C) and high (D) NT_{50} titers.

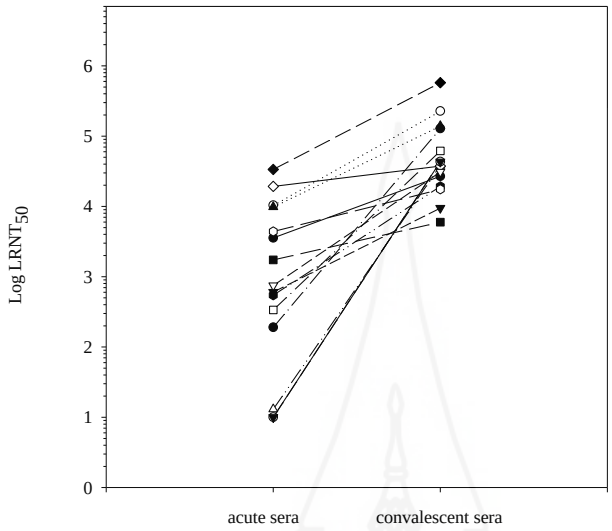

Figure 2. Neutralizing titers of human sera infected with acute and convalescent DENV-2 detected by LRNT.

Neutralizing activity of non-DENV-2 and JEV infected sera

Thirty-five clinical serum samples negative of current DENV infection were used to investigate the specificity of LRNT. Expectedly, no neutralizing activity was detected at a reciprocal dilution of <10 in all serum sample tested comparatively to the result obtained by FRNT. The pattern of neutralizing activity of JEV infected sera revealed 3 groups (Figure 3). All 3 groups of JEV infected sera displayed similar neutralizing activity between FRNT and LRNT. The first group displayed no neutralizing activity (Figure 3A). The second group displayed moderate neutralizing activity with $FRNT_{50}$ and $LRNT_{50}$ values of 1:626 and 1:606, respectively (Figure 3B). The third group displayed high neutralizing activity with $FRNT_{50}$ and $LRNT_{50}$ values of 1:12,856 and 1:10,559, respectively (Figure 3C). Among 6 specimens showed similar $FRNT_{50}$ and $LRNT_{50}$ value of ≥10, whereas the rest showed $FRNT_{50}$ and $LRNT_{50}$ value of <10. The level of agreement of neutralizing activity detected in JEV infected sera between LRNT compared to FRNT showed 0.996 (95% CI, 0.984 to 0.999%) of ICC value, indicating excellent reliability between 2 tests.

