Evaluation of three alternative methods to the plaque reduction neutralizing assay for measuring neutralizing antibodies to dengue virus serotype 2

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Abstract

Background Dengue is a global public health challenge which requires accurate diagnostic methods for surveillance and control. The gold standard for detecting dengue neutralizing antibodies (nAbs) is the plaque reduction neutralization test (PRNT), which is both labor-intensive and time-consuming. This study aims to evaluate three alternative approaches, namely, the MTT-based (or (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) microneutralization assay, the xCELLigence real-time cell analysis (RTCA), and the immuno-plaque assay-focus reduction neutralization test (iPA-FRNT).

Methods Twenty-two residual serum samples were tested for DENV-2 nAbs using all four assays at three neutralization endpoints of 50%, 70% and 90% inhibition in virus growth. For each neutralization endpoint, results were compared using linear regression and correlation analyses. Test performance characteristics were further obtained for iPA-FRNT using 38 additional serum samples.

Results Positive correlation of DENV-2 neutralization titers for the MTT-based microneutralization assay and the PRNT assay was only observed at the neutralization endpoint of 50% ($r = 0.690$). In contrast, at all three neutralization end points, a linear trend and positive correlation of DENV-2 neutralization titers for the xCELLigence RTCA and the PRNT assays were observed, yielding strong or very strong correlation ($r = 0.829$ to 0.967). This was similarly observed for the iPA-FRNT assay ($r = 0.821$ to 0.916), which also offered the added advantage of measuring neutralizing titers to non-plaque forming viruses.

Conclusion The xCELLigence RTCA and iPA-FRNT assays could serve as suitable alternatives to PRNT for dengue serological testing. The decision to adopt these methods may depend on the laboratory setting, and the utility of additional applications offered by these technologies.

Keywords Plaque reduction neutralization test, PRNT, Serology, Neutralizing antibodies, Dengue

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Background

Serological tests for virological infections are crucial for laboratory diagnosis, surveillance, and the management and control of viral diseases [1]. The assays are widely used for the detection of serum antibodies following a viral infection, and are often based on antigen-antibody interactions, including lateral flow-based rapid tests [2–5], immunoblots [6, 7], enzyme-linked immunosorbent assays (ELISA) [8–12], and other high-throughput chemiluminescence or electrochemiluminescence-based assays [13–15]. However, these serological methods may occasionally produce false-positive results due to non-specific reactivity or cross-reactivity with closely related viruses, and it is not possible to detect and quantify the level of neutralizing antibodies (nAbs) present in the serum [16–18]. The plaque reduction neutralization test (PRNT), which compares and differentiates infections caused by closely related viruses based on neutralizing titers, is often used as a benchmark against other serological tests and considered as the "gold standard" for the detection and quantification of virus nAbs in the serum of an infected person [19]. Notably, PRNT has been widely used in SARS-CoV-2 research, particularly for vaccine trials for the assessment of vaccine efficacy and protective immunity [20–25], and for dengue serological studies to distinguish infections caused by the four genetically similar but antigenically distinct serotypes of the dengue virus (DENV; serotypes 1 to 4) [26–29].

Dengue is a viral disease transmitted by the *Aedes* mosquito and is a significant public health challenge globally, with an estimated 100–400 million infections occurring yearly [30]. Infection by any of the four serotypes of DENV can result in a range of clinical symptoms, from mild fever to fatal dengue shock syndrome [31]. Secondary infections caused by a different dengue serotype may also cause challenges in diagnosis, as antibody profiles do not follow that of a primary infection, whereby after infection, anti-dengue IgG levels will peak instead of anti-dengue IgM [32]. Serological testing for dengue is therefore considered to be one of the most challenging, as cross-reactivity with other flaviviruses may occur; and the diagnosis of secondary and subsequent infections due to other DENV serotypes is also complex [17, 33, 34]. The PRNT assay is therefore an essential method for the diagnosis of dengue and immunity/pre-vaccination screening as it can both determine dengue serostatus and measure serotype-specific immunity [35, 36].

Although PRNT is a widely used method for the detection and quantification of virus nAbs in the serum of an infected person, it has its limitations. The method is labor-intensive, time-consuming, and has a low sample throughput, and is typically not used for screening large numbers of samples [19]. In addition, the use of plaque-forming viruses is a prerequisite for the PRNT, thus

