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Supplementary appendix

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Web Appendix to Effectiveness of ChAdOx1 nCoV-19 vaccine against SARS-CoV-2 infection during the B·1·617·2 (delta) variant surge in India: a test-negative case-control study and a Mechanistic Study of Post-Vaccination Immune Responses

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Supplementary methods

Assessment of clinical effectiveness of the vaccines:

The clinical effectiveness of the vaccines were assessed using a test-negative case control design during the period April 1, 2021 to May 31, 2021 when there was a massive surge of infection in India largely due to delta (B·1·617·2) (Figure S1). Our study population included all adults who underwent RT-PCR assay for SARS-CoV-2 at Translational Health Science and Technology Institute and ESIC Medical College and Hospital, the two large accredited laboratories in Faridabad district of the state of Haryana. Individuals visiting these health centres were tested if they had symptoms of influenza-like illness or have contact history with a SARS-COV-2 positive individual.[1] The samples were sent to the laboratories with a test requisition form that provided the clinical and contact details. The samples were analysed by RT-PCR assay which amplified ORF, E and S genes. The test results were reported in the format recommended by the Government of India. For the present study, we obtained all entries made by the two laboratories from April 1, 2021 till May 31, 2021. The study was approved by the Institute Ethics Committees of the partnering institutions.

Outcome assessment

From the database of study population, we selected all adults who tested positive as cases. If there was more than one test positive result for a person, the first instance of testing was selected as the date of testing. Individuals who negative results were considered for selection of controls. The control group was randomly sampled, aided by a computer program, in equal numbers as the case group matched for each calendar week of testing for the study period. We had 3695 and 2883 individuals with negative and positive tests respectively after we excluded 169 who refused consent for the interview, 194 who reported testing positive during the previous wave of infections in 2020 and 191 who were sampled before our study time frame of April 1, 2021. Additional 683 test negative individuals were excluded by random sampling as they were in excess of the test positives for each week window. Of the test negative individuals, 476 reported a positive result from tests done at a different laboratory. As per *a priori* study plan, these participants were excluded. (Figure S2, Table S1).

Sample size estimation – justification: We chose 60% VE for sample size estimation for the following reasons. The Phase 3 trial of ChAdOx1 nCoV-19 reported an overall efficacy of 70.4%. [2] Sub-analysis of the Phase III COV002 trial in the UK demonstrated vaccine efficacy of 70.4% (95% CI: 43.6% to 84.5%) at preventing symptomatic COVID-19 against the Alpha variant. [3] Since these were from RCTs, we expected a slight drop in the effectiveness in a real-world scenario as opposed to the ideal scenario in an RCT. We also expected some drop in the effectiveness against Delta variant as there were a few reports on the reduction in *in vitro* neutralization tests.[4] We, therefore, estimated our sample size for a 60% VE rather than 70% reported against previous variants but above the WHO requirement for approval of 50% to be clinically meaningful.

Figure S1: Period of study coinciding with the recent massive surge of SARS-CoV-2 infection in India

