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COVID-19 serum can be cross-reactive and neutralizing against the dengue virus, as observed by the dengue virus neutralization test

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ABSTRACT

Objectives: Observing the serological cross-reactivity between SARS-CoV-2 and dengue virus (DV), we aimed to elucidate its effect on dengue serodiagnosis and infectivity in a highly dengue-endemic city in India.

Methods: A total of 52 COVID-19 (reverse transcription-polymerase chain reaction [RT-PCR] positive) serum samples were tested in rapid lateral flow immunoassays and DV immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) to detect DV or SARS-CoV-2 IgG/immunoglobulin M. The COVID-19 antibody (Ab) positive samples were subjected to a virus neutralization test (Huh7 cells) using DV type 1 (DV1) clinical isolate.

Results: Most (93%) of the SARS-CoV-2 Ab-positive serum samples cross-reacted with DV in rapid or ELISA tests. All were DV RNA and nonstructural protein 1 (NS1) antigen-negative. COVID-19 serum samples that were DV cross-reactive neutralized DV1. Of these, 57% had no evidence of DV pre-exposure (DV NS1 Ab-negative). The computational study also supported potential interactions between SARS-CoV-2 Ab and DV1.

Conclusion: DV serodiagnosis will be inconclusive in areas co-endemic for both viruses. The COVID-19 pandemic appears to impart a protective response against DV in DV-endemic populations.

Introduction

In December 2019, the world faced the first wave of COVID-19. It was initially an endemic outbreak in the Hubei province, China. COVID-19 is an acute pneumonia-like respiratory illness in humans caused by a *betacoronavirus*, SARS-CoV-2. As even droplet nuclei can spread the virus, person-to-person transmission became rampant, augmented by super-spreader events. By the end of January 2020, the World Health Organization (WHO) declared the outbreak a Public Health Emergency of International Concern (World Health Organization, 2020). The COVID-19 pandemic completed its first year at the cost of 129 million infections and 2 million deaths globally and still counting (World Health Organization, 2022a). India reported over 34 million confirmed cases of COVID-19, with 461,000 deaths up to November 8, 2021 (World Health Organization, 2022b).

It was anecdotally reported that antibodies (Abs) elicited against SARS-CoV-2 cross-reacted with dengue virus (DV) and gave false-positive results in DV immunoglobulin G (IgG) and immunoglobulin M (IgM) rapid tests (Yan *et al.*, 2020). At about the same time, we started monitoring the onset and subsequent spread of COVID-19 over several months and observed that the highly dengue-endemic regions remained relatively less affected in terms of COVID-19 severity and mortality (Biswas *et al.*, 2020). Following up on this observation, we found that five of 13 archived DV serum samples (from 2017, pre-dating the pandemic) cross-reacted with SARS-CoV-2 antigen (Ags) and gave false-positive IgG and IgM results in Spike protein-based COVID-19 rapid tests (Nath et al., 2021b). However, other studies have shown that DV-infected serum did not cross-react with nucleocapsid-based SARS-

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CoV-2 Ab test kits (Faccini-Martínez *et al.*, 2020). Several publications by Lau *et al.* on SARS-CoV-2 Ab detection by highly sensitive chemiluminescent immunoassays stated that cross-reactivity with DV was not observed (Lau *et al.*, 2021, 2020a, 2020b).

Again, our computational docking studies detected a potential role of convalescent DV Abs in interacting with key angiotensin-converting enzyme 2 receptor-binding regions of SARS-CoV-2 Spike antigen and strongly supported our previous cross-reactivity results (Nath et al., 2021a). Finally, scientists from Israel extensively probed and confirmed the cross-reactivity between DV Abs and SARS-CoV-2 antigen(s) and *vice versa* using lateral flow immunoassay (LFIA)-based rapid tests and enzyme-linked immunosorbent assays (ELISAs) (Lustig *et al.*, 2021). They reported about 22% serological cross-reactivity both ways. Thus, there was ample evidence of serological cross-reactivity between the two distinct families of viruses (*Flaviviridae* and *Coronaviridae*). This aroused the question of whether they are also cross-protective (Biswas *et al.*, 2020).

Therefore, we investigated the reverse scenario, i.e., whether the increasing seropositivity for SARS-CoV-2 resulting from the ongoing pandemic has any impact on the dengue occurrences in highly dengue-endemic regions like Kolkata, India. Initially, we performed docking studies with SARS-CoV-2 Ab and DV envelope antigen. This was followed by checking COVID-19 Ab cross-reactivity in DV serological tests. Finally, the DV type 1 (DV1) neutralization test was conducted using the serum samples of SARS-CoV-2 infected patients.

Methods

Study subjects

Serum samples from 52 clinically and laboratory-confirmed (swab reverse transcription-polymerase chain reaction [RT-PCR] positive) patients with COVID-19 (Patients 1-52, Supplementary Table S1) were collected from Behala Balananda Brahmachari Hospital and Research Center in Kolkata from September 2020 to January 2021. All patients showed mild to severe COVID-19 symptoms but were discharged from the hospital eventually on recovery. The study was approved by the respective Institutional Ethical Committees of the previously mentioned hospital and Council of Scientific & Industrial Research-Indian Institute of Chemical Biology (CSIR-IICB), Kolkata. Written informed consent (in their native language) was obtained from all individual participants included in this study. All experiments were carried out per relevant guidelines and regulations.

Dengue and COVID-19 LFIAs

DV-specific IgG, IgM, and NS1 Ag detection were done using the Standard Diagnostics-Bioline Dengue Duo rapid test kit. As mentioned in the kit insert, the test kit was tested in four different study sites to determine its sensitivity and specificity. In brief, it showed 92.4% (95% confidence interval [CI]: 86.1-95.9%) sensitivity and 98.4% (95% CI: 95.5-99.5%) specificity for dengue NS1 Ag. For detection of dengue IgG/IgM, the kit showed 94.2% (95% CI: 88.5-97.2%) sensitivity and 96.4% (95% CI: 93.0-98.2%) specificity. This kit has also been used to test four mosquito-borne pathogens, e.g., Japanese encephalitis virus, Yellow Fever virus, *Plasmodium falciparum,* and *Plasmodium vivax,* but there was no cross-reactivity.

