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ABSTRACT The global spread and infective complications of Zika virus (ZKV) and dengue virus (DENV) have made them flaviviruses of public health concern. Serological diagnosis can be challenging due to antibody cross-reactivity, particularly in secondary flavivirus infections or when there is a history of flavivirus vaccination. The virus neutralization assay is considered to be the most specific assay for measurement of anti-flavivirus antibodies. This study describes an assay where the neutralization endpoint is measured by real-time PCR, providing results within 72 h. It demonstrated 100% sensitivity (24/24 ZKV and 15/15 DENV) and 100% specificity (11/11 specimens) when testing well-characterized sera. In addition, the assay was able to determine the correct DENV serotype in 91.7% of cases. The high sensitivity and specificity of the real-time PCR neutralization assay makes it suitable to use as a confirmatory test for sera that are reactive in commercial IgM/IgG enzyme immunoassays. Results are objective and the PCR-based measurement of the neutralization endpoint lends itself to automation so that throughput may be increased in times of high demand.

KEYWORDS dengue, flavivirus, serology, Zika

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Zika virus (ZKV) and dengue virus (DENV) are members of the family *Flaviviridae*, genus *Flavivirus*, which also includes yellow fever, Japanese encephalitis, and West Nile viruses. There are at least 4 closely related but serologically distinct DENV, designated DENV serotypes 1 through 4 (1). There is only transient and weak cross-protection among the 4 DENV serotypes, so individuals living in an area where DENV is endemic can be infected up to 4 times (2).

ZKV and DENV have spread across the tropics in Africa, Asia, the Pacific, and the Americas with their vector mosquitoes *Aedes aegypti* and *A. albopictus* (3, 4). Cocirculation of ZKV and DENV types 1 through 4 has been documented in both French Polynesia and Brazil (5, 6) and likely occurs in many other tropical countries. Incursions into temperate countries have also occurred, with autochthonous transmission observed in Europe (DENV in southeastern France, Croatia, and Madeira Island) and the United States (DENV and ZKV in Florida) (7–9).

Both ZKV and DENV infection can produce a wide spectrum of illnesses with many asymptomatic or subclinical infections (10, 11). ZKV's potential to cause fetal central nervous system abnormalities, growth restriction, and death (12) has necessitated ZKV testing of potentially exposed pregnant women, among whom nonspecific serological activity may occur, and asymptomatic individuals with low pretest probabilities of positive ZKV serology.

Direct detection of ZKV nucleic acids has limited utility in asymptomatic patients, as the periods of viremia and shedding in urine and saliva are relatively brief, occurring

TABLE 1 Real-time PCR neutralization assay for ZKV and a convalescent-phase serum specimen collected following a ZKV infection diagnosis by PCR[a]

Serum dilution	ZKV-positive serum[b]	ZKV-negative serum
1/20	**35.4**	27.1
1/40	**35.2**	28.1
1/80	**35.7**	28.8
1/160	**35.2**	27.0
1/320	**35.7**	26.8
1/640	29.5	29.1
1/1,280	29.8	29.2
1/2,560	28.6	28.7
Background	33.1	

[a]There is neutralizing antibody to ZKV in the known positive specimen (titer, 320) but not in the negative specimen (titer, <20).
[b]Real-time PCR cycle thresholds for each neutralization between the known positive serum, a negative serum specimen, and the ZKV stock virus at working dilution. The presence of neutralizing antibody is demonstrated by a cycle threshold greater than or equal to the background cycle threshold (in bold).

shortly after the onset of symptoms, if present (13–15). Consequently, we must rely on serology to diagnose infection in asymptomatic patients. A number of commercial enzyme immunoassays (EIAs) have been designed to detect IgM and IgG to ZKV and DENV. However, they are known to lack specificity, particularly when it comes to IgG. This tends to be more of a concern during secondary flavivirus infections, when there can be extensive IgG cross-reactivity with other flaviviruses, and in some cases, higher serological activity to the original infecting flavivirus (16, 17). Cross-reactivity can also occur in primary infection, particularly if there is a history of yellow fever, Japanese encephalitis, or tick-borne encephalitis virus vaccination (18, 19).