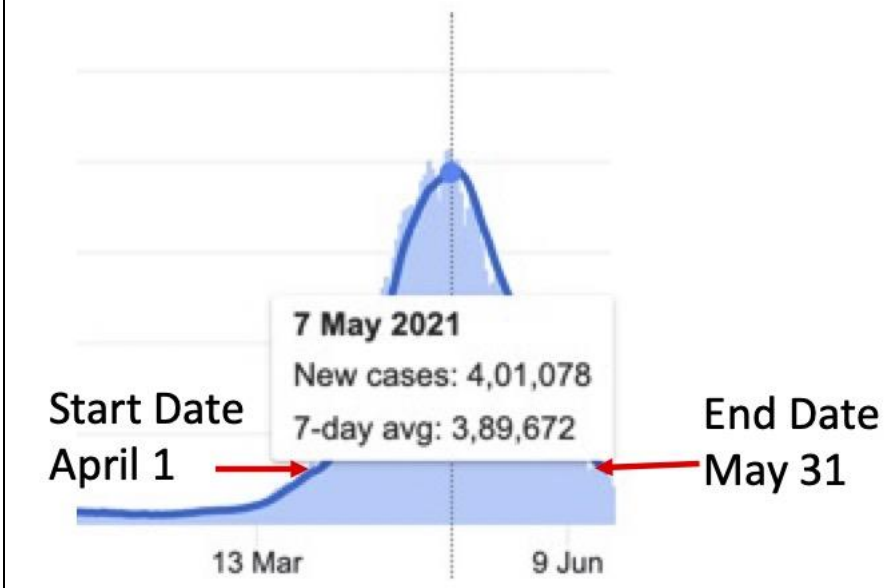


Figure S2. Participant flow in the study to estimate vaccine effectiveness

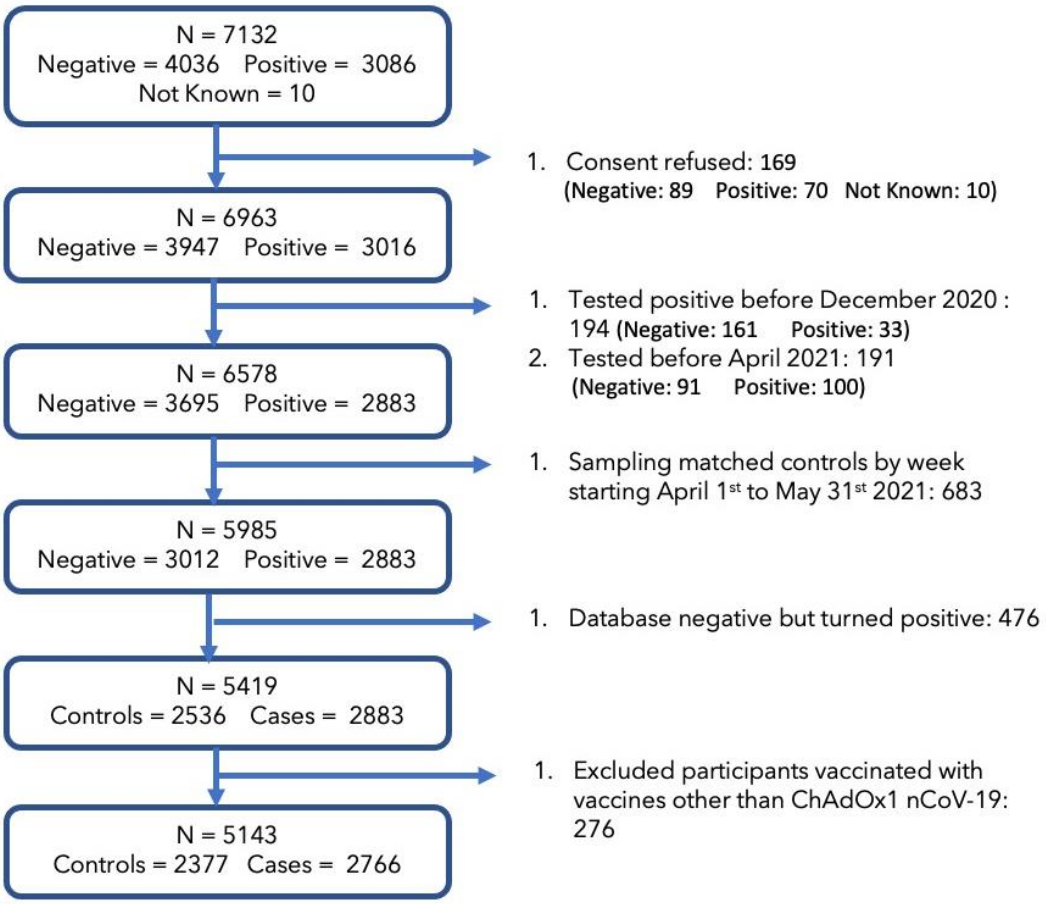


Table S1 Week-wise sampling numbers of cases and controls

Week starting from (YYYY-MM-DD)	Controls	Cases
2021-04-01	160	240
2021-04-08	599	588
2021-04-15	434	510
2021-04-22	250	306
2021-04-29	347	520
2021-05-06	382	405
2021-05-13	134	138
2021-05-20	71	59

Exposure assessment

We assessed the vaccination status of the study participants by a telephonic interview. The sampled participants, both test positive and negative individuals were reached out and a verbal informed consent was obtained before data collection. All those who consented were asked about their vaccination status, number of doses, dates of the doses, name of the vaccine and place of vaccination. Two types of vaccine are being given in India: ChAdOx1 nCoV-19, an adenovirus vectored, vaccine (Covishield) and a whole inactivated virion vaccine (Covaxin, Bharat Biotech, India). The type of vaccine received was confirmed based on the vaccination certificate or vaccination text message sent by the Government of India to the recipients. For 269 participants who were not aware or uncertain of the type of vaccine received, the name of the vaccine and dates of the vaccine were obtained from the immunization centres maintained by the district administration·

Assessment of covariates

We identified confounders, *a priori*, which needed adjustments in analysis based on the following rationale. Differences exist in the vaccination pattern based on age groups across different timepoints due to national vaccination policy to prioritise at-risk groups. Males are generally active outdoors and at risk of contracting infections and they tend to get vaccinated more than females. People who are frontline workers and at higher risk of exposure tend to get vaccinated more and they are the people who are more likely to get infected too. Therefore, we adjusted for these confounding factors - age, sex, and exposure.