excluding its applicability in many important viruses that are non-cytopathic [37, 38]. To address some of these limitations, a few alternative neutralization test methods have been developed, with improvements to the turnaround-time, throughput, and/or reduced hands-on time. After the antibody neutralization step, these assays measure virus infection and cell death via (i) colorimetric, (ii) electrical impedance, or (iii) antibody-based readouts [37, 39–43], instead of measuring plaque formation. Briefly, the colorimetric MTT-based (or (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a live-dead assay that indirectly measures cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity [44]. The assay has been previously adapted as a microneutralization test to quantify Zika virus nAbs in sera [41]. The xCELLigence real-time cell analysis (RTCA) system (Agilent Technologies) utilizes electrical impedance to continuously monitor changes in cell proliferation, morphology, adhesion, and viability caused by virus-induced cytopathic effects (CPE) and has been used to measure nAbs against West Nile virus, H1N1, and human enterovirus 71 [37, 39, 40]. The immuno-plaque assay-focus reduction neutralization test (iPA-FRNT) on the other hand, utilizes infrared conjugated virus-specific antibodies to detect focus-forming units (FFU) instead of plaque-forming units (PFU) with a reduced assay turnaround time from six days (for PRNT) to three days, and has been established in the quantification of both flaviviruses and SARS-CoV-2 [42, 43]. To visualize foci, an infrared imager is required to scan the iPA-FRNT plates, and the foci counting is performed on scanned images.

As these assays were not all tested against DENV, this study aimed to evaluate the utility of the assays, namely, (i) the MTT-based microneutralization assay; (ii) the xCELLigence RTCA; and (iii) the iPA-FRNT for the detection of DENV nAbs against the PRNT assay [19]. Through the measurement of DENV-2 nAbs, the performance of the assays were compared with PRNT using a panel of 22 serum samples. The iPA-FRNT was subject to further evaluation through measurement of DENV-4 nAbs and an additional panel of 38 serum samples. The test throughput, turnaround time, and other aspects affecting operational feasibility for all assays were also assessed. Overall, our study provided insights on the strengths and limitations of the assays as alternatives to the PRNT for the detection of DENV-2 nAbs.

Methods

Cells and viruses

Baby Hamster Kidney (BHK) cells (ATCC, CCL-10) were maintained in RPMI-1640 media supplemented with 5% heat-inactivated (HI) fetal bovine serum (FBS; Gibco, Brazil), 10 mM HEPES, 1mM sodium pyruvate, 100 U/

mL penicillin-streptomycin, and 2mM L-glutamine, and grown at 37 °C with 5% CO_2. C6/36 cells were cultured in Leibovitz's L-15 media supplemented with 10% HI FBS, 1mM sodium pyruvate, 100 U/mL penicillin-streptomycin, 2mM L-glutamine and grown at 28 °C. All virus stocks and serum samples were diluted in RPMI-1640 media supplemented with 2% HI FBS, 10 mM HEPES, 1mM sodium pyruvate, 100 U/mL penicillin-streptomycin, and 2mM L-glutamine.

Two dengue virus strains were selected for the evaluation. The DENV-2 strain, EHIE51858Y19 (Cosmopolitan genotype) was selected as the study focused on the measurement of DENV-2 nAbs in Singapore, where DENV-2 has been a common circulating strain which has caused large outbreaks [45]. Unlike the PRNT, MTT-based microneutralization and xCELLigence RTCA assays, the iPA-FRNT assay does not rely on CPE or plaque formation for viral detection. Therefore, to assess whether the iPA-FRNT could offer enhanced detection in measuring neutralizing antibodies to non-plaque forming viruses, a second DENV-4 strain, EHIE30932Y19 (Genotype II, the main DENV-4 strain circulating in Singapore from 2019 to 2020), which is unable to form plaques as demonstrated by prior testing, was also used in the study. Both viruses were isolated from clinical samples and propagated in C6/36 cells. DENV-2 was titrated by plaque assay, while DENV-4 was titrated by iPA (Supplemental Material). A standard curve of DENV-2 virus titration was carried out for the MTT and xCELLigence RTCA assays to determine an appropriate starting virus concentration for the neutralization tests. Due to differences in the analytical range between the two assays, the input virus concentration used for the MTT-based microneutralization assay and xCELLigence RTCA neutralization test were 10^4 and 10^5 PFU/mL, respectively.

Serum samples

Residual serum samples from a serosurvey collected from healthy blood donors by the Blood Services Group of the Singapore Health Sciences Authority from July to October 2021 were used in this study. Ethics approval was sought from the National Healthcare Group Domain Specific Review Board (NHG DSRB 2021/00257) and Bioethics Review Committee of National Environment Agency/Environmental Health Institute (NEA/EHI/IRB022). The serum samples were tested for the presence of dengue IgG antibodies using the PanBio™ Dengue IgG Indirect ELISA kit (Abbott) and 22 samples were randomly selected from the total number of serum samples collected (19 dengue IgG positive, 3 dengue IgG negative). The serum samples were heat-inactivated at 56 °C for 30 min and aliquoted to minimize freeze-thaw cycles. This serum panel was used for all three methods evaluated, including the PRNT assay (Supplemental Material).