COVID-19-specific IgG and IgM detection was carried out using the Abcheck kit. This kit detects Abs against the Spike protein of SARS-CoV-2. Per the kit brochure, the positive and negative coincident rates for SARS-CoV-2 IgM detection were 93.42% and 96%, respectively. In the case of SARS-CoV-2 IgG detection, the positive and negative coincident rates were 98.68% and 97.5%, respectively. In both cases, results were obtained considering viral RNA detection as the gold standard.

All tests were done per the manufacturer's instructions. In brief, each serum sample (20 µl) was added in a specified area of each test strip, followed by two drops (~80–100 µl) of kit-specific assay buffer in the designated spot, depending on the test kit. Appearances of "test line/control line" for all strip tests were confirmed to ensure the validity of the assay. In the case of the NS1 Ag detection strip, 100 µl of serum was added to the specified area. After 10-15 minutes, IgG- and IgM-specific lines were observed, confirming seropositive results.

SARS-CoV-2 Ab ELISA

All COVID-19 serum samples were also tested in the SARS-CoV-2 Spike protein IgG detection ELISA kit (CST #20154). The ELISA was performed per the manufacturer's protocol. In brief, serum samples were heat-inactivated at 53°C for 30 minutes and diluted (1:800) with sample diluent (provided in the kit) before running the assay. The absorbance of blank at 450 nm was subtracted from the optical density (OD)optical density of a sample, and also with positive and negative controls. Diluted samples were considered positive if OD(optical density) was more than 4.1 times the negative control OD(optical density). Samples were negative if OD(optical density) at 450 nm was less than three times the negative control OD. Per the kit insert, this kit has 100% specificity and 95.2% sensitivity.

DV IgG ELISA

The ELISA was performed according to the protocol provided in the kit (Euroimmun, Cat-EI 266b-9601G). Each patient's serum was diluted (1:101) in the sample buffer of the kit. The quantitative evaluation of reactive unit per ml (RU/ml) for each sample well of microplate was calculated by plotting the calibrator extinction coefficient with the calibrator's RU/ml. Samples with a cut-off value >22 RU/ml were considered dengue IgG ELISA-positive.

DV NS1 Ab ELISA

DV NS1 Ab ELISA was performed per the manufacturer's protocol (R&D Systems, Cat-DENG00). Recombinant NS1 antigens of DV types 1, 2, 3, and 4 were precoated onto microplate wells. This kit involves treatment of the samples to minimize false-positive results because of cross-reactive Abs to related flaviviruses, such as the Zika virus. Samples were diluted 50-fold before adding to the treatment plate and were, overall, diluted 100-fold before addition to the NS1 Ab detection plate.

RT-PCR for DV

RNA was extracted from 200 µl of COVID-19 serum samples using the High Pure Viral Nucleic Acid Extraction Kit (Roche), per the kit's prescribed manual. RT-PCR was done using primers, as described by Lanciotti *et al.* (Lanciotti *et al.*, 1992), to detect the presence of DV RNA.

Cell line

Huh7 cells were obtained from National Centre For Cell Science, India. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (D5796, Sigma) supplemented with 10% fetal bovine serum (Gibco) and Pen-Strep and L-Glutamine mix (Sigma) and Amphotericin B-2.5µg/ml (Gibco). Cell monolayers were grown at 37°C with 5%CO_2. During the passage, cells were washed with phosphate-buffered saline (PBS) (1X) and detached with Trypsin-ethylenediaminetetraacetic acid (1X) (Gibco).

Table 1
List of the serum samples used for virus neutralization tests.

DV-Ab+, CoV-Ab-, NS1+ (Archived pre-pandemic serums of 2017)	DV-Ab-, CoV-Ab-, NS1- (Archived pre-pandemic serums)	DV-Ab-, CoV-Ab+, NS1-(COVID-19 serums)	DV-Ab+, CoV-Ab+, NS1-(COVID-19 serums)
17-D-37	N-2	Sample No. 18	Sample Nos. 1-16;
17-D-50	N-4	Sample No. 19	20-38; 42-50
17-D-30	N-8	Sample No. 41	
17-D-68	N-10		

DV = dengue virus; Ab = antibody; No = number; Nos = numbers; NS1 = nonstructural protein 1.

Virus

DV1 was cultured from a serum sample collected during a 2017 dengue outbreak in Kolkata as described previously (Nath *et al.*, 2020). Briefly, the serum sample was filtered using a 0.22 µm PES syringe filter, inoculated in monolayer C6/36 cells, and incubated for five days. After three passages in C6/36 cells, the DV1 titer in the supernatant was determined and stored in aliquots as stocks. The NS1 gene of this virus was sequenced and deposited in the Genetic Codes Databank (GenBank) (MT072226). We passaged the virus only three times to keep it close to the clinical scenario. This virus is not plaque-forming, as reported previously in the case of other low passage clinical isolates (Raut *et al.*, 2019).

DV neutralization assay (VNT)

Serum samples were selected based on the results of LFIA and ELISA. Samples negative for DV Abs, SARS-CoV-2 Ab, and NS1 were considered DV-negative serum controls for neutralization. Predated COVID-19 pandemic samples, which were DV Ab-positive, SARS-CoV-2 Ab-negative, and NS1-positive were considered as positive serum controls for DV neutralization. Cross-reacting and non–cross-reacting COVID-19 serum samples were tested for DV-neutralizing activity (Table 1). Serum samples were inactivated at 56°C for 30 minutes and diluted with an equal volume of DMEM (supplemented with Pen-Strep, L-Glutamine mix, Sigma, and Amphotericin B, 2.5µg/ml). In each well of 96-well plates, 300 µl of diluted (1:1) serum was added. This was followed by adding 300 µl DV1 inoculum (100 X median tissue culture infectious dose) (World Organization for Animal Health, 2022). Diluted serum samples and DV1 inoculum were incubated at 37°C with 5%CO_2 for one hour. Then, 300 µl of Huh7 cell suspension was added to each well, and the plate was kept in an incubator. After 12 hours of incubation, 500 µl of DMEM was added to each well. At 48 hours after treatment, 600 µl of DMEM was added to maintain optimum pH. At 72 hours after incubation, the supernatant was aspirated from each well, and cells were washed with 1 ml of 1X PBS twice. After washing, 200 µl of fresh DMEM was added to each well, and cells were harvested; stored at -80°C until RNA was extracted.