The week-wise matching between cases and controls was done to ensure comparability between cases and controls. Both vaccination and infection by the rapidly evolving Delta strain present an evolving scenario over time and may lead to bias if the cases and controls are not similarly distributed over the study period. This could be due to many reasons. For instance, if we sample more cases than the controls in the later weeks of study period, the susceptibility period for acquiring an infection becomes imbalanced with the controls having a shorter one and if they had been given adequate time, they could have acquired the infection too and turned cases.

To account for confounders, we undertook matching and adjusting in a multivariable model as two strategies. We adjusted those confounders in the model for which we wanted to understand the impact on the effect estimate and matched those for which we didn't. Since the week of testing was of interest more from the point of preventing it from affecting the vaccine effectiveness (VE) estimate rather than as a biological covariate, we matched for it, and we adjusted for the other confounders in the model.

Quality control steps: Correction of discrepancy and missing data

For ensuring data quality, we instituted a series of quality assurance processes such as random real-time call monitoring. The real time monitoring of calls was scored for how the questions were put to the participants by the tele callers. Supervisors in the study dialled into the conversation between the interviewer and the participant, assessed how the questions on different parts of the questionnaire were framed by the interviewers and scored them on a scale of 1-5 (5 being the most accurate and 1 being the worst). The median score was 5 and almost 80% of the calls were scored 4 or more. To ensure that the data were consistently collected, nearly 5% participants were recalled by supervisors to check the essential variables (Table S2). The incoming data collected on Google Forms were checked real-time by the data management team to pick up inconsistencies with respect to age, dates of testing, vaccination, interval between the doses of vaccine, repeat calls due to call drops and manual errors in allotment and calling (Table S3).

Table S2. Agreement scores between responses elicited by the interviewer and the supervisor for important variables (N= 198)

Variables	Agreement (%)
Call check (to check if the participant received a call from our study team)	100
Name of the vaccine (Checked against vaccination portal)	92
Did you test positive on or before December 31, 2020	99
Oxygen supplementation	88

Table S3. Discrepancies identified during the process of data collection and resolution methods

Issues	Numbers	Solution
Sampling Duplicates (Same entries from the laboratory sampled on two dates)	214	Checked duplicates with respect to age, sex and phone number in sampling sheet These duplicate entries were removed at the time of data collection
Duplicate entries (Questionnaire administered twice due to call drops and manual errors in allotment and calling)	47 pairs	Checked for agreement between duplicates, Where all fields matched (13) - 1st entry was retained for analysis Where a few fields matched (8) - The entry with complete information was retained for analysis Where fields didn't match (26) - Call log was checked for correctness of calls and the first entry was retained for analysis.
ID-Name Mismatch (between the name in the laboratory database and response from participant)	104	Programmatically picked the mismatch and resolved manually
ID outside the range of allotment	190	If ID did not match with the allotted list, it was resolved manually

Neutralization assays:

Live Virus Focus Reduction Neutralization Assay:

Virus microneutralization assay titres were estimated as described previously·[5] Briefly, Vero E6 cells were seeded at 30,000 cells per well in a 96-well plate. Heat-inactivated serum samples (75 µl) were serially diluted from 1:20 to 1:640 using growth medium with 2% heat-inactivated FBS. To this, 75 µl of SARS-CoV-2 (dilution pre-determined to produce 50-150 microplaques) was added and kept for 1 h at 37ºC in 5% CO_2 incubator. The virus-serum mixtures were then added on Vero E6 cells and further incubated for 1 h at 37ºC for virus adsorption. After 1 h, viral inoculum was removed and cells were overlaid with 1·5% carboxymethylcellulose (Sigma-Aldrich) in growth medium with 2% heat-inactivated FBS and incubated at 37ºC in 5% CO 2 incubator. At 24 hours post infection, cells were fixed with formaldehyde solution and then stained with anti-spike RBD antibody at 1:4000 dilution (Sino Biologicals) for 1 hour, followed by HRP-conjugated anti-rabbit antibody at 1:4000 dilution (Invitrogen) for 1 hour. Cells were washed with PBS and incubated with TrueBlue substrate (Sera Care) for 10 minutes and washed with sterile MilliQ water. Plated were air-dried and microplaques were quantified by AIDiSPOT reader (AID GmbH, Strassberg, Germany) using AID EliSpot 8·0 software. 50% neutralization values were calculated with four-parameter logistic regression using GraphPad Prism 7·0e software. All virus-related experiments were performed in a biosafety level 3 lab.