Virus neutralization assay, most commonly in the form of a plaque reduction neutralization test (PRNT), is considered the most specific assay for measurement of anti-flavivirus antibodies (20). It detects the production of antibodies that functionally inhibit viral infection; significant plaque reduction is generally considered to have occurred when there is a 90% reduction in the number of viral plaques ($PRNT_{90}$) (20). In recent serological studies of ZKV the serological criteria for ZKV infection included a positive IgM ZKV EIA, ZKV $PRNT_{90}$ titers $\geq$20, and a ZKV $PRNT_{90}$/DENV $PRNT_{90}$ ratio $\geq$4 (11, 21). PRNT has a number of limitations, being expensive and labor intensive, requiring skilled scientists and a cell culture facility. It also has a turnaround time of up to 10 days and so does not allow for a real-time diagnosis.

The aims of this study were to develop and validate ZKV and DENV neutralization assays using real-time PCR to measure viral neutralization by antibody, thereby providing a faster turnaround time and developing an assay that is both more objective and scalable.

RESULTS

Optimization of stock virus-Vero cell incubation time. The duration of incubation of the virus-serum mix and Vero cell monolayer was the main determinant of the turnaround time for the real-time PCR neutralization assay. ZKV and DENV 1 to 4 stock viruses demonstrated adequate viral replication, as measured by real-time PCR, after 48 and 72 h incubation periods, but not 24 h (data not shown). An incubation period of 48 h was chosen as it provided a shorter assay turnaround time.

Example of the ZKV real-time PCR neutralization assay. An example of the real-time PCR neutralization assay results and analysis for ZKV is shown in Table 1. A convalescent-phase serum specimen collected after PCR-confirmed diagnosis of ZKV infection was tested against ZKV stock virus. Viral replication in the real-time PCR neutralization assay resulted in a real-time PCR cycle threshold that was lower than the background, nonreplicating cycle threshold. Inhibition of viral replication was demonstrated by a real-time PCR cycle threshold that was equal to or greater than the background cycle threshold. The neutralizing antibody titer was the reciprocal of the highest serum dilution, resulting in inhibition of virus replication compared to nonrep-

TABLE 2 Real-time PCR neutralization assay results for convalescent-phase sera from patients with proven ZKV infection compared to Institut Pasteur neutralization assay results

	ZKV neutralization by	
Serum sample no.[a]	Real-time PCR[b]	Institut Pasteur assay[c]
1	640	2,560
2	160	160
3	640	640
4	640	1,280
5	1,280	1,280
6	320	320
7	640	1,280
8	160	320
9	160	320
10	320	640
11	640	≥2,560
12	320	640
13	1,280	≥2,560
14	1,280	1,280
15	80	80
16	320	320
17	320	320
18	640	1,280
19	80	80
20	≥2,560	≥2,560
21	320	320
22	160	640
23	320	1,280
24	160	160

[a]Convalescent-phase serum from patient with PCR-proven ZKV infection.
[b]Real-time PCR neutralization titer expressed as the reciprocal of the highest dilution of patient serum that resulted in neutralization of the stock virus.
[c]Institut Pasteur neutralization titer expressed as the reciprocal of the highest dilution of patient serum that resulted in the absence of ZKV-specific cytopathic effect.

licating viral background. Results were considered valid when the virus back titration confirmed that approximately 100 infectious virus particles were present in the neutralization assay (virus nucleic acids were detectable in back titration dilutions of 1/10 and 1/100 but undetectable at a dilution of 1/1000) and when positive and negative real-time PCR controls gave expected results (data not shown).

Sensitivity of the real-time PCR-based neutralization assay. Only sera that had been characterized by the gold-standard diagnostic techniques of real-time PCR and/or PRNT were used to assess the sensitivity of the real-time PCR neutralization assay. The results of the real-time PCR ZKV neutralization assay and DENV serotype-specific neutralization assay, using convalescent-phase sera from patients with proven infection, are shown in Tables 2 and 3, respectively. Neutralizing antibody of various titers was detected in all cases, resulting in a sensitivity of 100% for both ZKV and DENV. In addition, when the infecting DENV serotype was known the neutralization assay detected neutralizing antibody to the correct serotype, either alone or at ≥4-fold higher titer compared to other DENV serotypes, in 11 out of 12 tests (91.7%). The infecting DENV serotype was not able to be determined for sample 1 (Table 3).

