25-Hydroxy Cholesterol Effectively Inhibits Japanese Encephalitis Virus infection in Vero Cells

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Abstract

The Japanese encephalitis virus (JEV) is a mosquito-borne, enveloped flavivirus that causes acute encephalitis. Although JEV is increasingly recognized as a global threat, there is currently no FDA-approved treatment available for JEV. 25-hydroxycholesterol (25-HC) is an oxysterol produced through the oxidation of cholesterol, a process catalyzed by cholesterol 25-hydroxylase (CH25H), which is an interferon-stimulated gene that is upregulated during viral infections. In this study, we report for the first time that 25-HC effectively prevents JEV infection in cells. Our results show that 25-HC inhibits JEV infection in Vero cells with an IC50 of ~ 3.7 μM, while displaying no cell toxicity (CC50 > 50 μM). Given the antiviral effects of 25HC against Zika and Dengue viruses, our findings suggest that 25-HC could be a promising candidate for the development of broad-spectrum anti-flaviviral therapeutics.

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Keywords: Japanese encephalitis virus, 25-hydroxycholesterol, flavivirus, membranes, cholesterol

The Japanese encephalitis virus (JEV) is a member of the Flaviviridae family that is transmitted via mosquitoes. JEV mainly infects various cell types in the CNS and causes acute encephalitis. Its primary distribution areas are tropical parts of Asia, such as Japan, China, Taiwan, Korea, the Philippines, India, and all of Southeastern Asia. JEV is becoming more widespread throughout Asia due to the increase in mosquito population and is also rapidly spreading worldwide. More than 50,000 cases of JEV infection with a 20–30% mortality are reported annually. Most of the JEV-infected cases remain asymptomatic. Survivors suffer neurological or neuropsychiatric after effects that mimic Parkinsonian movement disorders, paralysis similar to that of poliomyelitis, or cognitive impairment (Misra and Kalita, 2010). Children under the age of 14 are most commonly affected by the disease. However, there are no FDA-approved drugs against JEV and available vaccines are not completely effective (Erraab and Kantelebc, 2015, Srivastava et al. 2023).

JEV is an enveloped, positive-sense, single-stranded RNA virus with a diameter of around 50 nm with spheroid cubical symmetry. Its 11000 nucleotides genome is translated in a single polyprotein precursor, which is then cleaved into three structural [Capsid(C), pre-membrane (prM), and Envelope(E)] and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins by host and viral proteases (Zhu et al. 2023). Receptor-mediated endocytosis and low pH-triggered membrane fusion within endosomes allow JEV to transfer their genome inside the host cells (Yun and Lee, 2018). Subsequently newly synthesized viral components are assembled and packaged at the host endoplasmic reticulum (ER) membrane and released by exocytosis. Host membranes play crucial roles in different stages of the JEV infection, particularly during the entry processes (Das et al. 2010).

25-hydroxycholesterol (25-HC) is an oxysterol that is produced by oxidation of cholesterol catalyzed by cholesterol 25-hydroxylase (CH25H). CH25H is an interferon-stimulated gene that is upregulated upon viral infection (Liu et al. 2013). CH25H and its product 25HC regulate the cholesterol content of membranes in different subcellular compartments. 25HC promotes cholesterol trafficking into the endoplasmic reticulum (Kandutsch and Chen, 1975), and 25 HC treatment decreases plasma membrane cholesterol level (Heisler et al. 2023) by increasing the accessibility of membrane cholesterol and inducing lipid droplet formation (Nguyen et al. 2024). Moreover, 25 HC alters the plasma membrane properties such as domain organizations and fluidity (Domingues et al. 2021). 25-HC treatment leads to antiviral activity against a wide range of viruses such as SARS CoV2, Zika, Ebola virus (EBOV), Nipah virus (NiV), and human immunodeficiency virus (HIV). On the other hand, 25 HC does not affect Adeno virus infection (Liu et al. 2013). Although the antiviral effect of 25 HC against Zika and Dengue have been reported (Liu et al. 2013), the antiviral effect of 25HC against JEV has not been explored. Since plasma membrane cholesterol plays an important role in the entry of JEV (Das et al. 2010), and 25 HC modulates cholesterol organization in plasma membranes, we hypothesize that 25HC treatment will inhibit JEV infection. Our results show that 25 HC is a potent inhibitor of JEV infection in cells.