An additional 11 dengue IgG positive, and 27 dengue IgG negative serum samples were tested for DENV-2 nAbs by PRNT and iPA-FRNT. The PanBio Dengue IgG Indirect ELISA results for the evaluation panel and all serum samples are available in Tables S1 and S2, respectively (Supplemental Material).

Neutralization test using the MTT-based microneutralization assay

96-well plates were seeded with 4.0×10^3 BHK cells/well on the same day of the experiment and allowed to adhere for a minimum of 4 h before use. Serial dilutions of serum samples (an initial dilution of 1:10, followed by serial 4-fold dilutions from 1:40 to 1:640) were mixed with an equal volume of DENV-2 suspension containing 10^4 PFU/mL and incubated at 37 °C for 1 h. The media from the cell monolayer plate was replaced with 50 µL/well of each virus-serum mixture and incubated at 37 °C with 5% CO_2 for 1 h, followed by the addition of 50 µL/well of RPMI-1640 supplemented with 2% FBS. After 6 days incubation, the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, USA) was performed according to the manufacturer's instructions using the same day method.

Neutralization test using the xCELLigence real-time cell analyzer (RTCA)

E-Plates were seeded with 1.5×10^4 BHK cells/well, and the assay was carried out following the protocol described by Suryadevera and colleagues [46]. Serial dilutions of serum samples as described above were mixed with an equal volume of DENV-2 suspension containing 10^5 PFU/mL and incubated at 37 °C for 1 h. Subsequently, the media in the E-Plate containing the cell monolayer was replaced with 50 µL/well of each virus-serum mixture and the plate was reloaded on the RTCA workstation. After 1 h incubation at 37 °C with 5% CO_2, 100 µL of RPMI-1640 supplemented with 2% FBS was added to each well. The E-Plate was returned to the workstation and electrical impedance was measured every 15 min for 7 days. The area under the curve (AUC) data were obtained using the RTCA Software Pro.

Focus-reduction neutralization test using iPA (iPA-FRNT)

96-well plates were seeded with 1.5×10^4 BHK cells/well a day before the experiment. Serial dilutions of serum samples as described above were mixed with an equal volume of DENV-2 or DENV-4 suspension containing 144 PFU or FFU in 120 µL, and incubated at 37 °C for 1 h. Subsequently, 25 µL/well of each virus-serum mixture was added to the cell monolayer and incubated at 37 °C for 1 h. The inoculum were replaced with 150 µL/well of overlay media (1:1 mixture of 2% carboxylmethyl cellulose and 2X RPMI-1640 supplemented with 4% FBS)

and further incubated for 42 h and 48 h for DENV-2 and DENV-4, respectively, before removing the overlay. The fixing and antibody probing steps were performed as described by Amarilla and colleagues [43]. The plates were scanned on the Odyssey® M imaging system (LI-COR Biosciences) and foci were counted manually.

Calculation of percentage inhibition and derivation of neutralization titers

For the MTT-based microneutralization and xCELLigence RTCA assays, the optical density (O.D.) values and AUC values were used for calculating percentage inhibition, respectively. The difference between the cell control value and the virus control value was interpreted as a 100% inhibition effect. Percentage inhibition of the serum samples were determined as follows: (100/Cell Control Result – Virus Control Result) × (Sample Result – Virus Control Result). For iPA-FRNT, the percentage inhibition of the serum samples were determined as follows: [1 – (Number of Foci in Sample Well/Average No. of Foci in Virus Control Wells)] × 100%. With the percentage inhibition obtained, neutralization titers for each method were derived using a four-parameter logistic curve calculator [47].

Statistical analyses

Data was visualized and statistical analyses were carried out using GraphPad Prism 9.0 (GraphPad Software, Boston, Massachusetts USA). For each level of inhibition, PRNT results were compared with the respective alternative method using linear regression and correlation analyses. The strength of correlation was assessed to be negligible ($r=0.00-0.10$), weak ($r=0.10–0.39$), moderate ($r=0.40–0.69$), strong ($r=0.70–0.89$) and very strong ($r=0.90-1.00$) based on the guide provided by Schober et al. [48]. The Area under Receiver-Operating Characteristics curve (AUROCC) was also determined (Supplemental Material). The 2×2 contingency table and test performance characteristics including test sensitivity and specificity, comparing the iPA-FRNT and PRNT assays were analyzed using the QuickCalcs online calculator (Graphpad, USA).