RNA extraction and intracellular virus quantification

As described before, RNA was isolated from 200 µl of Huh7 cell lysate of each well. RNA quantity was determined using Nanodrop One (Thermo). Virus titer was determined using SYBR Green dye-based one-step quantitive (q) RT-PCR with Luna Universal One-Step qRT-PCR reagent (New England Biolabs). qRT-PCR was done using an equal quantity of RNA from each experimental condition. Quantstudio 5 (Applied Biosystems, Thermo) was used to run the qPCRs. Primers for qRT-PCRs were the same as described for DV1 serotyping by Lanciotti *et al.* (Lanciotti *et al.*, 1992).

In silico *docking experiment*

Protein Data Bank (PDB) structure files of SARS-CoV-2 Ab, DV1, and West Nile Virus (WNV) envelope antigen were retrieved from the Research Collaboratory for Structural Bioinformatics PDB; PDB ID: 7BWJ (SARS-CoV-2 receptor-binding domain [RBD] neutralizing Ab), 3G7T (DV1 envelope protein in the postfusion conformation), 2HG0 (WNV envelope glycoprotein). The SARS-CoV-2 Ab structure was in neutralizing condition with SARS-CoV-2 Spike protein RBD. A separate PDB structure file of the Ab was created consisting of only the paratope i.e. the fragment antigen-binding part, using PyMOL Molecular Graphics System Version 2.3.3. (Schrödinger, LLC software). Subsequent processing of these structure files, such as solvent deletion, deletion of alternate positions (retaining only the highest occupancy positions), hydrogen addition, and partial charge assignment, were done using the Dock Prep plugin of Chimera software (Pettersen *et al.*, 2004). Standard residues (receptor amino acids) were assigned AMBER ff14sb partial charges (Cornell *et al.*, 1995; Maier *et al.*, 2015). For the receptor cofactors with ANTECHAMBER, AM1-BCC charges were computed, which is included in Chimera (Jakalian *et al.*, 2000; Wang *et al.*, 2006). After all the modifications, the modified Ab structure was uploaded as a receptor PDB file in each of the two Fast Fourier Transform (FFT) algorithm-based docking servers, i.e., ClusPro (Kozakov *et al.*, 2017) and ZDOCK (Pierce *et al.*, 2014), along with DV or WNV envelope antigen as ligand input PDB file. For ClusPro, Ab mode was enabled for docking. A total of 10 antigen-antibody complex structures were predicted by each of the servers. All the interaction interfaces of 20 complex structures were thoroughly analyzed in PyMol using the "find any interaction within 3.5Å cut-off" plugin. Each amino acid of antigen(s) in the interaction interface was identified and marked. The marked amino acids were listed and analyzed in Microsoft Excel (Supplementary Table S2).

Results

The patients with COVID-19 serum samples were highly false-positive in DV LFIAs

Serum samples from 52 clinically and laboratory-confirmed (swab RT-PCR-positive) patients with COVID-19 (Patients 1-52, Supplementary Table S1) were collected from Behala Balananda Brahmachari Hospital and Research Center in Kolkata from September 2020 to January 2021. All patients showed mild to severe COVID-19 symptoms but were discharged from the hospital eventually on recovery.

All the serum samples were tested for SARS-CoV-2 Abs (IgG and IgM) using the Abcheck COVID-19 rapid strip test. It was found that 47 of 52 samples were positive for IgG, IgM, or both. Therefore, 90% of serum samples contained a detectable amount of SARS-CoV-2 Abs (Table 2). The previously mentioned results were confirmed using the SARS-CoV-2 Spike protein serological IgG ELISA kit. The ELISA results corroborated precisely with the results of the LFIA diagnostic kit.

These serum samples were tested on lateral flow-based Standard Diagnostics-Bioline Dengue Duo rapid strip test detecting DV IgG, IgM, and NS1 antigen per the manufacturer's instructions. Samples from 34 of 47 patients with COVID-19 were only DV IgG-positive, seven were IgG and IgM dual positive, and one

Table 2
List of 52 COVID-19 NAT-positive serum samples along with the test results in AbCheck COVID-19 rapid Ab test; SARS-CoV-2 Spike IgG ELISA, CST; Standard Diagnostics-Bioline Dengue Duo (IgG, IgM, and NS1), Euroimmun DV IgG ELISA, and R&D Systems DV NS1 Ab ELISA.