We wanted to investigate whether 25 HC inhibits JEV infection in cells. We used Vero cells for this purpose which is used to study JEV infection (Kiesslich and Kamen, 2020). First, we evaluated the cytotoxicity of 25HC in Vero cells by the MTT assay. We did not observe any significant change in cell viability in the presence of 25 HC up to 50 µM in 72 hours (Figure 1).

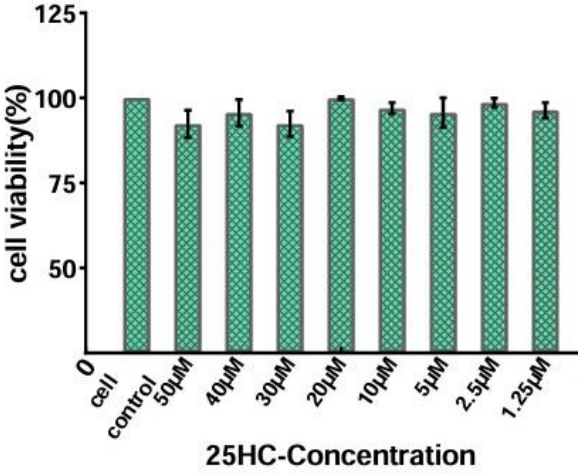


Figure 1. Cell viability as a function of 25 HC concentration. Vero cells were incubated with increasing amounts of 25 HC in DMSO (up to 50 µM) for 72 hours. Cell viability was determined by MTT assay. Data represent means ± SD of three replicates. See the methods section for details.

Next, we evaluated the effect of 25 HC on the infectivity of JEV in Vero cells by estimating the intracellular viral load by FACS and extracellular release of viral antigen in the supernatant by ELISA. We incubated Vero cells with varying amounts of 25 HC for 14 hours before infection according to previous reports (Liu et al. 2013). Figure 2 shows Alexa 488-4G2 positive cells in the scatter plot as a function of 25 HC concentration. Flow cytometry data reveals that the intracellular viral load decreased by about 87% in 25-HC pre and post-treated Vero cells at 20µM with an IC_{50}

of 3.70μM ± 0.14 (n = 3) (Figure 2). Next, we wanted to evaluate whether 25HC has any effect on virus release. For this, we determined the level of viral antigen (the Envelope protein) released in