Specificity of the real-time PCR-based neutralization assay. The specificity of the neutralization assay was assessed using sera positive for a non-ZKV and/or non-DENV flavivirus, either by serological or molecular testing, collected from individuals with a clinical history of infection or vaccination (Table 4). A serum specimen positive for Chikungunya IgG antibodies was also included since this alphavirus is often a differential diagnosis to ZKV and DENV due to its similar geographic distribution and clinical presentation (5, 6, 22). The real-time PCR ZKV and DENV neutralization assays produced a neutralization titer of <20 in 11/11 cases (100%) when the serum was known to be negative for that virus.

TABLE 3 Real-time PCR neutralization assay results for convalescent-phase sera from patients with proven DENV infection

Patient sample no.	DENV type as per real-time PCR	Time post PCR detection	Stock virus[a]			
			DENV 1	DENV 2	DENV 3	DENV 4
1	1	20 days	**160**	80	40	20
2	1	24 days	**20**	<20	<20	<20
3	1	35 days	**160**	<20	<20	<20
4	1	18 mo	**40**	<20	<20	<20
5	2	12 days	<20	**20**	<20	<20
6	2	44 days	<20	**320**	<20	<20
7	2	4 mo	<20	**320**	<20	<20
8	2	6 mo	<20	**640**	20	<20
9	3	12 days	<20	<20	**20**	<20
10	3	18 days	<20	20	**160**	<20
11	3	2 years	<20	<20	**80**	<20
12	4	36 days	20	<20	<20	**160**
13[b]	Undetermined	5 years	**80**	<20	<20	<20
14[b]	Undetermined	—	**160**	40	20	<20
15[b]	Undetermined	—	**1,280**	80	320	20

[a]Real-time PCR neutralization titer expressed as the reciprocal of the highest dilution of patient serum that resulted in neutralization of the stock virus. The highest real-time PCR neutralization titer for each assay is indicated (in bold).

[b]DENV serotype had not been determined previously for these specimens. When there was no history of nucleic acid confirmation of DENV infection (—), DENV-neutralizing antibody was detected using the PRNT.

A third specimen from a patient with ZKV infection confirmed by PCR was tested but not included in the specificity data. This specimen was from a patient with likely secondary ZKV infection on a background of previous DENV infection. Cross-reactivity was shown in the gold-standard PRNT, where neutralizing antibody to both DENV and ZKV was detected. The serum specimen demonstrated cross-reactivity in the real-time PCR neutralization assays, with the detection of high-titer neutralizing antibody to both DENV (serotype 1 titer, 160; serotype 2 titer ≥2,560; serotype 3 titer, 160; and serotype 4 titer, 320) and ZKV (titer, 1,280). The highest titer of neutralizing antibody to DENV by ≥4-fold was to DENV serotype 2, making it the most likely previously infecting DENV serotype. These results demonstrate that the real-time PCR neutralization assay is not immune to the difficulties posed by secondary flavivirus infections.

Reproducibility of the real-time PCR-based neutralization assay. Results were consistent when a selection of known positive serum specimens were tested in the

TABLE 4 Specificity testing of ZKV and DENV real-time PCR neutralization assays

Infection or vaccination type and serum	Verification method[b]	Neutralization titer with stock virus[a]				
		ZKV	DENV 1	DENV 2	DENV 3	DENV 4
Flavivirus infection						
ZKV	Real-time PCR	**1,280**	<20	<20	<20	<20
ZKV	Real-time PCR	**160**	<20	<20	<20	<20
DENV 1	Real-time PCR	<20	**80**	<20	<20	<20
DENV 1	Real-time PCR	<20	**160**	80	40	20
DENV 2	Real-time PCR	<20	<20	**320**	<20	<20
DENV 3	Real-time PCR	<20	<20	20	**80**	<20
DENV 4	Real-time PCR	<20	20	<20	<20	**160**
DENV type unknown	PRNT	<20	**1,280**	80	320	20
Alphavirus infection						
Chikungunya	Real-time PCR	<20	<20	<20	<20	<20
Flavivirus vaccination						
YF and JEV[c]	EIA	<20	<20	<20	<20	<20
YF	EIA	<20	<20	<20	<20	<20