SL no.	Sample Name	AbCheck COVID-19 IgG	AbCheck COVID-19 IgM	SARS-CoV-2 Spike IgG ELISA	SD-BIOLINE Dengue IgG	SD-BIOLINE Dengue IgM	SD-BIOLINE Dengue NS1 Ag	EUROIMMUN DV IgG ELISA	NS1 Ab ELISA
1	Patient-1	+	+	+	+	+	-	+	+
2	Patient-2	+	+	+	+	-	-	+	-
3	Patient-3	+	+	+	+	+	-	+	+
4	Patient-4	+	+	+	+	-	-	+	-
5	Patient-5	-	+	+	+	-	-	+	-
6	Patient-6	+	+	+	+	-	-	+	-
7	Patient-7	+	+	+	+	-	-	+	+
8	Patient-8	+	+	+	+	-	-	+	-
9	Patient-9	+	+	+	+	-	-	+	+
10	Patient-10	+	+	+	+	-	-	+	+
11	Patient-11	+	-	+	+	+	-	+	+
12	Patient-12	+	+	+	+	-	-	+	-
13	Patient-13	+	-	+	+	-	-	+	-
14	Patient-14	+	+	+	+	-	-	+	-
15	Patient-15	+	+	+	+	-	-	+	-
16	Patient-16	+	+	+	+	+	-	+	+
17	**Patient-17**	-	-	-	-	-	-	+	-
18	Patient-18	+	+	+	-	-	-	-	-
19	Patient-19	+	+	+	-	-	-	-	-
20	Patient-20	+	-	+	+	+	-	+	+
21	Patient-21	+	+	+	+	-	-	+	-
22	Patient-22	+	+	+	+	-	-	+	-
23	Patient-23	+	+	+	+	-	-	+	+
24	Patient-24	+	+	+	+	-	-	+	-
25	Patient-25	-	+	+	+	-	-	+	-
26	Patient-26	+	+	+	+	-	-	+	-
27	Patient-27	+	+	+	+	-	-	+	+
28	Patient-28	+	+	+	+	+	-	+	+
29	Patient-29	+	+	+	+	-	-	+	-
30	Patient-30	+	+	+	+	-	-	+	-
31	Patient-31	+	+	+	+	+	-	+	+
32	Patient-32	+	+	+	+	-	-	+	-
33	Patient-33	+	+	+	-	+	-	-	-
34	Patient-34	+	+	+	+	-	-	+	-
35	Patient-35	-	+	+	+	-	-	+	-
36	Patient-36	+	+	+	-	-	-	+	-
37	Patient-37	+	+	+	+	-	-	+	+
38	Patient-38	+	+	+	+	-	-	+	+
39	**Patient-39**	-	-	-	+	-	-	+	-
40	**Patient-40**	-	-	-	-	-	-	-	-
41	Patient-41	+	+	+	-	-	-	-	-
42	Patient-42	+	+	+	+	-	-	+	-
43	Patient-43	+	-	+	+	-	-	+	+
44	Patient-44	+	+	+	+	-	-	+	-
45	Patient-45	-	+	+	+	-	-	+	+
46	Patient-46	+	+	+	+	-	-	+	-
47	Patient-47	+	+	+	-	-	-	+	-
48	Patient-48	+	+	+	+	-	-	+	+
49	Patient-49	+	+	+	+	-	-	+	-
50	Patient-50	+	+	+	+	-	-	+	-
51	**Patient-51**	-	-	-	+	-	-	+	+
52	**Patient-52**	-	-	-	+	-	-	-	+
		n = 43/52	n = 43/52	n = 47/52	n = 34/47	n = 8/47	n = 0/52	n = 43/47	n = 19/52
		IgG/IgM (n = 47/52)			IgG/IgM (n = 42/47)				

The samples in bold, were negative in SARS-CoV-2 Ab tests. The cross-reactivity with DV Ab tests was considered only for the SARS-CoV-2 Ab-positive samples.
The "+" sign signifies that the test result was positive; the "-" sign signifies that the test result was negative.
Ab = antibody; Ag = antigen; CST = Cell Signaling Technology; DV = dengue virus; ELISA = enzyme-linked immunosorbent assay; IgG = immunoglobulin G; IgM = immunoglobulin M; NAT = Nucleic acid testing; NS1 = nonstructural protein 1; R&D = Research & Diagnostics; SD-BIOLINE = Standard Diagnostics-Bioline; SL = serial.

was only IgM-positive. All the samples tested negative for NS1 Ag (Table 2). It is interesting to observe that overall, 42 samples (34 IgG-positive, 7 IgG/IgM-positive, 1 IgM-positive = 42) constituted about 89% (42/47) of the COVID-19 seropositive cases that "cross-reacted" in DV serological strip tests (Supplementary Figure S1). Serum samples from patients 33 and 52 were respectively DV IgM-positive and IgG-positive in rapid Ab tests but negative in DV IgG ELISA. One sample (patient 40) was all negative, whereas some COVID-19 samples (patients 17, 39, 51, and 52) were SARS-CoV-2 Ab-negative but DV IgG-positive by LFIA (patient 52) or ELISA (patient 17) or both (patient 39, 51) (Table 2).

Dengue IgG ELISA on serum samples from patients with COVID-19 confirmed the high degree of cross-reactivity

A total of 43 of 47 SARS-CoV-2 Ab-positive samples were found DV IgG ELISA-positive, constituting about 91% of all the samples having detectable SARS-CoV-2 Ab (Table 2). This result coincided

Table 3
List of COVID-19-predated healthy control serum samples tested using the Abcheck COVID-19 IgG/IgM and Standard Diagnostics-Bioline Dengue Duo rapid test kits. A total of 10 of 32 samples were positive.

SL No.	Sample Name	SD-BIOLINE Dengue IgG	SD- BIOLINE Dengue IgM	SD-BIOLINE Dengue NS1 Ag
1	P-H-1	+	-	-
2	P-H-2	+	-	-
3	P-H-3	-	-	-
4	P-H-4	-	-	-
5	P-H-5	+	-	-
6	P-H-6	-	-	-
7	P-H-7	-	-	-
8	P-H-8	+	-	-
9	P-H-9	-	-	-
10	P-H-10	-	-	-
11	P-H-11	-	-	-
12	P-H-12	-	-	-
13	P-H-13	+	-	-
14	P-H-14	+	-	-
15	P-H-15	-	-	-
16	P-H-16	-	-	-
17	P-H-17	-	-	-
18	P-H-18	-	-	-
19	P-H-19	-	-	-
20	P-H-20	+	-	-
21	P-H-21	-	-	-
22	P-H-22	-	-	-
23	P-H-23	-	-	-
24	P-H-24	-	-	-
25	P-H-25	+	-	-
26	P-H-26	-	-	-
27	P-H-27	-	-	-
28	P-H-28	+	-	-
29	P-H-29	+	-	-
30	P-H-30	-	-	-
31	P-H-31	-	-	-
32	P-H-32	-	-	-

The "+" sign signifies a positive result; the "-" sign signifies a negative result.
Ag = antigen; IgG = immunoglobulin G; IgM = immunoglobulin M; SD-BIOLINE = Standard Diagnostics-Bioline; SL = serial.

well with the strip test results (89%). Serum samples from patients 17, 36, and 47 were negative in DV rapid tests but positive in DV IgG ELISA.

COVID-19 serum samples did not contain DV RNA

RT-PCR of extracted RNA from sera revealed that all samples were negative for DV RNA.

DV lateral flow-based strip test of COVID-19 predated healthy control samples indicated lower DV seroprevalence

A total of 32 healthy sera (P-H-1 to 32), predating the COVID-19 outbreak, collected from October 2016 to July 2017 from Kolkata, were tested using the Standard Diagnostics-Bioline Dengue Duo rapid test. A total of 10 samples gave positive results in only dengue IgG, which is about 31.25 % of the sample size (Table 3). No serum tested positive for dengue IgM or NS1 Ag.