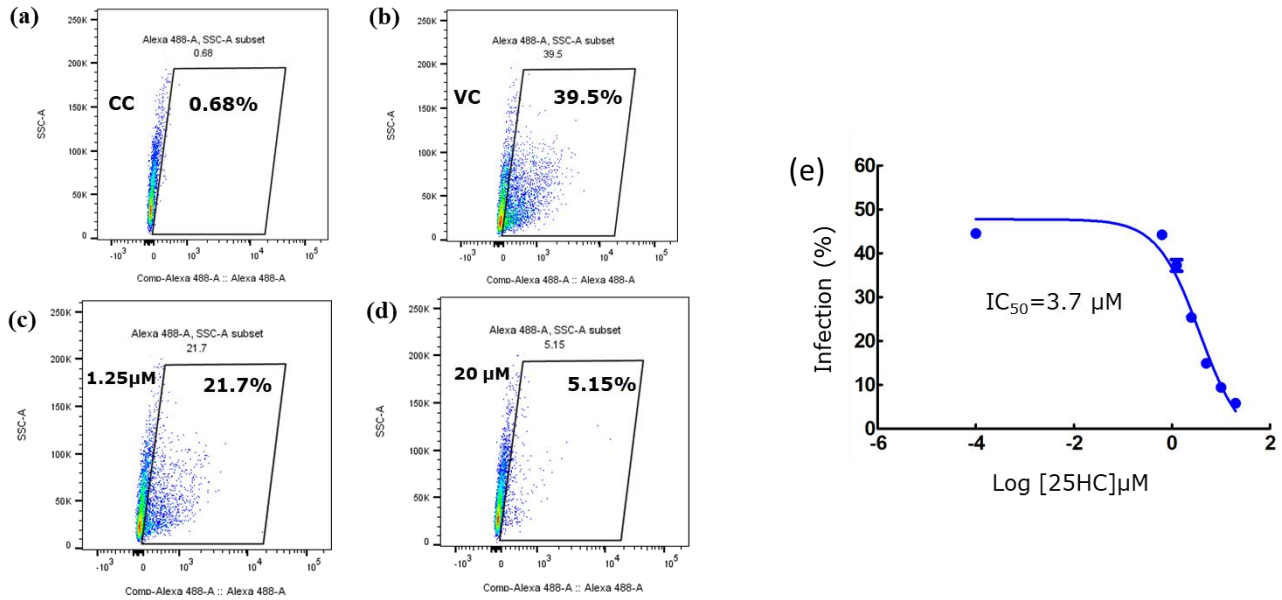


Figure 2. Effect of Pre and post-treatment 25HC on JEV infection. Representative flow cell scatter diagrams show the reduction in JEV infections in Vero cells in the presence of 25 HC. Vero cells were pre-treated with varying amounts of 25 HC followed by infection with JEV at 0 .01 MOI. Cells were grown for 72 hours in the presence of (same) varying amounts of 25 HC. Cells were stained with Alexa488 labeled 4G2 Antibody against the fusion domain of envelope protein. Numbers indicate the percentage of Alexa 488 + virus-infected cells within the entire population (a) Cell control, (b) Virus control without 25 HC treatment, (c) sample treated with 1.25μM 25HC, (d) sample treated with 20μM 25HC. Data was analyzed using Flowjo software. (e) A dose-response curve showing the effect of 25 HC on JEV infection. Data represent mean ± SD from at least three independent experiments. See the methods section for details.

the media in the presence of varying amounts of 25 HC by ELISA (See Figure 3). Our results show that 25 HC treatment reduced viral release from Vero cells quite effectively with an IC_{50} of 7 ± 0.27 μM. There is no significant difference between the IC50 values for infectivity measured by FACS and virus release determined by ELISA. Our data therefore suggest that 25 HC likely influences the early stages of infection, with its effect on viral release being a consequence of its

impact on infectivity. In other words, 25 HC may not directly affect the egress of the virus from infected cells.

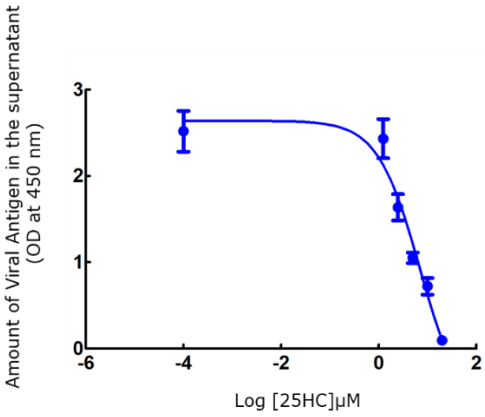


Figure 3. The inhibitory effect of 25HC on JEV release. Vero cells were pre-treated with varying amounts of 25 HC followed by infection with JEV at 0 .01 MOI. Cells were grown for 72 hours in the presence of (same) varying amounts of 25 HC. The amount of spike protein released in the media was determined by ELISA using HRP conjugated 4G2 antibody against the envelope protein. The figure shows the amount of antigen as a function of 25 HC. Data represent mean ± SD from at least three independent experiments. See the methods section for details.

Next, we wanted to check whether 25HC has any impact on the first round of infection. For this, we monitored intracellular viral load after 12 hours of infection. Our results show that even at early time point 25 HC reduced (relative) infection to ~ 24 % in the presence of 20 μM 25 HC.

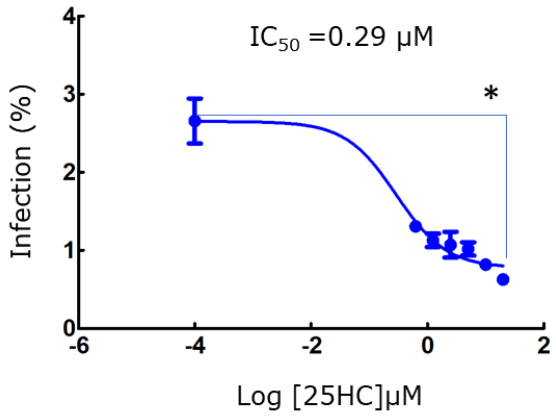
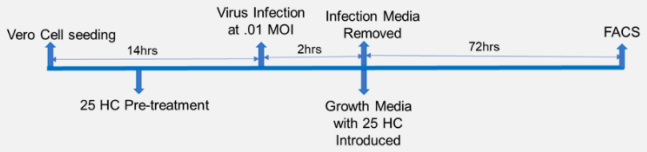
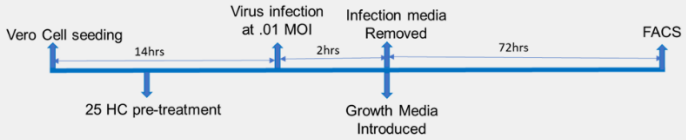
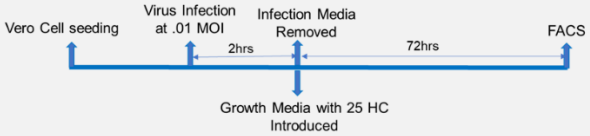