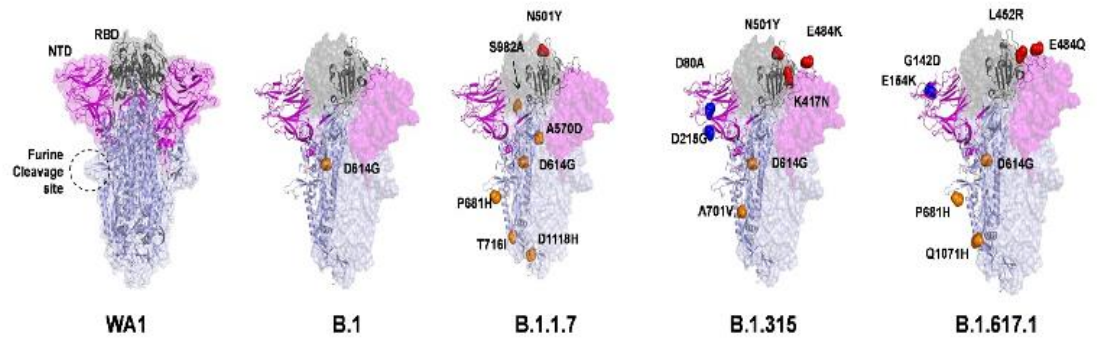
Table S4. Details of the live viruses used in the assays:

S. No.	Virus name	Virus details	Accession No.
1	SARS-CoV-2 Wuhan Isolate	Isolate USA-WA1/2020, NR-52281	GenBank: MN985325, GISAID: EPI_ISL_404895, GenBank: MT020880
2	SARS-CoV-2 UK	Isolate USA/CA_CDC_5574/2020, NR-54011	GISAID: EPI_ISL_751801
3	SARS-CoV-2 SA	Isolate hCoV-19/South Africa/KRISP-K005325/2020, NR-54009	GISAID: EPI_ISL_678615
4	SARS-CoV-2 (delta)	THSTI_287	GenBank: MZ356566.1

Quantitative antibody measurement:
The antibody concentration against receptor binding domain of Spike protein of SARS-CoV-2 was measured by enzyme linked immunosorbent assay (ELISA). National Institute for Biological Standards and Control 20/130 research reagent (assigned concentration of 502 ELISA Units/ml (ELU/mL)) was used as standard primary reference material. Lower limit of detection for the assay was 8 ELU/ml. Antibody concentrations were calculated for each sample dilution by interpolation of the OD values on the 4-parameter logistic (4-PL) standard curve from positive control and adjusted according to their corresponding dilution factor using Gen5 software (BioTek Instruments, USA).

Expression and purification of RBD protein of wildtype SARS CoV-2 and Variants:
We expressed and purified recombinant RBD protein of wild type SARS-CoV-2, and RBD proteins of mutant viruses from Expi293 cells (ThermoFisher) by introducing site-directed mutagenesis one by one for E484Q, L452R (typical of B·1·617), N501Y (typical of alpha (B·1·1·7)), and K417N, E484K and N501Y (typical of beta (B·1·351)) as described earlier·[6] The desirable mutations were introduced utilizing primers designed to introduce requisite changes. (Figure S3) The transfected supernatants with secreted protein were harvested 6 days post-transfection by centrifugation of the culture and loaded onto Ni-NTA agarose (Qiagen) for purification. Ni-NTA purified fractions were then pooled and concentrated using Amicon centricons (10kDa cut off) and further purified through size exclusion chromatography using a Superdex 200 Increase 10/300 GL column (GE Healthcare), equilibrated in PBS. The purified RBD was snap-frozen in liquid nitrogen and stored at −80 °C for further use.

Figure S3: Structural presentation of SARS-CoV-2 spike protein WT and the different circulating mutants·



The structure of spike was created using pymol and the protein data bank ID: 7DDD·[7] The N terminal domain (NTD) is shown in colour magenta, RBD in grey and the S2 domain in blue colour. The residues that are mutated are shown as spheres in a single monomer in cartoon representation (blue sphere in NTD, red sphere in RBD and yellow sphere in S2 domain). The other two monomers are shown in surface representation.