Results

Serum samples screened by PRNT for DENV-2 nAb

DENV-2 neutralizing antibody (nAb) titers were obtained for 22 serum samples at three levels of virus neutralization endpoints (PRNT$_{50}$, 50%; PRNT$_{70}$, 70%; and PRNT$_{90}$, 90%; reduction in plaque counts) using the PRNT assay (Fig. 1). Overall, the serum panel yielded a range of antibody neutralizing titers which would be used
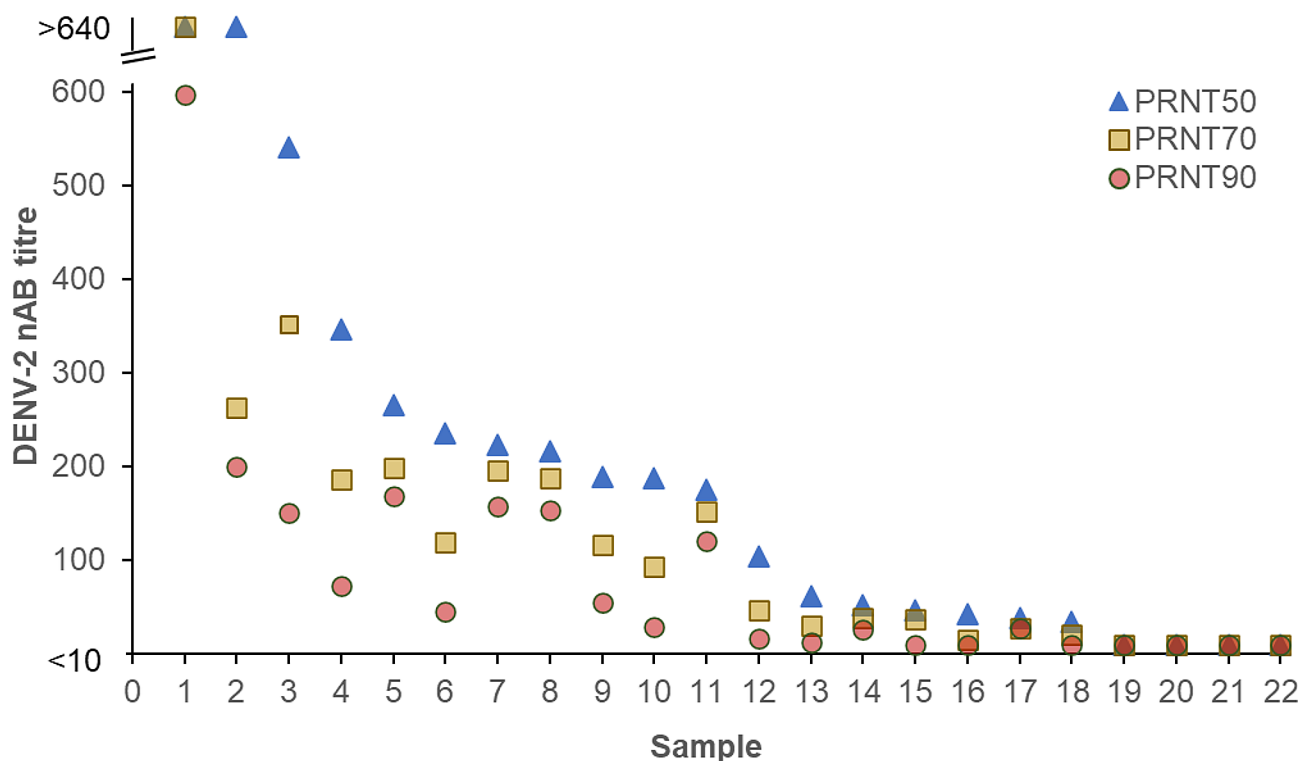

Fig. 1 Neutralizing DENV antibody titers against DENV-2 Cosmopolitan genotype virus strain using the PRNT assay. DENV-2 neutralizing antibody (nAb) titers for 22 serum samples at three levels of virus neutralization endpoints (samples 1–19: Dengue IgG positive; samples 20–22: Dengue IgG negative)

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as a reference for comparison with the three alternative PRNT methods evaluated (Table S1).

No significant correlation between DENV-2 neutralizing antibody titers for the MTT-based microneutralization assay and the PRNT assay for two of three neutralization endpoints