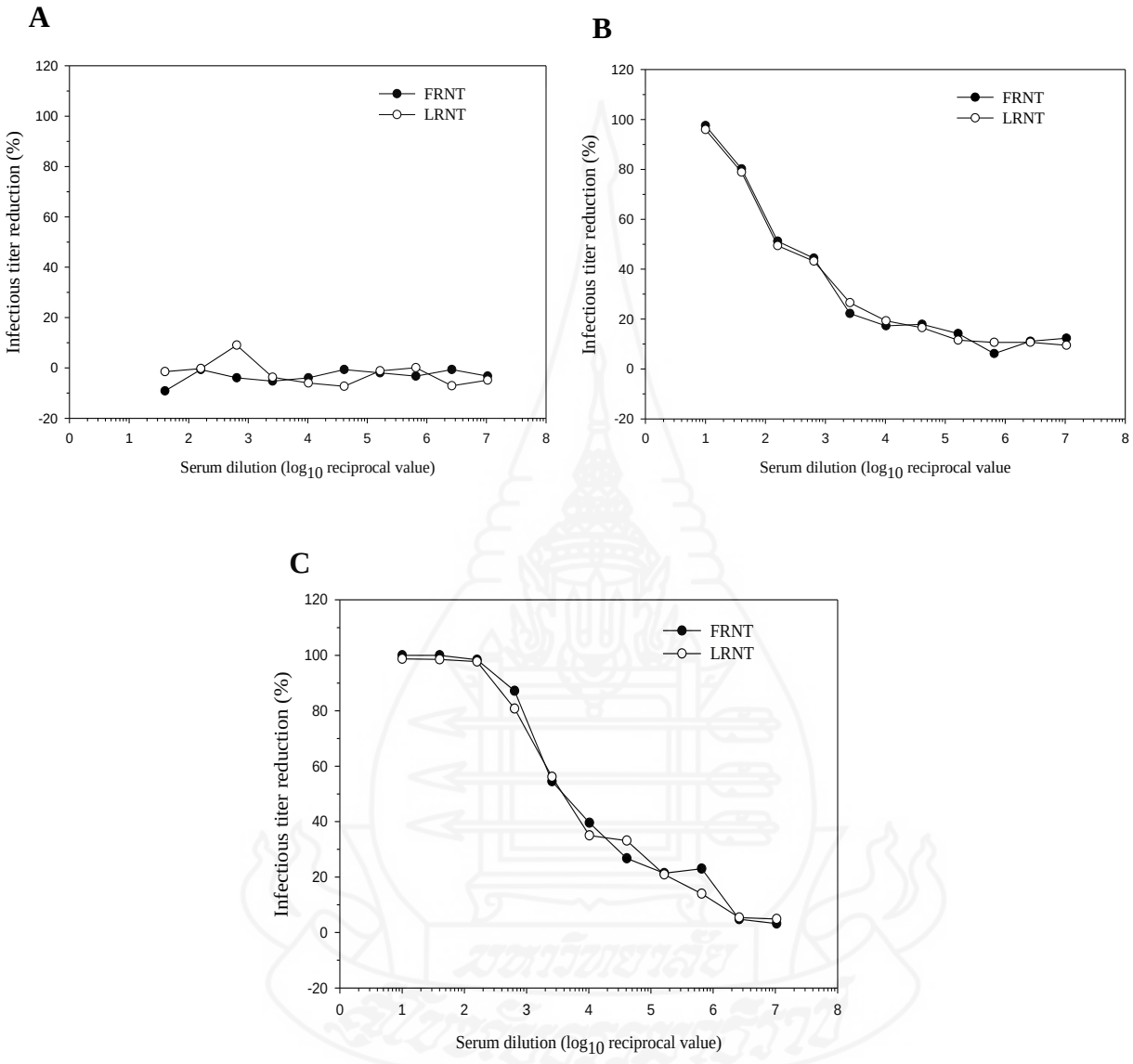

Figure 3. Neutralizing antibody assays of human sera infected with JEV. JEV infected sera were reacted with DENV-2, strain 16681 or virus reporter at 37°C. After 48 hours of infection in Vero cells, focus-based detection method (closed circle) and luciferase-based detection method (opened circle) were employed to determine neutralizing activity of the sera. A). non neutralizing antibody activity was detected in JEV patient sera. Dose-dependent neutralizing patterns were observed by moderate (B) and high (C) NT_{50} titers.

Discussion

A reliable high-throughput neutralization assay is critical for DENV diagnosis and vaccine studies. A number of Luc-based neutralization assays have been improved and validated with clinical serum samples to overcome the limitation of the conventional PRNT assay. One of these used luciferase dengue virus reporter for measuring neutralizing antibodies with rapid, high throughput providing a useful tool for laboratory detection and epidemiological investigation (Song et al., 2014). Several DENV reporters have been developed and evaluated in clinical samples from infected animal and patients demonstrating in respect to agreement of 76.4% correlation with conventional plaque-based assay (Shan et al., 2017). To quantitatively compare the traditional PRNT, neutralization results showed no statistically different (ANOVA, $p>0.05$) of NT_{50} titers obtained from PRNT for each of the sera tested (Mattia et al., 2011). In the sense of correlation with the PRNT assay, $LRNT_{50}$ titers are well correlated ($R^2 > 0.95$) with $PRNT_{50}$ titers (Song et al., 2014). These results indicate the corresponding neutralizing activity with the traditional PRNT. Accordingly, LRNT evaluation in our study revealed strong correlation of neutralizing titers in DENV-2 (ICC=0.994) and JEV (ICC=0.996) infected sera equivalent to the reference method.