Peripheral Blood Mononuclear Cells isolation:
PBMC isolation was performed using the Ficoll-Paque density gradient centrifugation method. The blood sample was diluted with PBS and layered on top of Lymphoprep™ in a ratio of 1:1:1. The tubes were centrifuged at 800g, at 25°C, for 30 minutes without brakes. PBMC layer was transferred to 50ml tube by pipetting along with remaining plasma. The tubes were then filled up to 50ml with complete media and centrifuged at 300g, at 4°C, for 15 minutes [8]. The supernatant was discarded, and the cells were resuspended in 5ml of complete RPMI (RPMI 1640, 10% heat-inactivated FBS, 2 mM L-glutamine, 1 % nonessential amino acids, 1 % sodium pyruvate, 5×10^{-5} M β-mercaptoethanol). The cells were then counted in a hemocytometer by Trypan Blue staining, and the number of cells was noted. Five million cells were separated for stimulation and were given a second wash with RPMI in a 15ml falcon. The remainder of the cells were resuspended in freezing media containing 90% FBS + 10% DMSO and were stored at -80°C for two weeks before transferring to liquid nitrogen.

Ex-vivo T cell Stimulation:
T cell stimulation was performed unless mentioned otherwise by incubating PBMCs in complete RPMI media at 37°C in a 96 well plate with peptide pools at a concentration of 2 µg/ml/peptide. Stimulation with an equimolar concentration of dimethyl sulfoxide (DMSO) was used as a negative control. In all the T cell assays, phytohemagglutinin (PHA, Roche; 5µg/ml) was used as a positive control. Any sample with a low PHA signal was removed for quality control. For cytokine profiling, PBMCs were stimulated with spike peptide pools for 48h and the culture supernatant was analysed by cytokine bead assay. ICS was performed for PBMCs after stimulation with spike peptide pools or DMSO or PHA for 18-20 hours with the presence of monesin (GolgiStop™, BD Bioscience) during the last 6h. AIM assay was performed as described previously.[9] Briefly, the PBMCs were cultured for 24h in the presence of spike peptide pool or DMSO or PHA. For IFN-γ ELISA, PBMCs were stimulated with whole RBD protein as described earlier.[2] Briefly, 0·2-0·5 million cells were stimulated with either WT or mutant recombinant RBD protein at a concentration of 1µg/well in 96 well plate in 200 µl of complete media. A similar number of PBMCs were seeded with complete media alone without any stimulation. The unstimulated cells served as negative control, and T cells immune response was calculated by subtracting the readings of unstimulated PBMCs from the stimulated PBMCs.

Cytokine Profiling:
After ex-vivo stimulation of PBMCs with antigens for 48h, cell culture supernatant was collected and cytokines, TNFα, IFNγ and IL-2, concentrations in the culture supernatants were determined using a bead-based multiplex TH1/TH2 human LEGENDplex™ immunoassay (Biolegend) as per the manufacturer's instructions. The bead's fluorescence was measured with a BD FACSymphony (BD Biosciences) and analysed with the cloud based LEGENDplex™ Data Analysis Software Suite. Spike-specific T cell cytokine production was obtained by subtracting background values of DMSO-stimulated control media. Negative values were set to zero.

Intracellular staining and cell surface staining: Spike peptide pool stimulated PBMCs were stained with live/dead marker and T-cell surface markers (CD4 FITC, 1:100; CD8 BV510, 1:100; all BD Biosciences) in FACS buffer for 30 min at 4 °C. Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences)

according to the manufacturer's instructions. Intracellular staining was performed in Perm/Wash buffer for 30 min at 4 °C (IFNγ PE-Cy7, 1:50; IL-2 BB700, 1:50; TNFα BB700, 1:50; GranzymeB (GZB) PE 1:50; Perforin APC 1:100 all BD Biosciences). For AIM assay, spike peptide pools stimulated PBMCs were stained for live/dead marker and surface markers (CD4 FITC, 1:100; CD8 BV510, 1:100; CD69 PE-CF594 1:100; CD137 BV-605 1:100; OX-40 PE-Cy7 1:100, all BD Biosciences) in FACS buffer (PBS supplemented with 2% FBS (Gibco)) for 30-40 min at 4 °C. Samples were then acquired on a FACS Symphony™ instrument (BD Biosciences) using BD FACSuite software version 1.0.6 and analysed with FlowJo software version VX (FlowJo LLC, BD Biosciences). Spike-specific cytokine production was background subtracted by the values obtained with DMSO-containing medium. Negative values were set to zero.