[a]Neutralization titer expressed as the reciprocal of the highest dilution of patient serum that resulted in neutralization of the stock virus. The highest real-time PCR neutralization titer for each assay is indicated (in bold).

[b]Real-time PCR performed on earlier acute-phase serum specimen. EIA, enzyme immunoassay.

[c]YF, Yellow fever; JEV, Japanese encephalitis virus.

TABLE 5 Reproducibility of ZKV and DENV real-time PCR neutralization assays

Virus type per real-time PCR	Day tested[c]	Stock virus[a]				
		ZKV	DENV type 1	DENV type 2	DENV type 3	DENV type 4
ZKV	1	1,280	—[b]	—	—	—
	29	≥2,560	—	—	—	—
	147	1,280	—	—	—	—
	307	1,280	—	—	—	—
DENV 1	1	—	40	<20	<20	<20
	68	—	80	<20	<20	<20
	150	—	40	—	—	—
	171	—	40	—	—	—
DENV 2	1	—	<20	640	<20	<20
	68	—	<20	320	<20	<20
	150	—	—	320	—	—
	197	—	—	320	—	—
DENV 3	1	—	<20	20	80	<20
	168	—	<20	<20	80	<20
	194	—	—	—	80	—
DENV 4	1	—	20	<20	<20	160
	35	—	20	<20	20	320
	148	—	—	—	—	160
	194	—	—	—	—	160

[a]Real-time PCR neutralization titer expressed as the reciprocal of the highest dilution of patient serum that resulted in neutralization of the stock virus.
[b]—, assay was not performed.
[c]Relative to the first date tested, in days.

neutralization assay against ZKV and DENV 1 to 4 stock viruses on multiple occasions over a minimum period of 6 months (Table 5).

DISCUSSION

This study describes the development and validation of a real-time PCR-based neutralization assay for ZKV and DENV, where the neutralization endpoint is measured by real-time PCR assay instead of by the counting of viral plaques. Few studies have used PCR as an approach to measure virus neutralization assay endpoints (23–25). As far as we are aware, this is the first report of a real-time PCR-based neutralization assay being used in the diagnosis of a flavivirus infection.

The initial steps in the assay, including making virus and serum dilutions and transferring virus-serum mixtures onto cell monolayers, are currently performed manually and so remain relatively labor-intensive. However, the RNA extraction and real-time PCR steps can be automated, allowing throughput to be scaled up in periods of increased demand. In addition, the RNA extraction and real-time PCR protocols described in this study are already used as part of routine clinical diagnostics at the Victorian Infectious Diseases Reference Laboratory (VIDRL) and so the real-time PCR-based neutralization assay can be readily incorporated into the current laboratory work flow. Finally, the high sensitivity of PCR allows the detection of virus replication and the neutralization endpoint to be assessed at 48 h postinfection of the cell monolayer. This results in an overall test turnaround time of less than 72 h.

Real-time PCR amplifications curves were easy to interpret and comparison to background (nonreplicating virus) provided an objective way in which to determine the neutralizing antibody titer. The sensitivity and specificity data generated in this study suggest that the ZKV and DENV real-time PCR neutralization assays will be suitable as confirmatory tests applied to serum specimens that are reactive in an IgM/IgG EIA screen. The sensitivity of the real-time PCR neutralization assay was excellent at 100% for both ZKV and DENV. In addition, the DENV serotype could be correctly discerned in 91.7% of the cases where the infecting DENV serotype was known and in a majority of cases there was minimal or no reactivity with the other DENV serotypes. This provides

confidence in reporting the DENV serotype when the neutralizing titer for one serotype is ≥4-fold higher than for the other three DENV serotypes. The specificity of the assay was 100% for ZKV and DENV, respectively, when testing sera from patients with no history of that particular flavivirus infection. A high specificity is important in a neutralization assay as its primary role is to confirm true-positive, and rule out false-positive, EIA results.