Present study demonstrated the performance of LRNT along with FRNT in 35 non-dengue, 35 dengue and 10 JEV-infected patient sera for use as alternative method for dengue neutralizing antibody detection. Neutralizing activity in non-dengue and 4 JEV-infected sera displayed no neutralizing titers in all samples as expected, indicating the specificity of LRNT in parallel with traditional PRNT (Song et al., 2014; Shan et al., 2017; Mattia et al., 2011; Zhang et al., 2016; Kato & Hishiki, 2017; Shan et al., 2017). However, 6 samples of JEV-infected sera displayed neutralizing activity against DENV-2. These was likely due to potential interfering by the presence of dengue-IgM and IgG antibodies developed by previous DENV exposed (no data show). To further improve for DENV-2 serotype specificity of the LRNT, a large number of sera infected with DENV-1, DENV-3, and DENV-4 and other flavivirus-cross reactive group like Zika virus should be employed in neutralization assay.

Suggestions

Measurement of neutralizing antibody in DENV-infected patient sera employing SIP^{sLuc} by LRNT offers advantages for robust detection of neutralizing antibody in dengue immune sera. However, this study demonstrated the specificity of LRNT using few numbers of non-DENV and JEV-infected patient sera. Expanded number and type of human serum samples, including primary and secondary DENVs-infected sera and other flaviviruses infected serum samples should be included in the performance testing to further improve the reliability of LRNT assay.

References

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R., Simmons, C. P., Scott, T. W., Farrar, J. J., & Hay, S. I. (2013). The global distribution and burden of dengue. *Nature*, *496*(7446), 504–507.

Borisevich, V., Nistler, R., Hudman, D., Yamshchikov, G., Seregin, A., & Yamshchikov, V. (2008). A highly sensitive and versatile virus titration assay in the 96-well microplate format. *Journal of virological methods*, *147*(2), 197–205.

CDC. (2020). Dengue Retrieved on October 5, 2020, from https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/dengue

Dejnirattisai, W., Wongwiwat, W., Supasa, S., Zhang, X., Dai, X., Rouvinski, A., Jumnainsong, A., Edwards, C., Quyen, N., Duangchinda, T., Grimes, J. M., Tsai, W. Y., Lai, C. Y., Wang, W. K., Malasit, P., Farrar, J., Simmons, C. P., Zhou, Z. H., Rey, F. A., Mongkolsapaya, J., & Screaton, G. R. (2015). A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nature immunology*, *16*(2), 170–177.

Gubler, D. J. (1998). Dengue and dengue hemorrhagic fever. *Clinical microbiology reviews*, *11*(3), 480–496.

Guzman, M.G., & Kouri, G. (1996). Advances in dengue diagnosis. *Clin diagn lab immunil*, *3*(6), 621-627.

Henchal, E. A., Gentry, M. K., McCown, J. M., & Brandt, W. E. (1982). Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *The American journal of tropical medicine and hygiene*, *31*(4), 830–836.

Innis, B. L., Nisalak, A., Nimmannitya, S., Kusalerdchariya, S., Chongswasdi, V., Suntayakorn, S., Puttisri, P., & Hoke, C. H. (1989). An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *The American journal of tropical medicine and hygiene*, *40*(4), 418–427.

Junjhon, J., Panyasu, K., Kaewsod, S., Saipin, k., Somasa, P., Sangiambut, S., Sriburi, R., Keelapang, P., Puttikhunt C., Kasinrerk, W., Hirunpetcharat, C., & Sittisombut, N. Generation and characterization of luciferase-secreting, single-round infectious DENV reporter for functional antibody assays. Unpublished results.

Kato, F., & Hishiki, T. (2016). Dengue Virus Reporter Replicon is a Valuable Tool for Antiviral Drug Discovery and Analysis of Virus Replication Mechanisms. *Viruses*, *8*(5), 122.

Koo, T. K., & Li, M. Y. (2016). A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. *Journal of chiropractic medicine*, *15*(2), 155–163.