While qualitative results were generally comparable for all three methods evaluated (Supplemental Material, Figure S1, Table S3), quantitatively, linear regression analyses comparing the MTT-based microneutralization assay with the PRNT assay yielded near-zero slopes for the neutralization endpoints of 70% and 90%, suggesting that the neutralization titers obtained by both assays were not comparable (Fig. 2B-C; Table 1). However, at the neutralization endpoint of 50%, there was a linear trend between the neutralization titers obtained for the MTT-based microneutralization and the PRNT assays (slope 0.976 ± 0.228), with a co-efficient of determination of $r^2 = 0.477$ (Table 1). Similarly, positive correlation of DENV-2 neutralization titers for both assays was observed only for the neutralization endpoint of 50%, with moderate correlation (NT$_{50}$: $r = 0.690$, 95% CI [0.397, 0.861]) (Table 1). At the higher endpoints of 70% and 90%, there was no significant correlation between neutralizing titers for the MTT-based microneutralization assay and the PRNT assay ($p > 0.05$). Additionally, the wide confidence interval ranges suggests variability in the neutralizing titers obtained (NT$_{70}$: $r = 0.261$, 95%
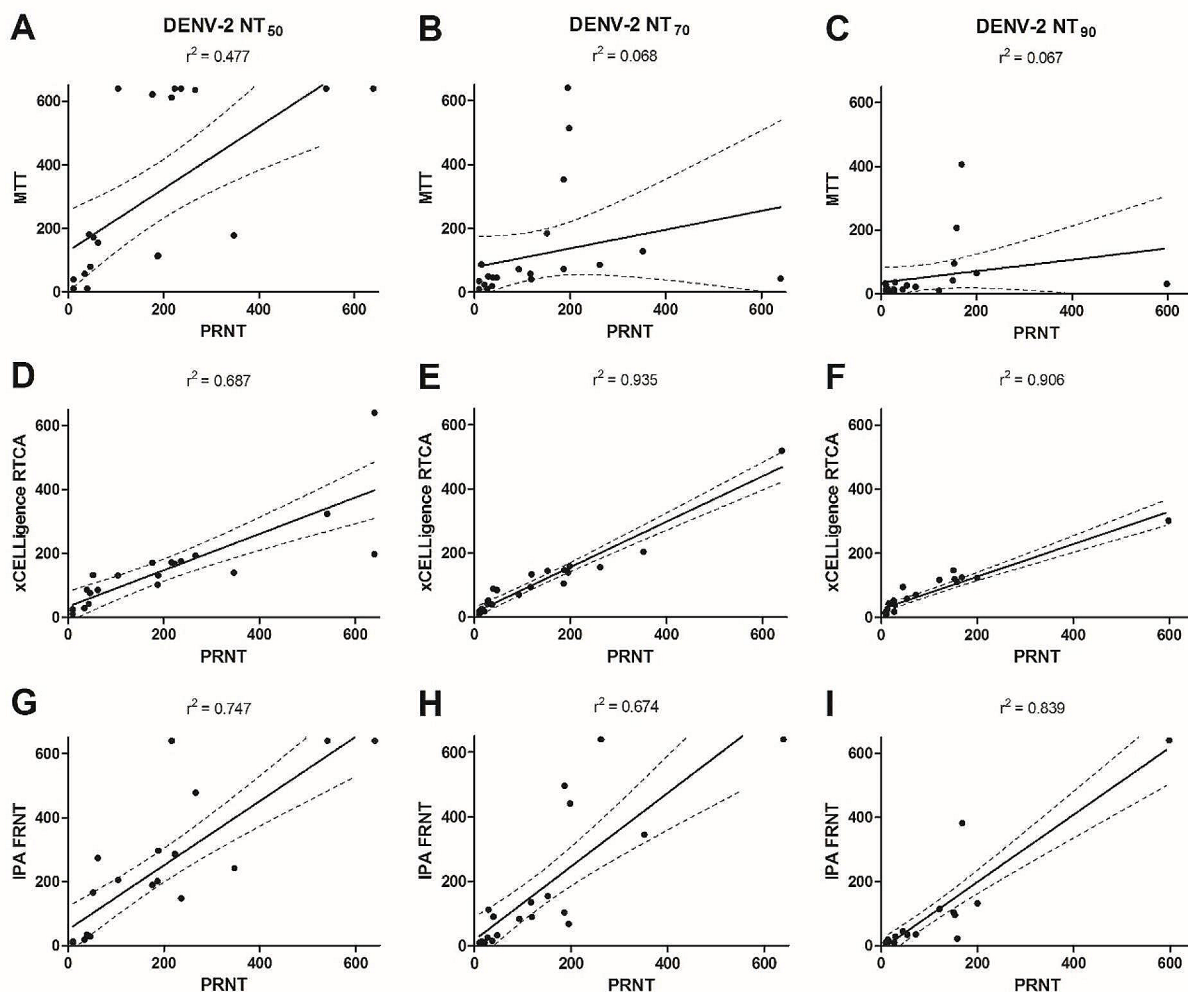

Fig. 2 Comparison of DENV-2 neutralization titers obtained by the MTT-based microneutralization, the xCELLigence RTCA and the iPA-FRNT assays with the standard PRNT assay. Linear regression and scatterplot of neutralizing titers comparing the evaluated assay with standard PRNT at neutralization endpoints of 50% (left panel), 70% (middle panel) and 90% (right panel). **Panel A-C**: MTT-based microneutralization assay. Non-linearity was observed for two of three neutralization endpoints (NT$_{70}$ and NT$_{90}$). (**D-F**) xCELLigence RTCA assay. (**G-I**) iPA-FRNT assay. At all three neutralization endpoints, positive correlation was observed for both the xCELLigence RTCA and iPA-FRNT assays