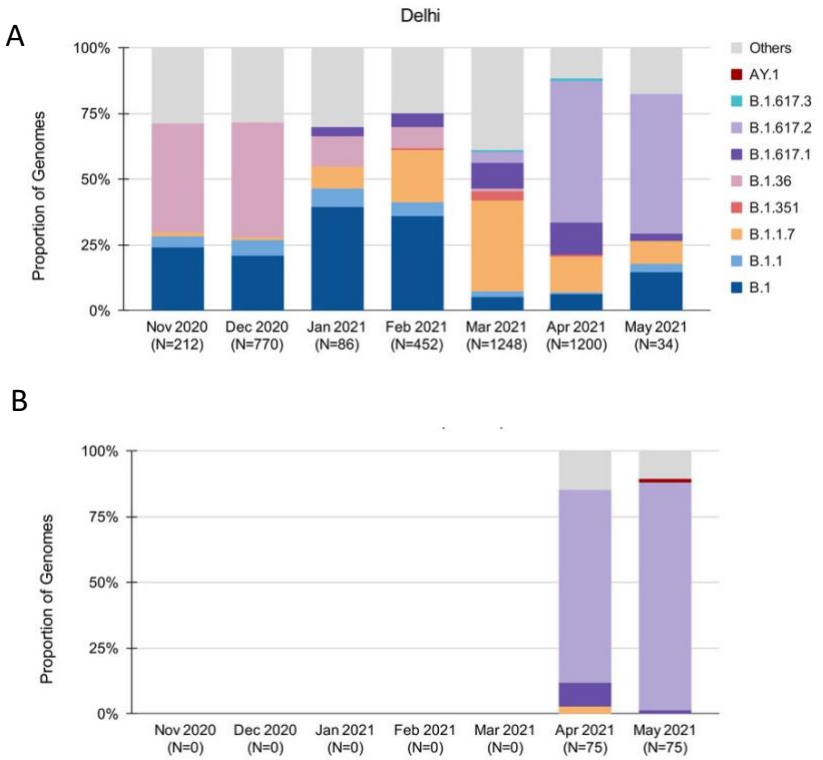
Ex-vivo IFN-γ ELISPOT Assay: PBMC were thawed in complete medium and treated with Benzonase (50 IU/mL; Sigma) at 37°C for 60 min. Subsequently, 0.25×10^6 PBMC were stimulated with SARS-CoV-2 PepMix or PepTivator® peptide pools at 2µg/mL per peptide in 100µl in precoated and blocked ELISPOT plates (MabTech) at 37°C for 20 hours. As a positive control PHA was used. After 20h, cells were removed and plates were developed as per the manufacturer's guidelines (MabTech Cat. No. 3420-4AST). Subsequently, the plates were analysed using AIDiSPOT reader (AID GmbH, Strassberg, Germany) using AID EliSpot 8·0 software. Number of spots per million cells were calculated after subtraction of the spots in DMSO treated control wells (Figure S6e).

ELISA for IFN-γ: ELISA was performed to detect antigen-specific IFN-γ production from culture supernatant from RBD-stimulated PBMCs on day three of cell culture. As a positive control, PHA was used, samples that did not exhibit positive IFN gamma response in PHA treated wells, were omitted from the analysis. BioLegend® ELISA MAX™ Deluxe Human IFN-γ kit (BioLegend, Cat. No. 430104) was used, and the manufacturer's protocol was followed. The cell supernatant was diluted with 1X Assay Diluent in a ratio of 1:4. A standard curve ranging from 2000pg/ml to 31·25pg/ml was set up to quantify results. The standards were added in duplicates, and the mean OD of the duplicates was used for generating the standard curve. Absorbance at 450nm was read on BioTek Synergy HT Microplate reader and was analysed using BioTek Gen5 software. The RBD-specific IFN-γ amounts were corrected for background by subtraction of values obtained from unstimulated PBMCs. Negative values were set to zero. Based on the IFN-γ production, samples were classified as 'responder' in case PBMCs stimulated with WT RBD protein secreted higher IFN-γ as compared to the unstimulated PBMCs.