The real-time PCR neutralization assay, like PRNT, is expected to have reduced specificity in the setting of secondary flavivirus infections. Significant cross-reactivity was observed in the DENV and ZKV PCR-based neutralization assays on testing a serum specimen collected from a patient with proven current ZKV infection and a background of likely prior DENV infection. The dominant epitopes responsible for highly potent, serotype-specific humoral immunity to DENV appear to be located in the hinge region of the E glycoprotein (26, 27). However, a significant proportion of human anti-DENV antibodies seem to be serotype cross-reactive, with many directed to the prM/M protein and the fusion loop of the E protein (28, 29). During the acute phase of secondary infection, there is a high degree of serotype cross-reactivity among the antibodies produced by memory B cells (30). This means that, when measured by PRNT, high-titer neutralizing antibodies are produced against two, and often all four, DENV as well as against other non-DENV flaviviruses (17). The interaction of virus with serum-neutralizing antibody in the real-time PCR-based neutralization assay occurs in the same manner as in PRNT and so does not eliminate this issue.

Detection of flavivirus-neutralizing antibody activity by PRNT is generally thought to coincide with the detection of seroconversion by commercially available IgM and IgG EIAs (31). For primary ZKV and DENV infection, this is expected to occur from day 10 following the onset of illness (17, 32, 33). The earliest specimen from which DENV-neutralizing antibody was detected in this study was collected 12 days post positive PCR. However, the titer of neutralizing antibody does not appear to be very high at this stage of illness and collection of a convalescent specimen at least 1 month post onset of illness may provide more clear-cut results. A prospective study of children aged between 6 months and 15 years in Thailand showed that while the kinetics of neutralizing antibody produced following primary infection varied somewhat depending on infecting serotype, in general there was increase in neutralizing antibody titer from convalescence to 6 months postinfection (34). Increased antibody titers after this period were attributed to boosting of the immune response due to subsequent infection. This trend is borne out in the data presented in this study, with the highest titers detected between 1 month and 6 months post DENV-positive PCR. Boosting of antibody titers is unlikely to occur in a majority of the Australian population, where acquisition of DENV infection occurs primarily in the setting of travel to tropical countries.

One of the limitations of this study is the small number of samples used. This reflects limited access to well-characterized sera for an infection that is diagnosed relatively uncommonly in the southern Australian population. While our small sample size may have an impact on precision, the use of only well-characterized sera provides the optimal means to compare the real-time PCR neutralization assay to another gold-standard assay and so produce quality sensitivity and specificity data.

The emergence of ZKV has led to the need to diagnose infection in asymptomatic individuals that may be in the convalescent phase of infection. Diagnosis in this setting relies on the use of serology and has prompted the development of a more rapid, gold-standard serological platform for flavivirus diagnosis. It is envisaged that the DENV and ZKV real-time PCR neutralization assays will be performed alongside each other as a confirmatory step for serum specimens that are reactive for ZKV and/or DENV in commercial IgM/IgG EIAs. The demand for this type of testing is expected to continue as these arboviruses continue to spread globally. The real-time PCR-based approach to the virus neutralization assay should be generalizable to other viruses.

MATERIALS AND METHODS

Viruses and cell culture. Vero cells were grown in 96-well culture plates in minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO) supplemented with 2% fetal bovine serum (Bovogen, VIC, Australia) (MEM-2).

The African lineage MR766 prototype ZKV strain was kindly provided by PathWest (WA, Australia). DENV serotypes 1 to 3 were isolated from patient specimens at the Victorian Infectious Diseases Reference Laboratory (VIDRL; VIC, Australia). DENV serotype 4 was obtained from the QIMR Berghofer Medical Research Institute (QLD, Australia). All viruses were amplified in Vero cells and MEM-2 to generate virus stocks. Extracellular virus was harvested when the observed cytopathic effect reached 4 or greater and the presence of high-titer virus was confirmed by real-time PCR. Virus stock was stored at −80°C in aliquots until use.