Table 1 Linear regression and correlation analyses

	MTT NT$_{50}$	MTT NT$_{70}$	MTT NT$_{90}$	xCELLigence RTCA NT$_{50}$	xCELLigence RTCA NT$_{70}$	xCELLigence RTCA NT$_{90}$	iPA-FRNT$_{50}$	iPA-FRNT$_{70}$	iPA-FRNT$_{90}$
Linear Regression									
Co-efficient of determination (r^2)	0.477	0.068	0.067	0.687	0.935	0.906	0.747	0.674	0.839
Slope	0.976±0.228	0.293±0.243	0.179±0.149	0.568±0.086	0.709±0.042	0.507±0.037	1.00±0.130	1.139±0.177	1.048±0.103
Deviation from zero	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Correlation									
Pearson r (95% CI)	0.690 (0.397–0.861)	0.261 (-0.181–0.615)	0.259 (-0.183-0.614)	0.829 (0.626–0.927)	0.967 (0.921–0.987)	0.952 (0.885–0.981)	0.864 (0.697–0.943)	0.821 (0.611–0.923)	0.916 (0.805–0.965)
P value	0.0004	0.2412	0.2447	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Significant correlation	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes

Table 2 Test performance characteristics of iPA-FRNT using PRNT as a reference

Test results*		Standard PRNT$_{50}$ (Positive cut-off titer ≥ 10)		Total
		Positive	**Negative**	
iPA-FRNT$_{50}$ (Positive cut-off titer ≥ 10)	Positive	29	2	31
	Negative	0	29	29
	Total	29	31	60

*The lower limit titer of quantification for the iPA-FRNT and PRNT were 14 and 34, respectively

CI [-0.181,0.615]; NT$_{90}$: r=0.259, 95% CI [-0.183,0.614]) (Fig. 2A-C; Table 1).

Linear trend and positive correlation of DENV-2 neutralizing antibody titers for the xCELLigence RTCA and the PRNT assay for all three neutralization endpoints

At all three neutralization endpoints, a linear trend and positive correlation of DENV-2 neutralization titers for the xCELLigence RTCA and the PRNT assays were observed, yielding strong (NT$_{50}$: r=0.829, 95% CI [0.626,0.927]) or very strong correlation (NT$_{70}$: r=0.967, 95% CI [0.921,0.987]; NT$_{90}$: r=0.952, 95% CI [0.885,0.981]) (Fig. 2D-F; Table 1). The narrow confidence interval ranges further highlight the comparability between the neutralizing antibody titers for both assays. However, it was observed that DENV-2 neutralizing samples obtained by the xCELLigence RTCA generally yielded a lower slope, and in turn lower titer and sensitivity when compared with standard PRNT at all neutralization cut-offs (Slope < 1.00, NT$_{50}$: 0.568±0.086, NT$_{70}$: 0.709±0.042, NT$_{90}$: 0.507±0.037) (Fig. 2D-F; Table 1).

Linear trend and positive correlation of DENV-2 neutralizing antibody titers for the iPA-FRNT and the PRNT assay for all three neutralization endpoints

Similar to the xCELLigence RTCA assay, a linear trend and positive correlation of DENV-2 neutralization titers for the iPA-FRNT and the PRNT assays were observed for all three neutralization endpoints, yielding strong (NT$_{50}$: r=0.864, 95% CI [0.697,0.943]; NT$_{70}$: r=0.821, 95% CI [0.611,0.923]) or very strong correlation (NT$_{90}$: r=0.916, 95% CI [0.805,0.965]) (Fig. 2G-I; Table 2). DENV-2 neutralizing titers obtained by the iPA-FRNT assay were also generally comparable to standard PRNT at all neutralization endpoints (Slope ~ 1, NT$_{50}$:1.00±0.130, NT$_{70}$:1.139±0.177, NT$_{90}$:1.048±0.103) (Fig. 2G-I; Table 2).

Test performance characteristics of iPA-FRNT compared to standard PRNT

Based on the comparable correlation and titers obtained, the iPA-FRNT was further evaluated with an additional 11 dengue IgG positive and 27 dengue IgG negative

serum samples, totaling 60 samples (30 IgG positive and 30 IgG negative) (Table S2, Supplementary Material). Using the neutralization endpoint of 50%, strong correlation was similarly observed ($r=0.875$, 95% CI [0.798,0.924]) (Figure S2, Supplementary Material). A 2×2 contingency table was constructed comparing the iPA-FRNT$_{50}$ and PRNT$_{50}$ results (Table 2). The iPA-FRNT yielded 100% sensitivity, correctly identifying all 29 PRNT-positive samples, and 93.5% specificity, identifying 29 out of 31 PRNT-negative samples. The positive predictive value was 93.5% while the negative predictive value was 100%.