Whole Genome sequencing: The biospecimens for sequencing were sampled randomly from those who came for RT-PCR testing at our testing centres distributed across the study period as part of the SARS-CoV-2 genomic surveillance program and are reliable representation of the study population and period. Sequencing ready libraries were prepared from the archived SARS-CoV-2 RNA samples using the amplicon-based COVIDSeq (Illumina Inc, USA) test kit.[10] All the synthesized libraries were sequenced on the NovaSeq 6000 platform with a read length of 100x2 base pairs.The raw data generated by sequencing in binary base call format was demultiplexed to FASTQ files using bcl2fastq (Illumina, v2·20). The reads were trimmed at a base quality cut-off of Q30 using Trimmomatic (v0·39)[11] The trimmed reads were aligned to the SARS-CoV-2 reference genome (NC_045512·2) using Hisat2 (v2·1·0).[12] The aligned data files were used for further analysis including variant calling and consensus sequence generation. Lineages were assigned using pangolin (v3·0·5, pangoLEARN 2021-06-05)[13]

Supplementary results

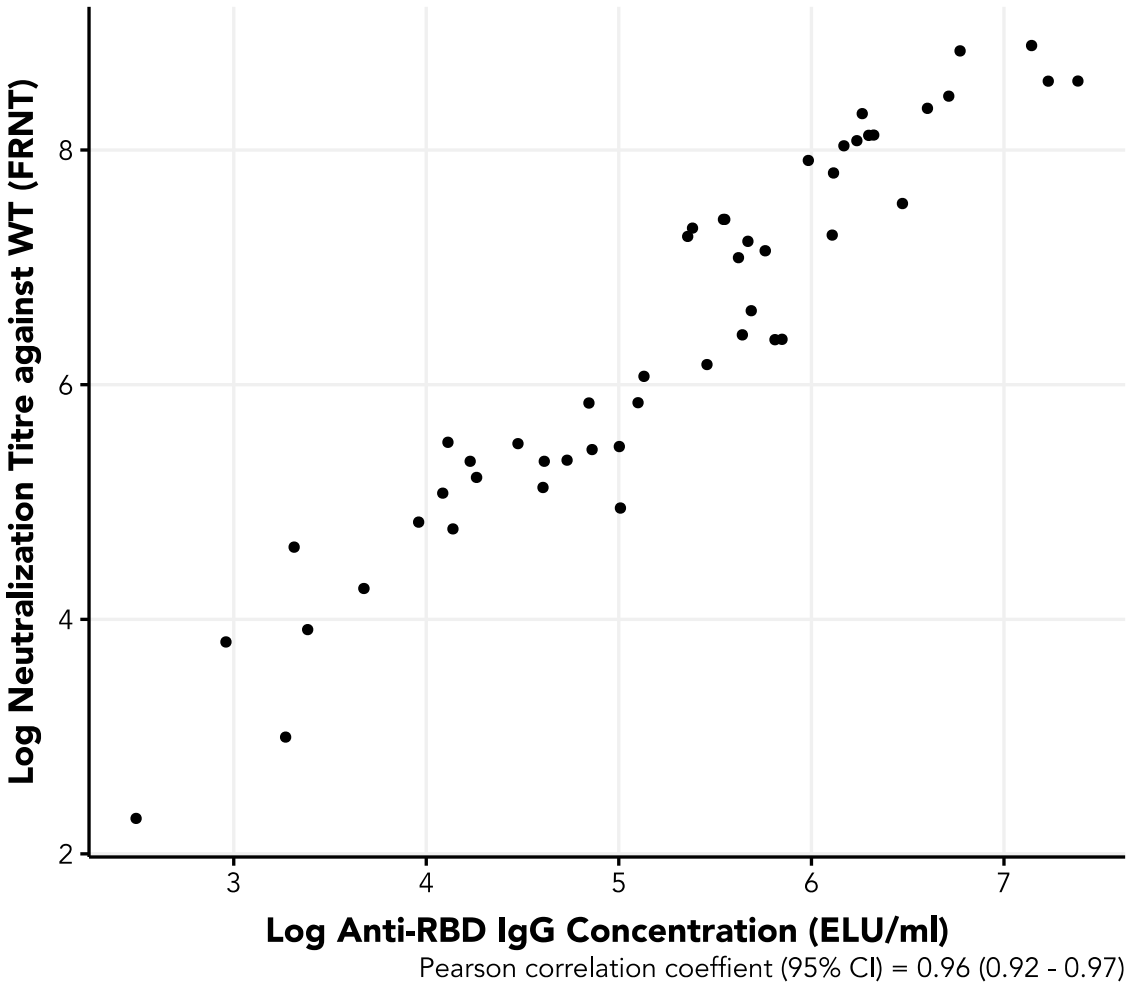
Fig S4. Month wise distribution of VoC in Delhi National Capital Region



The top graph (A) shows the dominant strains of SARS-CoV2 in national capital, Delhi. The bottom graph (B) demonstrates the dominance of delta in our study population during the study period.