Virus stocks were titrated in 150 μl of MEM-2 and inoculated onto Vero cells. Culture supernatant was harvested at 48 h postinfection and virus assayed by real-time PCR. The endpoint dilution, the highest dilution of stock virus to result in detection of nucleic acid by real-time PCR, was determined for each virus (ZKV and DENV types 1 to 4). The neutralization assay virus working dilution, determined to deliver approximately 100 infective virus particles to each well, was then calculated as 200-fold more concentrated than this endpoint dilution.

Serum samples. Deidentified human adult diagnostic sera collected between 2011 and 2016 and stored at −20°C were used to assess the performance of the neutralization assay. In order to define the sensitivity and specificity of the neutralization assay, only well-characterized sera were employed. All sera used to test the sensitivity of the neutralization assay had been confirmed as ZKV- or DENV-positive by real-time PCR on a previous specimen from the same patient or by PRNT at a reference laboratory (Pathology West, Westmead, NSW, Australia). Additionally, neutralization titers from the ZKV PCR-based neutralization assay were able to be compared to those produced in a ZKV neutralization assay performed at the Institut Pasteur, Noumea, New Caledonia, and adapted from Gallian et al. (35). Sera used to test the specificity of the real-time neutralization assay had been shown to be negative for the flavivirus of interest and positive for another arbovirus by real-time PCR, EIA, and/or PRNT.

The use of human serum samples was approved by the Melbourne Health Human Research Ethics Committee (QA2017026).

Virus RNA extraction and reverse transcription. For RNA extraction, 150 μl of virus supernatant was mixed with 120 μl of lysis buffer (ATL buffer, Proteinase K; Qiagen, Hilden, Germany) and incubated at room temperature for 10 min. Total RNA was prepared using a robotic column extraction system (QIAcubeHT; Qiagen) according to the manufacturer's instructions.

Reverse transcription (RT) was performed using the SensiFAST cDNA synthesis kit (Bioline, London, UK) using a reduced reaction volume so that each reaction mixture contained 5 μl of extracted RNA, 2.5 μl of nuclease free water (Promega, Maddison, WI), 2 μl of TransAmp buffer, and 0.5 μl of reverse transcriptase. Reduced reaction volumes had previously been validated (36). RT was performed using the following conditions: 25°C for 10 min, 42°C for 15 min, 85°C for 5 min, and a 4°C hold. The cDNA samples were stored at 4°C until required.

ZKV and DENV serotype-specific real-time PCR. The ZKV real-time PCR assay amplified a region of the NS5 gene using the forward primer CCTCAAGGAYGGGAGATCCA and reverse primer RGCTCGGCCA ATCAGTTCAT, with detection using a TaqMan probe (TGGTCCCYTGCCGCCACCAA). The DENV real-time PCR assay comprised four singleplex TaqMan real-time PCRs using primers and probes for DENV types 1, 2, 3, and 4 and has been described previously (37).

Each real-time PCR mixture contained 3 μl of cDNA, 5.9 μl of nuclease-free water, 10 μl of 2× PerfeCTa qPCR FastMix (Quanta BioSciences, Beverly, MA), 0.36 μl virus-specific primer pool (final concentration, 0.9 μM), and 0.8 μl virus-specific TaqMan probe (final concentration, 0.2 μM). Individual reactions were performed in a 96-well plate using the 7500 Fast real-time PCR system instrument and software (Applied Biosystems, Carlsbad, CA) and the following conditions: 95°C for 20 s, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. ZKV and serotype-specific DENV positive controls and nucleic-acid-free negative controls were used to verify the performance of each PCR run.

Real-time PCR-based neutralization assay. Serial 2-fold dilutions of patient sera were incubated with an equal volume of predetermined virus stock working dilution (calculated to deliver 100 infectious particles) for 2 h at 37°C. Subsequently, 150 μl of the virus/serum mixtures were transferred in duplicate to the corresponding wells of Vero cell monolayers and incubated for a further 2 h at 37°C. The supernatant was removed and replaced by 150 μl of MEM-2 and the Vero cell culture plate incubated at 37°C. After 48 h the supernatant was harvested and duplicates combined for subsequent virus RNA extraction, RT, and detection of virus by real-time PCR.