Antibody neutralization against non-plaque forming virus with the iPA-FRNT

Using DENV-4 (EHIE30932Y19; Genotype II) as the challenge virus, reliable neutralization titers could not be obtained using the standard PRNT, MTT-based neutralization and xCELLigence RTCA assays as the strain was unable to produce CPE and in turn, form plaques. However, neutralizing titers were obtained using iPA-FRNT for 8 out of 22 samples (titer $\geq$10, Table 3). This could be explained since iPA-FRNT is based on dengue antigen-specific immunostaining rather than a measurement of CPE or cell death. As all four dengue serotypes circulate in Singapore [49], it is possible that the serum panel comprises of samples that have antibodies reactive to individual dengue serotypes, or a combination of serotypes due

Table 3 Neutralizing DENV antibody titers against non-plaque forming DENV-4 virus strain using the iPA-FRNT assay

IPA-FRNT titers: DENV-4		
Sample	FRNT$_{50}$	FRNT$_{70}$	FRNT$_{90}$
1	<10	<10	<10
2	<10	<10	<10
3	<10	<10	<10
4	27	13	<10
5	<10	<10	<10
6	17	12	<10
7	11	10	<10
8	<10	<10	<10
9	<10	<10	<10
10	14	<10	<10
11	10	<10	<10
12	<10	<10	<10
13	19	12	<10
14	<10	<10	<10
15	47	17	<10
16	<10	<10	<10
17	<10	<10	<10
18	<10	<10	<10
19	<10	<10	<10
20	<10	<10	<10
21	<10	<10	<10
22	27	12	<10

to secondary infection or cross-protection. As there were no PRNT titers obtained, a comparison could not be performed between iPA-FRNT and PRNT.

Discussion

The performance of three alternative methods to the standard PRNT, namely the MTT-based microneutralization assay, the xCELLigence RTCA system, and the iPA-FRNT, were evaluated for the detection of DENV-2 serotype-specific neutralizing antibodies. Overall, both the xCELLigence RTCA and iPA-FRNT assays yielded strong or very strong positive correlation with PRNT for all three neutralization endpoints tested. Although the xCELLigence RTCA assay generally yielded higher correlation co-efficients (Pearson's r), the absolute titers from the iPA-FRNT were more comparable with standard PRNT (slope $\sim$1). The iPA-FRNT also offers the added advantage of assessing neutralization to non-CPE and plaque-forming viruses. In contrast, no significant correlation was observed between DENV-2 nAb titers for the MTT-based microneutralization assay and the PRNT assay for two of three neutralization endpoints, with only moderate correlation for the neutralization endpoint at 50% inhibition.

The test throughput, turnaround time, recurring cost and operational considerations of the assays were also assessed (Table 4). The MTT-based microneutralization assay has a simple workflow which can be easily adapted for high-throughput screening and does not require specialized equipment except for an absorbance plate reader which is found in most serological laboratories. While the MTT-based microneutralization assay has been routinely used in antiviral compound screening and cytotoxicity tests as a "live-dead" assay to assess the rate of cell proliferation and cytotoxicity [50–52], and had been previously adapted to microneutralization assays; results from the present study suggests only moderate correlation with the PRNT assay at the neutralization endpoint of 50%, with significant decline in correlation at more stringent endpoints (70% and 90%). As DENV do not necessarily induce lytic cell death, this could have resulted in the reduced sensitivity of the MTT-based microneutralization assay, and in turn, the observed weak correlation between the MTT-based microneutralization assay and the PRNT. Caution is thus advised if the MTT-based neutralization assay is to be used as an alternative to PRNT for DENV.

The xCELLigence RTCA system offers an automated workflow and quantitative readout, which reduces hands-on time and makes the process less laborious. However, the analyzer has a maximum running capacity of up to one or six 96-well plates per run (equivalent to 16 or 96 samples, using the Single Plate and Multiple Plates model, respectively), and may not be suitable for

Table 4 Comparison of alternative methods to the plaque reduction neutralizing assay for measuring neutralizing antibodies to DENV-2

Factor	Standard PRNT	MTT-based microneutralization	xCELLigence RTCA	iPA-FRNT
Correlation with PRNT	Not applicable	Weak ($PRNT_{50}$) or no correlation ($PRNT_{70/90}$)	Strong ($PRNT_{50}$) or very strong ($PRNT_{70/90}$)	Strong ($PRNT_{50/70}$) or very strong ($PRNT_{90}$) correlation
Throughput	4 samples per 24-well plate	16 samples per 96-well plate	16 samples per 96-well plate (Up to six plates)	16 samples per 96-well plate
Ability to detect non-plaque forming viruses	No	No	No	Yes
Turnaround time	5–7 days	6 days	7 days	3 days
Specialized equipment required	No	Absorbance plate reader	RTCA reader	Infra-red imager
Consumables	• Virus, cell culture reagents & consumables	• Virus, cell culture reagents & consumables	• E-plates • Virus, cell culture reagents & consumables	• Infra-red dye conjugated virus-specific antibodies • Virus, cell culture reagents & consumables
Recurring cost per sample[1]	• $	• $$	• $$$	• $
Equipment cost[1]	• Not applicable	• $$$$	• $$$$$$$	• $$$$$