NS1 Ab ELISA of COVID-19 samples to determine the previous dengue-exposure

All 52 COVID-19 samples were tested in NS1 Ab Capture ELISA, and 19 samples were positive for DV NS1 Ab. Among SARS-CoV-2 Ab-positive samples, 17 tested positive for NS1 Ab (17/47 = 36.2%).

Computational docking studies with flavivirus envelope antigen and SARS-CoV-2 antibody predicted cross-reactive neutralization potency against DV

The structural PDB file of DV1 envelope antigen (PDB ID: 3G7T) was dock-prepared in Chimera software along with the SARS-CoV-2 RBD neutralizing Ab PDB file (PDB ID: 7BWJ). After adding additional rotamers, charges, and forcefield in the structural files, the DV antigen and SARS-CoV-2 Ab PDB files were docked using two FFT-based docking servers, i.e., ClusPro and ZDOCK. Each docking server provided 10 predicted antigen-antibody complex structures. A total of 20 predicted structures were analyzed in PyMOL software, and the antigen-antibody interacting surface was mapped. Only the common interactions predicted by both ClusPro and ZDOCK were considered to increase the specificity. It was observed that among the 20 predicted structures, SARS-CoV-2 Ab interacted 46 times with the DV1 neutralization epitope region (aa 295-395) (Chen *et al.*, 2017) (Figure 1) (Supplementary Table S2). To compare the interaction frequency, we also set a control docking experiment with another flavivirus envelope antigen, i.e., WNV envelope antigen (PDB ID: 2HG0). After similar structural refinement and docking with the SARS-CoV-2 Ab, it was found that the SARS-CoV-2 Ab interacted only nine times (Supplementary Table S2) in the neutralization epitope site of WNV envelope antigen (aa 295-395) (Beasley and Barrett, 2002).

SARS-CoV-2 infected patients' serum samples can neutralize DV1 clinical isolate (VNT)

NS1 and DV Ab-positive serum samples (predating COVID-19) were considered positive controls. Healthy serum samples (predating the COVID-19 pandemic), negative for DV Abs, SARS-CoV-2 Ab, and DV NS1 were used as negative serum controls. In this study, samples that were positive for SARS-CoV-2 and DV Abs but NS1-negative were tested for neutralization potential. Samples that were not cross-reacting, i.e., SARS-CoV-2 Ab-positive but DV Ab-negative, were also tested (Table 1).

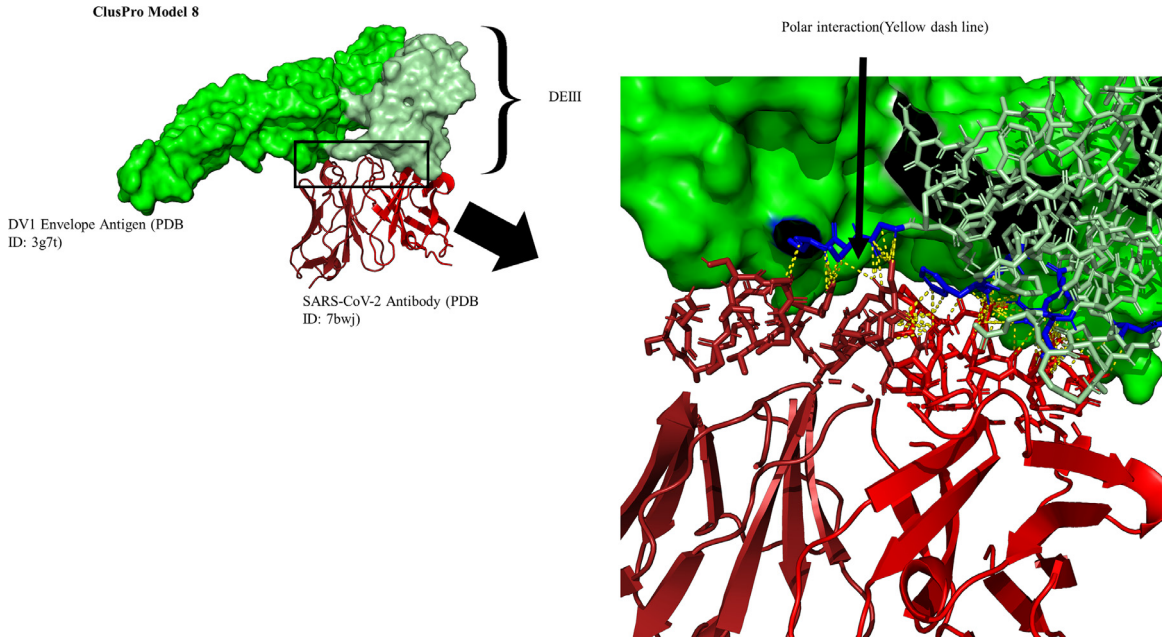

Figure 1. Representative docking complex of DV antigen and SARS-CoV-2 antibody. Among the 10 docked models of the ClusPro server, model 8 showed the highest number of interactions by SARS-CoV-2 Ab in the DEIII region of DV type 1 envelope antigen. The SARS-CoV-2 Ab is colored red and the envelope antigen is colored green. The amino acids in the epitope region of DEIII interacting with the Ab are colored blue. Polar interactions between amino acids are marked as yellow dotted lines. Ab = antibody; DEIII = dengue virus envelope protein domain III; DV = dengue virus.

Intracellular DV genome equivalents were highest for the negative serum controls. In the case of positive serum controls, DV1 yield was much reduced ($P = <0.0001$) than in negative serum controls, as expected. The COVID-19 serum samples (those were also DV Ab-positive but NS1-negative) effectively neutralized DV ($P = 0.0001$) (Figure 2, Supplementary Table S3). Only three serum samples were SARS-CoV-2 Ab-positive but DV Ab-negative (Table 2). All three samples (Nos. 18, 19, 41) were unable to neutralize DV1 (Figure 2).