Figure 4. Antiviral effect of JEV at early time point. The figure shows the reduction in infection (determined by FACS) in the presence of 25 HC, assessed after 12 hours of infection. Vero cells were pre-treated with varying amounts of 25 HC followed by infection with JEV at 0 .01 MOI. Cells were grown for 12 hours in the presence of (same) varying amounts of 25 HC. Cells were stained with Alexa488 labeled 4G2 Antibody against the envelope protein. Data represent mean ± SD from at least three independent experiments. See the methods section for details (* implies $p < 0.05$).

To delineate the mechanism of the antiviral effect of 25HC against JEV, we carried out the time of addition assay (Ojha et. al. 2021). We compared the effect of pre-and post-treatment with 25 HC on the JEV infectivity with the corresponding effect of only pre-treatment and only post-treatment (Table 1). Interestingly, only the pre-treatment condition exhibited the highest IC_{50} of 6.2 μM, where pre-and post-treatment yielded an IC_{50} of 3.7 μM, while only post-treatment inhibited infection with an IC_{50} of 4.6 μM. (Table 1). This suggests that pre and post-treatment brought about the most effective inhibition and 25 HC might affect multiple stages of the infection, e.g., viral entry and fusion and packaging.

Table 1: Comparison of the anti-JEVeffects of 25 HC under different conditions

Treatment		IC_{50} (µM)	Maximum Inhibition (%) at 20µM
Pre and post-treatment		3.70	84
Only pre treatment		7.71	63
Only post treatment		4.62	83

25HC could inhibit viral infection by directly inhibiting endocytosis or by preventing fusion of viral envelope with endosomal membranes (Zang et al. 2020; Li et al 2017). It was earlier shown that lipid rafts play a crucial role in JEV entry and replication, and cholesterol depletion by mβCD (disruption of lipid rafts) reduced JEV infection in cells (Das et al. 2010). Interestingly, 25 HC is known to alter cholesterol organization in the plasma membranes by increasing cholesterol accessibility and reducing plasma membrane cholesterol. Therefore, it is plausible that 25 HC exerts its antiviral effect against JEV by inhibiting raft-dependent endocytosis via altered cholesterol organization in the plasma membrane. Previously, it was shown that 25HC enhances cholesterol esterification and promotes cholesterol transport from the plasma membrane to the ER (Kandutsch and Chen, 1975). 25HC depletes accessible cholesterol on the plasma membrane through activating acyl-CoA: cholesterol acyltransferase (ACAT) (Wang et al. 2020b) and leads to enhanced lipid droplet formation. To test whether 25HC treatment has any effect on membrane

cholesterol level, we checked the level of lipid droplets by Nile red staining upon 25 HC treatment. Our results show (Figure 5) a significant increase in lipid droplet (staining) in the presence of 25 HC, as reported earlier (Wang et al. 2020b). Therefore, it is likely that 25 HC might impair the endocytosis by JEV by depleting accessible cholesterol at the plasma membrane, thereby altering the preexisting domain or raft organizations at the plasma membrane. In addition, 25 HC may alter the organization and dynamics (e.g., the fluidity) of the plasma membrane due to the presence of the additional OH group (Domingues et al. 2021). However, our time-point data indicate that 25 HC impacts multiple stages of the JEV life cycle, warranting further investigation. Taken together, we show that 25 HC is an efficient inhibitor of JEV infection.