A background sample was included in each assay as a measure of nonreplicating virus background. This was performed using the working dilution of virus incubated on Vero cells in the absence of patient sera. In brief, after the working dilution of virus was incubated on the Vero cell monolayer for 2 h, the supernatant was removed and replaced by 150 μl of MEM-2. This volume of MEM-2 was then collected as the background sample and subjected to virus RNA extraction, RT, and real-time PCR, as described previously. The neutralization titer was determined as the highest serum dilution resulting in inhibition of virus replication compared to background. The inclusion of background samples in each assay also controlled for interassay variability in virus replication.

For each assay, a virus back titration was performed for ZKV and DENV types 1 to 4 in order to show that the amount of infectious virus added to each well was correct. This comprised addition of the virus stock to wells at the working dilution (approximately 100 infectious virus particles per well) and at 1/10, 1/100, and 1/1000 dilutions of the working dilution, in the absence of patient serum.

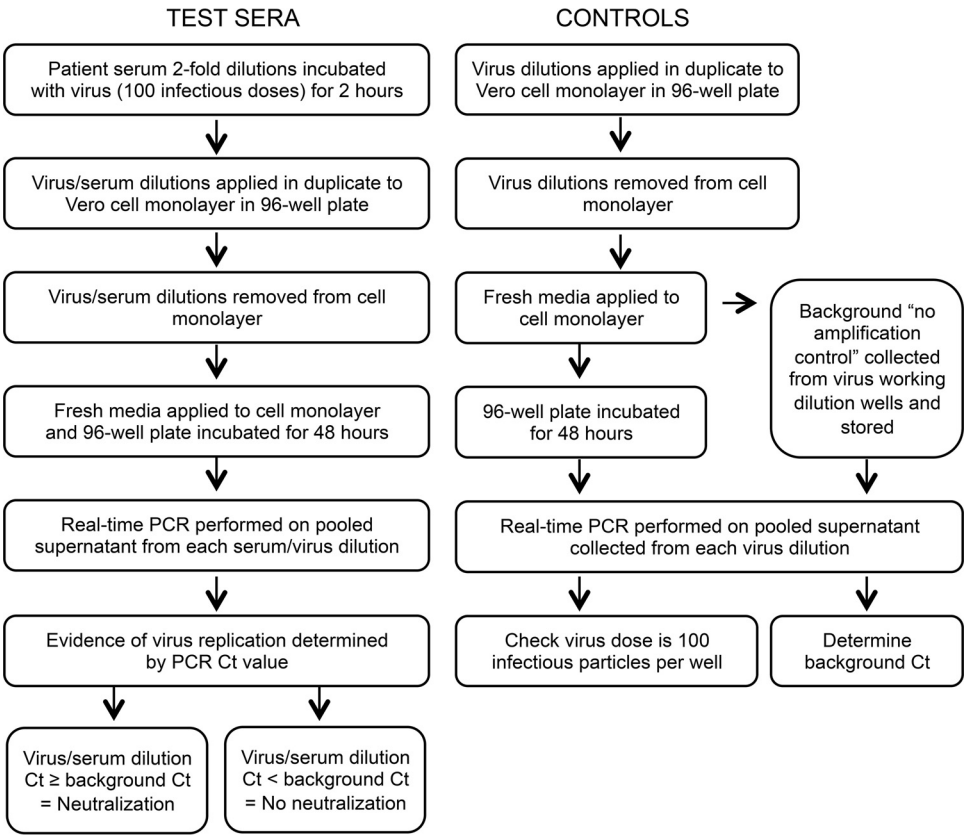

FIG 1 Real-time-PCR-based neutralization assay algorithm. Virus control dilutions included the working dilution (100 infectious virus particles per well) and 1/10, 1/100, and 1/1000 dilutions. C_T, cycle threshold.

An algorithm demonstrating the steps of the real-time PCR neutralization assay is provided in Fig. 1.

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