Discussion

Approximately 89-91% of COVID-19 Ab-positive serum samples cross-reacted with DV in LFIA (42/47) or ELISA tests (43/47) (Table 2). Overall, 44 of 47 COVID-19 Ab-positive samples (93%) gave evidence of DV seropositivity (Table 2). This starkly contrasts with the COVID-19 samples from Israel showing 22% cross-reactivity (Lustig *et al.*, 2021) with DV, Israel being a dengue non-endemic region. The observed DV seropositivity was notably higher than the prepandemic (2017) dengue seroprevalence in India (Murhekar *et al.*, 2019). The latter was estimated at 48.7%.

Serum samples from 32 apparently healthy patients (without any history of dengue), collected from 2016 to 2017 in Kolkata, showed 32% seropositivity for DV IgG (Table 3). Thus, the serological cross-reaction of over 93% of SARS-CoV-2 infected patients' serum samples could not be explained by the background DV seroprevalence of 32% for Kolkata or 49% for India (Murhekar *et al.*, 2019). Only 36.2% of samples tested positive for NS1 Ab. Therefore, the remaining (93-36) = 57% DV cross-reacting COVID-19 Ab-positive serum samples had no evidence of previous DV exposure.

The high percentage of DV cross-reactivity in the COVID-19 serum samples also did not corroborate that India experienced a much smaller number of dengue cases in 2020 (National Vector Borne Disease Control Program, 2022). Interestingly, none of the cross-reacting COVID-19 serum samples were DV NS1-positive or RNA-positive.

To study this cross-reactivity further, we docked SARS-CoV-2 Ab (isolated from patients) with DV1 envelope antigen and found noticeable interactions (46) in the neutralization epitope of the dengue virus envelope protein domain III (DEIII) region. In addition, we analyzed docking of WNV envelope antigen and SARS-CoV-2 Ab, but the number of interactions was only nine. This indicated the potential of SARS-CoV-2 Ab to neutralize DV.

To functionally assess the impact of the COVID-19 pandemic on DV host-pathogen interaction, we performed VNT of DV1 clinical strain using COVID-19 serum samples. In support of our hypothesis (that COVID-19 is protective against dengue), SARS-CoV-2 infected patients' serum samples that cross-reacted with DV envelope had been found to neutralize and restrict DV1 entry into host cells significantly. Samples that were negative for NS1 Ab (i.e., without a traceable history of DV infection) also successfully neutralized DV1. There was no significant difference in the degree of virus neutralization between the NS1 Ab-positive/negative COVID-19 serum samples, as evident from the low SD among the samples. We used DV serum samples from 2017, i.e., predating the COVID-19 pandemic, as positive controls for DV neutralization. These serum samples significantly reduced the intracellular virus yield, confirming the approach and VNT assay conditions. During the period of sample collection (September 2020 to January 2021), four variants of concern (VOC) were reported in India and Kolkata. These were Alpha (B.1.1.7), Beta (B.1.351), Gamma (P1), and Delta (B.1.617.2). Among them, the highest number of reports were of the Delta variant, followed by Alpha, Beta, and Gamma VOC (GISAID, 2022 https://www.gisaid.org/hcov19-variants). Thus, our observations were relevant to the subjects under study and may not apply to all diagnostic tests and all SARS-CoV-2 variants identified to date.

Three COVID-19 serum samples (No. 18, 19, 41) that were not cross-reactive with DV were also considered for the neutralization test. All these samples were RT-PCR positive for SARS-CoV-2 but negative for DV in all respects (Table 2). All three samples could not neutralize DV1: perhaps these antibodies were generated against different emerging variants of SARS-CoV-2. The Spike