[1] "$" represents approximately 10-fold increase in cost

high-throughput screening. Besides the limited throughput, the initial cost of the analyzer and the recurring cost of the E-Plates should be considered before adopting the method. Optimization and validation of this method would also be required for each new virus (e.g. dengue serotypes) before implementation. Additionally, the system should also be tested on its compatibility with viruses that do not cause CPE, since the assay relies on impedance readings to monitor changes in cell viability. Nevertheless, results from this study showed that the xCELLigence RTCA neutralization test is a promising alternative to the standard PRNT, yielding higher correlation values compared with the other assays evaluated. Besides its utility for virus neutralization tests, the xCELLigence RTCA have also been used in the determination of virus titers [37, 53, 54], viral growth kinetics [39], and antiviral compound screening [55–57].

The iPA-FRNT workflow offers the shortest turnaround time amongst the methods evaluated and is useful where rapid results are essential for timely patient management and outbreak control. The assay turnaround time could be significantly reduced by half, taking three days instead of 6–7 days required by PRNT and other methods evaluated. Notably, the assay also yield test sensitivity and specificity values of above 90% when compared with PRNT. While the ability of the virus to induce CPE is a prerequisite for most cell-based virology assays, the iPA-FRNT does not have this requirement as it relies on foci detection based on antibody-antigen interactions. Therefore, among the assays evaluated, it is the only method which could detect and quantify non-plaque forming viruses, as demonstrated by the DENV-4 strain used in the evaluation. Furthermore, the iPA-FRNT has been reported to be further optimized on 384-well plates,

with comparable nAb levels between 96- and 384-well plates observed [43], offering increased scalability. The assay therefore has potential use in seroprevalence studies, where large populations are screened for DENV serotype-specific immunity investigations [42, 43, 58–63]. To date, iPA-FRNT has also been used in the detection of antibodies to human respiratory syncytial virus [61], Zika virus [42] and SARS-CoV-2 [43]. Although the evaluation showed that the iPA-FRNT is an efficient high-throughput screening method and a promising alternative to the standard PRNT, similar to the xCELLigence RTCA, initial equipment cost of the infrared imager for foci visualisation, and recurring consumables costs for specific antibodies would be required. The need for specific antibodies may also limit its applicability for screening novel viruses.

This study has several limitations. We were limited by the small sample size evaluated, and the number of DENV serotypes tested. Dengue infection status of the donors were also not available. In future studies, a larger sample size tested against all four serotypes would provide a more robust assessment of alternative PRNT methods and their cross-neutralization. Additionally, wild-type DENV strains instead of laboratory strains were used. While well-established laboratory strains offer consistent plaque formation and assay reproducibility, they may not be relevant in the local epidemiological context. The decision to use either wild-type or laboratory (or both) DENV strains would therefore depend on the objectives of the study. While the PRNT assay was carried out based on guidelines from WHO [19], the assay also could be further adapted for non- or slow-CPE forming DENV strains, such as through extending the adsorption time or the use of different cell lines. Limited

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access to equipment, particularly the xCELLigence RTCA system, which was only available on loan, also limited the number of samples and technical replicates evaluated in this study.

Conclusions

In conclusion, this study found that both the xCELLigence RTCA and iPA-FRNT assays can serve as viable alternatives to PRNT for dengue serological testing. They offer comparable results while also providing additional benefits such as automation (xCELLigence RTCA) or faster turnaround time and increased throughput (iPA-FRNT). Although these assays may require specialized equipment and consumables, they have the potential to reduce hands-on time and manpower. The decision to adopt either method may depend on factors such as the laboratory's needs and the relevance of other applications offered by the respective technologies.

Abbreviations
BHK	Baby Hamster Kidney
CPE	Cytopathic effects
DENV	Dengue virus
ELISA	Enzyme-linked immunosorbent assay
FFU	Focus-forming units
iPA	FRNT-immuno-plaque assay-focus reduction neutralization test
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nAb	Neutralizing antibodies
PFU	Plaque-forming units
PRNT	Plaque reduction neutralization test
RTCA	Real-time cell analysis

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12985-024-02459-y.

Supplementary Material 1

Data availability
No datasets were generated or analysed during the current study.

Declarations

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