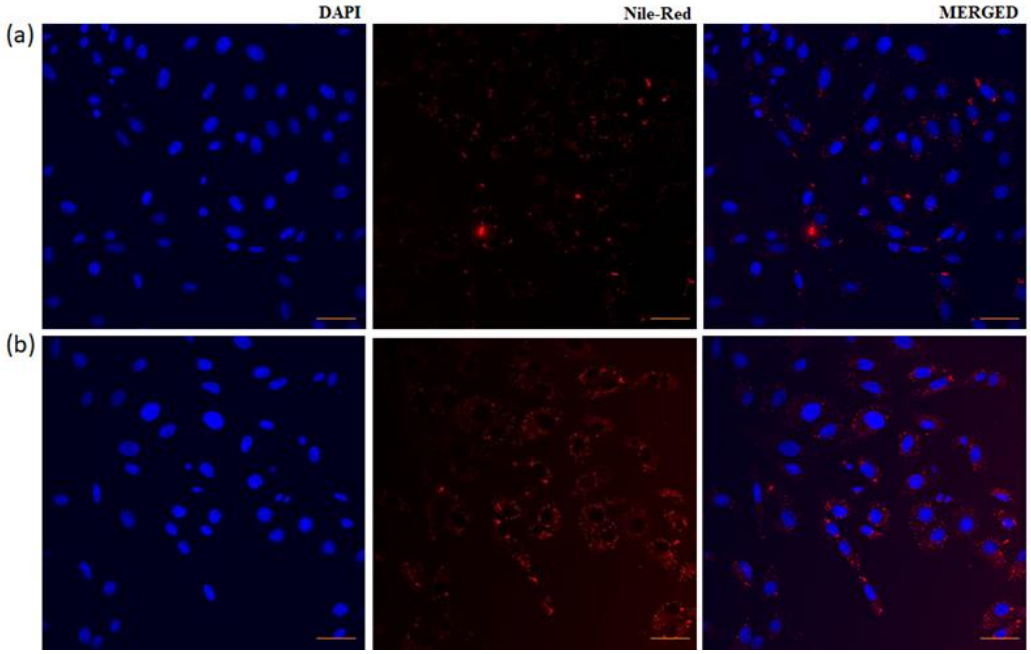


Figure 5: Distribution of lipid droplets in the cell in the presence and absence of 25HC. Panel (a) Less number of lipid droplets proclaim more cholesterol in the cell membrane in the absence of 25HC. Panel (b) the presence of 25HC marks depletion of cholesterol indicating more lipid droplets, stained with Nile-red. Scale bar is 40µm.

25-HC is the product of the interferon-inducible enzyme CH25H. It was earlier reported that CH25H expression is significantly upregulated upon infection by human parainfluenza virus type 3 (HPIV3) and respiratory syncytial virus (RSV), SARS Cov2 and Zika virus (Li et al. 2017, Wang et al. 2020). 25-HC demonstrates a broad range of antiviral activity; however, it's in vitro antiviral activity against JEV has not been reported. Our results show that 25 HC efficiently inhibits JEV infection in Vero cells and warrants further investigation. Antiviral drug development against JEV is rather sluggish and has only limited success (Guo et al. 2024). Due to the increase in mosquito population, JEV is rapidly spreading to various parts of the world and, therefore there is an urgent unmet need to develop sustainable antiviral strategies against JEV. In this context, our finding may lead to a new treatment against JEV as well as a novel pan-antiviral strategy against flaviviruses.

Author Contributions: KA carried out experiment, collected and analyzed data, and wrote the first draft, BB generated and characterized the antibody. SH conceptualized, supervised, analyzed data, wrote and edited the manuscript. RKT supervised, edited the manuscript and secured funding.

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Supplementary Information

Materials and methods:

Cytotoxicity assay. Vero Cells (Vero Cells C1008, cat- CRL-1586 ATCC) were cultured in growth medium [DMEM media (D5648-L, Sigma) with 10% FBS (10270-106, GIBCO)] under 5% CO_2 at 37°C. Cells were seeded in a 96well cell culture plate ($1*10^4$/well) in 200 µl/well. After 12 hours of seeding, 25-Hydroxycholesterol (H1015-25MG, SIGMA) was added at different concentrations in triplicates with vehicle (DMSO) control. The plate was incubated at 37°C with 5% CO_2. At 68 hrs, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, CAS298-93-1, Sigma) was added. The plate was incubated at 37 °C with 5% CO_2. At 72 hrs, the supernatant was removed carefully and DMSO (100 µl/well) was added to dissolve crystals. The plate was kept at shaking condition for 10-15 minutes. Absorbance was measured at 570 nm using a microplate reader.