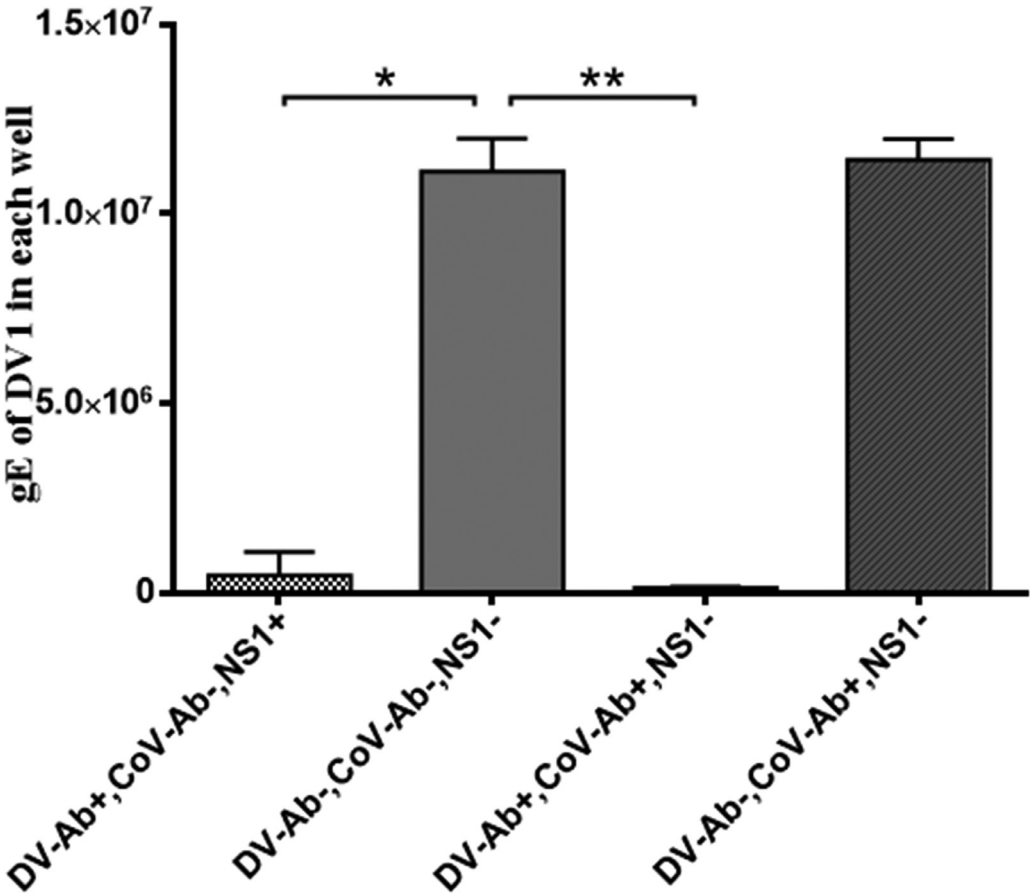

Figure 2. DV neutralization assay result using SARS-CoV-2 infected patients' serum samples. DV Ab+, SARS-CoV-2 Ab-, and NS1+ serum samples were considered positive controls. They significantly reduced (*P = <0.0001) the virus yield concerning negative controls (DV Ab-, SARS-CoV-2 Ab-, NS1-). Mean DV yield (gE) for all the DV Ab+, SARS-CoV Ab+, NS1- serum samples was low (**P = 0.0001) compared with the negative serum controls. In the case of these samples, there was no significant difference in DV neutralization capacity between the NS1 Ab + and NS1 Ab - COVID-19 samples. However, for the three DV Ab-, SARS-CoV-2 Ab+, NS1- samples (Nos. 18, 19, 41), DV1 titer was quite similar to negative controls. Error bars indicate SD. Ab = antibody; DV = dengue virus; gE = genome equivalent; Nos = numbers; NS1 = nonstructural protein 1; SD = standard deviation.

protein of such variants may not share enough antigenic similarity with DV to elicit cross-reactivity/DV neutralization.

We thought of two possible explanations for our previously mentioned observations. First, SARS-CoV-2 has some antigenic similarities with DV (Lustig *et al.*, 2021). This led to cross-reaction of Abs elicited against one another (Lustig *et al.*, 2021; Nath et al., 2021b). This is possibly the case for the 57% COVID-19 Ab-positive serum samples that were cross-reactive in DV Ab tests but showed no evidence of DV exposure (DV RNA-negative; DV NS1-negative and DV NS1 Ab-negative).

Secondly, because of antigenic similarity, SARS-CoV-2 infection may stimulate existing DV memory cells (from previously DV-infected individuals, including DV asymptomatic cases), resulting in a boost in DV Abs production (Nath *et al.*, 2021a). This is supported by the fact that all DV seropositive samples were DV RT-PCR and NS1 Ag negative, suggesting that none were from recently DV-infected subjects. Eight samples showed DV IgM, but, as mentioned earlier, this could be nonspecific. This explanation holds true for the 17 cross-reacting COVID-19 Ab-positive samples that were DV NS1 Ab-positive but DV RNA and DV NS1 antigen-negative. Either way, the COVID-19 pandemic appears to immunologically stimulate a large part of the population against DV, as evident from our findings.

A study from India included 44 DV-infected children grouped based on clinical severity and mortality. The same children were then screened for SARS-CoV-2 infection and serological evidence. It was found that previous exposure to SARS-CoV-2 had resulted in a less severe outcome with no death (Ravikumar *et al.*, 2021). These observations serve as further circumstantial/epidemiological evidence of our observations that SARS-CoV-2 serum samples can neutralize and protect against dengue. In addition, all the seropositive/cross-reactive patients with COVID-19, although hospitalized, finally recovered. Notably, many other highly dengue-endemic regions (e.g., Guangzhou in China and Sri Lanka) also reported significantly reduced dengue outbreaks in 2020 during the pandemic (Jiang *et al.*, 2021; Liyanage *et al.*, 2021). These observations serve as further circumstantial /epidemiological evidence of our observations.

Conversely, high DV Ab prevalence also appears to reduce severity and mortality because of COVID-19 as the mortality per million populations in India (July-August, 2021), despite a strong second wave, was about 300 compared with around 2000 in many dengue non-endemic countries (Worldometer, 2021). A study comprising 2351 participants from the Brazilian Amazon basin reported that COVID-19 was associated with a higher risk of death in those who did not have previous DV exposure (Silvestre *et al.*, 2021).

Several reports stated that SARS-CoV-2 Ab tests cross-reacted with DV; others did not. Because of these contradictory findings, we performed DV VNT using COVID-19 serum samples and discovered that COVID-19 serum samples (even with no evidence of DV pre-exposure, i.e., DV RNA-negative; DV NS1-negative, and DV NS1 Ab-negative) could, indeed, neutralize DV. The present study's

findings also indicate that the existing DV serological tests may no longer be conclusive for DV diagnosis in highly dengue-endemic countries where both the viruses co-exist.

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Ethical approval

This study was performed in accordance with the ethical standards (on par with the 1964 Declaration of Helsinki and its later amendments) of the ethical committees on human subjects of relevant institutions. SSG obtained ethical approval (Ref no. BBBH/0164/2021-2022) from Behala Balananda Brahmachari Hospital and Research Center. SB obtained ethical approval (Ref No. IICB/IRB/2021/4) from CSIR-IICB. All experiments were carried out in accordance with the relevant guidelines and regulations.

Author contributions

SB, SS, and HN conceived and designed the study. HN, AM, SR, and TK performed the experiments. Clinical sample collection and management was done by SRN, SSG, HN, SR, AM, and TK. SB acquired the funding. SB, SS, HN, and AM wrote the original draft of the manuscript. SB, SS, and HN performed the critical analysis of data. All the authors did a formal analysis of the data. The final manuscript was reviewed and edited by all the authors.

Extended data

The structures of docking models, both in raw and analyzed format, are available at Mendeley Data (https://data.mendeley.com/datasets/y6xjbjcgz8/1).

Data sharing

Further information and resource requests should be directed to and will be fulfilled by the corresponding author, Dr. Subhajit Biswas (subhajit.biswas@iicb.res.in).

Competing interests

The authors have no competing interests to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.07.013.