Mattia, K., Puffer, B. A., Williams, K. L., Gonzalez, R., Murray, M., Sluzas, E., Pagano, D., Ajith, S., Bower, M., Berdougo, E., Harris, E., & Doranz, B. J. (2011). Dengue reporter virus particles for measuring neutralizing antibodies against each of the four dengue serotypes. *PloS one*, *6*(11), 27252.

Rothman A. L. (2011). Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nature reviews. Immunology*, *11*(8), 532–543.

Russell, P.K., Nisalak, A., Sukhavachana, P., & Vivona, S. (1967). A plaque reduction test for dengue virus neutralizing antibodies. *J Immunol*, *99*(2), 285-90.

Sangiambut, S., Suphatrakul, A., Sriburi, R., Keelapang, P., Puttikhunt, C., Kasinrerk, W., Malasit, P., & Sittisombut, N. (2013). Sustained replication of dengue pseudoinfectious virus lacking the capsid gene by trans-complementation in capsid-producing mosquito cells. *Virus research*, *174*(1-2), 37–46.

Shan, C., Ortiz, D. A., Yang, Y., Wong, S. J., Kramer, L. D., Shi, P. Y., Loeffelholz, M. J., & Ren, P. (2017). Evaluation of a Novel Reporter Virus Neutralization Test for Serological Diagnosis of Zika and Dengue Virus Infection. *Journal of clinical microbiology*, *55*(10), 3028–3036.

Shan, C., Xie, X., Ren, P., Loeffelholz, M. J., Yang, Y., Furuya, A., Dupuis, A. P., 2nd, Kramer, L. D., Wong, S. J., & Shi, P. Y. (2017). A Rapid Zika Diagnostic Assay to Measure Neutralizing Antibodies in Patients. *EBioMedicine*, *17*, 157–162.

Song, K. Y., Zhao, H., Jiang, Z. Y., Li, X. F., Deng, Y. Q., Jiang, T., Zhu, S. Y., Shi, P. Y., Zhang, B., Zhang, F. C., Qin, E. D., & Qin, C. F. (2014). A novel reporter system for neutralizing and enhancing antibody assay against dengue virus. *BMC microbiology*, *14*,(44), 44-52.

Summers, P. L., Cohen, W. H., Ruiz, M. M., Hase, T., & Eckels, K. H. (1989). Flaviviruses can mediate fusion from without in *Aedes albopictus* mosquito cell cultures. *Virus research*, *12*(4), 383–392.

Vorndam, V., & Kuno, G. (1997). Laboratory diagnosis of dengue virus infections. *CAB International London United Kingdom*, *44*(2), 313-333.

Wahala, W. M., & Silva, A. M. (2011). The human antibody response to dengue virus infection. *Viruses*, *3*(12), 2374–2395.

Watanabe, K., Hirokawa, C., Kon, M., Tamura, T., & Nishikawa, M. (2008). Estimation of focus reduction neutralization test for measurement of neutralizing antibody titer against Japanese encephalitis virus. *Japanese journal of infectious diseases*, *61*(5), 424–425.

WHO. (2009). Dengue guidelines for diagnosis, treatment, prevention and control Retrieved on Mar 3, 2020 from: http://www.who.int /tdr/publications/documents/dengue-diagnosis.pdf.

Yasumura, Y., & Kawakita, M. (1963). The research for the SV40 by mean of tissue culture technique. *Nippon Rinsho*, *21*(63), 1201-1219.

Yenchitsomanus, P. T., Sricharoen, P., Jaruthasana, I., Pattanakitsakul, S. N., Nitayaphan, S., Mongkolsapaya, J., & Malasit, P. (1996). Rapid detection and identification of dengue viruses by polymerase chain reaction (PCR). *The Southeast Asian journal of tropical medicine and public health*, *27*(2), 228–236.

Zhang, P. T., Shan, C., Li, X. D., Liu, S. Q., Deng, C. L., Ye, H. Q., Shang, B. D., Shi, P. Y., Lv, M., Shen, B. F., Qin, C. F., & Zhang, B. (2016). Generation of a recombinant West Nile virus stably expressing the Gaussia luciferase for neutralization assay. *Virus research*, *211*, 17–24.