Flow cytometry

Vero cells were seeded in a 96 well plate. Cells were pretreated with 25-HC at different concentrations before 14hrs of infection followed by infection with JE virus [Japanese Encephalitis Virus (Vellore Strain) was a kind gift from Dr Vikas Agrawal at the Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow] at .01 MOI. The plate was incubated for 2 hrs under humid conditions in 5%CO_2. At 2 hrs post infection, infection media was removed and fresh media at 200µl/well (DMEM with 2 %FBS) with 25 HC (at the same concentrations of the pre-treatment) was added. The plate was incubated under humid condition in 5% CO_2 for 72 hrs.

Overlay media was aspirated and cells were washed with 1x PBS, 150 µl/well. Cells were harvested by adding 0.25% Trypsin-EDTA (25 µl/well). Next, cells were transferred to a U-bottom 96 well plate and were fixed with 4% paraformaldehyde (50 µl/well). After proper mixing, and the plate was incubated for 10 mins at RT to fix the cells. Cells were permeabilized with 1x permeabilization buffer at 150 µl/well (2%BSA+ 0.1%Saponin in PBS). Next, 40 µl/well blocking solution (1% normal mouse serum in 1x permeabilization buffer) was added. After 30 minutes incubation at RT, 4G2-alexa488 mAb (20 µl/well) was added, antibody dilution was prepared in blocking buffer (1:400). The Plate was incubated at 37°C with shaking. After that the cells were washed with 1X permeabilization and buffer, and pelleted by centrifugation at 1000g. The cell pellet was resuspended in 1x PBS (200 µl/well). The samples were read in a flow cytometer (BD Bioscience-FACS LYRIC). Data was analyzed using FlowJo (BD Bioscience).

Confocal microscopy

Cells were seeded on coverslips and treated with 25-HC for 24 hours. The cell monolayer was washed with 1X PBS and was fixed with 4% PFA. Next, cells were permeabilized using TritonX100 (0.1%) followed by washing with PBS. Cells were stained with Nile red (N30/3-1G, sigma) by incubating with Nile red. After that the cells were washed with PBS. The coverslips were mounted on a glass slide with a mounting media containing DAPI (F6057, Sigma). The slides were imaged using an Olympus confocal microscope.

Antigen release assay

Vero Cells were cultured in a growth under 5% CO_2 at 37°C. After 14 hours of seeding cells were pretreated with 25-HC at different concentrations (20µM, 10µM, 5µM, 2.5µM, and 1.25µM). After 14 hrs, Cells were infected with the Japanese Encephalitis Virus at 0.01 MOI. 2% FBS was used in infection media. The plate was incubated at 37°C with 5% CO_2, during incubation plate was Shaken at 30-minute intervals to avoid drying. At 2 hours postinfection, the infection media with virus was replaced with fresh DMEM containing 2% FBS along with varying concentrations of 25HC. At 72 hrs post-infection, the supernatant was collected to check viral (antigen) release in the supernatant using ELISA. 96 well, high binding ELISA Plate (Corning-3590) was coated with 100µl/well coating buffer [0.1M Sodium Bicarbonate solution, pH-9.6 with 2.5 µg/ml 4G2 antibody (against envelope fusion domain of JEV) (Wang et. al. 2020a). The plate was incubated overnight at 4°C, followed by three times 1XPBS washing. For blocking, 5% skim milk in 1xPBS was used. The plate was again washed with 1x PBS three times (150 µl/well). Each sample was added at 100 µl/well in triplicates. The plate was incubated at 37°C for one hour followed by washing with PBST (0.1% tween20). HRP-tagged 4G2 antibody (1:4000),100 µl/well was added. After one hour of incubation, three times PBST washing was done. TMB substrate (TMBW-0100-01, Surmodics) was added (100 µl/well) in the dark. The plate was incubated at 37°C for 30 minutes. In the end, 7% (v/v) H_2SO_4 was added (50 µl/well). The plate was read at 450 nm in an ELISA plate Reader in chemiluminescence mode.