References

Beasley DWC, Barrett ADT. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. J Virol 2002;76:13097–100.
Biswas S, Sukla S, Biswas S. COVID-19 virus infection and transmission are observably less in highly dengue-endemic countries: is pre-exposure to dengue virus protective against COVID-19 severity and mortality? Will the reverse scenario be true? Clin Exp Investig 2020:1–5.
Chen WH, Chou FP, Wang YK, Huang SC, Cheng CH, Wu TK. Characterization and epitope mapping of dengue virus type 1 specific monoclonal antibodies. Virol J 2017;14:189.
Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM, Ferguson DM, et al. A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. J Am Chem Soc 1995;117:5179–97.
Faccini-Martínez ÁA, Rivero R, Garay E, García A, Mattar S, Botero Y, et al. Serological cross-reactivity using a SARS-CoV-2 ELISA test in acute Zika virus infection, Colombia. Int J Infect Dis 2020;101:191–3.
GISAID, Tracking of variants, https://www.gisaid.org/hcov19-variants/, 2022 (accessed 2 June 2022).
Jakalian A, Bush BL, Jack DB, Bayly CI. Fast, efficient generation of high-quality atomic charges. AM1-BCC model: I. Method. J Comput Chem 2000;21:132–46.
Jiang L, Liu Y, Su W, Liu W, Yang Z. Decreased dengue cases attributable to the effect of COVID-19 in Guangzhou in 2020. PLoS Negl Trop Dis 2021;15.
Kozakov D, Hall DR, Xia B, Porter KA, Padhorny D, Yueh C, et al. The ClusPro web server for protein-protein docking. Nat Protoc 2017;12:255–78.
Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 1992;30:545–51.
Lau CS, Hoo SP, Liang YL, Phua SK, Aw TC. Performance of two rapid point of care SARS-COV-2 antibody assays against laboratory-based automated chemiluminescent immunoassays for SARS-COV-2 IG-G, IG-M and total antibodies. Pract Lab Med 2021;24:e00201.
Lau CS, Hoo SP, Yew SF, Ong SK, Lum LT, Heng PY, et al. Evaluation of an electrochemiluminescent SARS-CoV-2 antibody assay. J Appl Lab Med 2020a;5:1313–23.
Lau CS, Oh HML, Hoo SP, Liang YL, Phua SK, Aw TC. Performance of an automated chemiluminescence SARS-CoV-2 IG-G assay. Clin Chim Acta 2020b;510:760–6.
Liyanage P, Rocklöv J, Tissera HA. The impact of COVID-19 lockdown on dengue transmission in Sri Lanka; A natural experiment for understanding the influence of human mobility. PLoS Negl Trop Dis 2021;15.
Lustig Y, Keler S, Kolodny R, Ben-Tal N, Atias-Varon D, Shlush E, et al. Potential antigenic cross-reactivity between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and dengue viruses. Clin Infect Dis 2021;73:e2444–9.
Maier JA, Martinez C, Kasavajhala K, Wickstrom L, Hauser KE, Simmerling C. ff14SB: improving the accuracy of protein side chain and backbone parameters from ff99SB. J Chem Theor Comput 2015;11:3696–713.
Murhekar MV, Kamaraj P, Kumar MS, Khan SA, Allam RR, Barde P, et al. Burden of dengue infection in India, 2017: a cross-sectional population based serosurvey. Lancet Glob Health 2019;7:e1065–73.
Nath H, Basu K, De A, Biswas S. Dengue virus sustains viability of infected cells by counteracting apoptosis-mediated DNA breakage. bioRxiv. 8 Feburary 2021 https://www.biorxiv.org/content/10.1101/2020.06.19.162479v2. accessed 8 July 2022.
Nath H, Mallick A, Roy S, Sukla S, Basu K, De A, et al. Archived dengue serum samples produced false-positive results in SARS-CoV-2 lateral flow-based rapid antibody tests. J Med Microbiol 2021b;70.
Nath H, Mallick A, Roy S, Sukla S, Biswas S. Computational modelling supports that dengue virus envelope antibodies can bind to SARS-CoV-2 receptor binding sites: is pre-exposure to dengue virus protective against COVID-19 severity? Comput Struct Biotechnol J 2021a;19:459–66.
National Vector Borne Disease Control Program (NVBDCP). Dengue/dhf situation in India. https://nvbdcp.gov.in/index4.php?lang=1&level=0&linkid=431&lid=3715, 2022 (accessed 15 May 2021).
Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera—A visualization system for exploratory research and analysis. J Comput Chem 2004;25:1605–12.
Pierce BG, Wiehe K, Hwang H, Kim BH, Vreven T, Weng Z. ZDOCK server: interactive docking prediction of protein-protein complexes and symmetric multimers. Bioinformatics 2014;30:1771–3.
Raut R, Corbett KS, Tennekoon RN, Premawansa S, Wijewickrama A, Premawansa G, et al. Dengue type 1 viruses circulating in humans are highly infectious and poorly neutralized by human antibodies. Proc Natl Acad Sci U S A 2019;116:227–32.
Ravikumar N, Randhawa MS, Nallasamy K, Angurana SK, Kumar M, Mohi GK, et al. Impact of recent SARS-CoV-2 infection on the course and severity of dengue in children: a prospective observational study from North India. Am J Trop Med Hyg 2021;105:751–5.
Silvestre OM, Costa LR, Lopes BVR, Barbosa MR, Botelho KKP, Albuquerque KLC, et al. Previous dengue infection and mortality in coronavirus disease 2019 (COVID-19). Clin Infect Dis 2021;73:e1219–21.
Wang J, Wang W, Kollman PA, Case DA. Automatic atom type and bond type perception in molecular mechanical calculations. J Mol Graph Model 2006;25:247–260.
World Health Organization. COVID-19 Public Health Emergency of International Concern (PHEIC) Global research and innovation forum. https://www.who.int/publications/m/item/covid-19-public-health-emergency-of-international-conce

rn-(pheic)-global-research-and-innovation-forum, 2020 (accessed 15 May 2021).
World Health Organization. WHO Coronavirus (COVID-19) Dashboard. https://covid19.who.int/, 2022a (accessed 8 Nov 2021).
World Health Organization. WHO India Coronavirus (COVID-19) Dashboard with vaccination data]. https://covid19.who.int/region/searo/country/in, 2022b (accessed 8 Nov 2021).
World Organization for Animal Health. Terrestrial manual online access. https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/, 2022 (accessed 21 May 2021).
Worldometer, COVID Live Update. https://www.worldometers.info/coronavirus/, 2021 (accessed 5 November 2021).
Yan G, Lee CK, Lam LTM, Yan B, Chua YX, Lim AYN, et al. Covert COVID-19 and false-positive dengue serology in Singapore. Lancet Infect Dis 2020;20:536.