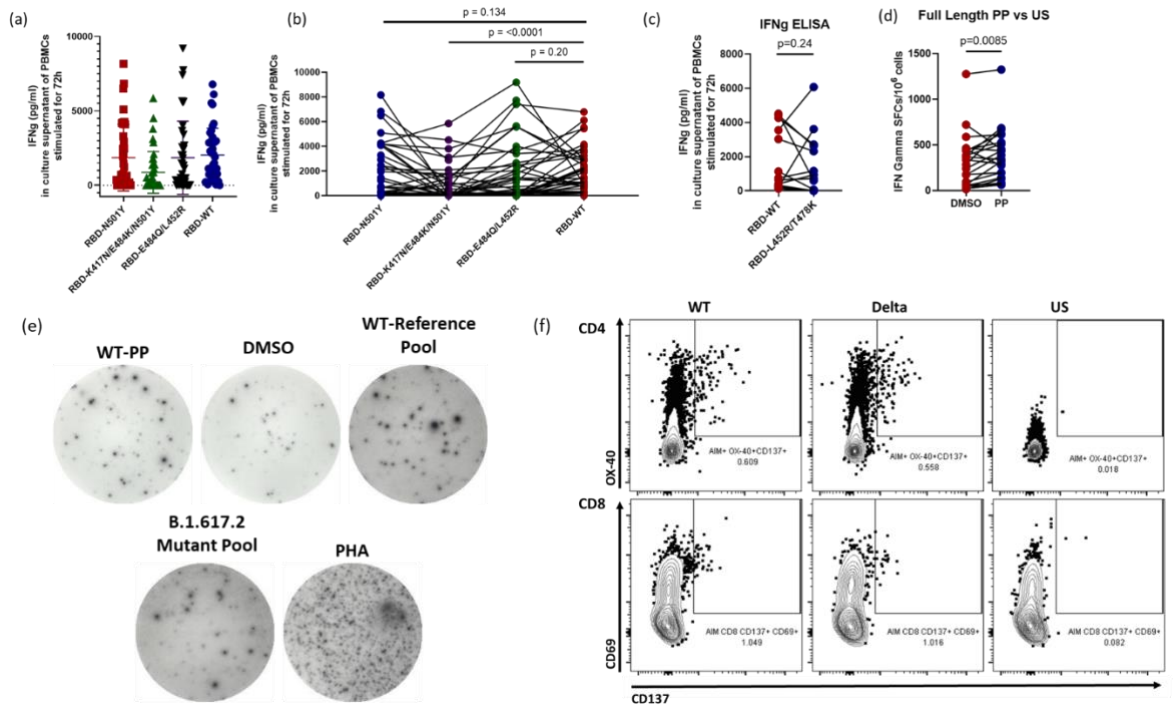
These biospecimens were sampled randomly from those who came for RT-PCR testing at our testing centres distributed across the study period as part of the SARS-CoV-2 genomic surveillance program and are reliable representation of the study population and period (http://clingen.igib.res.in/covid19genomes/).

Fig. S5: Correlation between anti-RBD SARS-CoV-2 IgG concentration and neutralization titre against SARS-CoV-2 WT



The logarithmic values of anti-RBD SARS-CoV-2 IgG concentration and neutralization titres against SARS-CoV-2 WT are plotted on X- and Y-axis respectively. N=49

Fig. S6: SARS-CoV-2 Specific T cell response in vaccinated individuals



(a-b) IFN-γ response by PBMCs upon stimulation with wild type and VoCs RBD protein (mean IFN-γ secretion of WT-RBD = 2032·6 pg/ml; alpha = 1858·8 pg/ml; beta = 869·0; kappa = 1853). (c) IFN-γ response by PBMCs upon stimulation with wild type and delta RBD protein (mean IFN-γ secretion of WT-RBD = 1605.7 pg/ml; and delta = 1389.5 pg/ml). (d) Antigen-specific T cell immune response was evaluated by stimulating 0·25 million PBMCs with WT overlapping peptide pool of 15mers with 11 amino acid overlap of full-length WT spike protein. As a negative control, the PBMCs were treated with equal volume of DMSO. Each data point represents the spot count from wells for one study participant (e) Representative ELISPOT image of stimulated PBMCs (f) Representative flow cytometry plots of SARS-CoV-2 specific AIM+ CD4+ and CD8+ T cells.; PP = Peptide Pool; SFCs = Spot Forming Cells.

Table S5. Clinical characteristics of the healthy vaccinated participants included for immunological analysis (N= 59)

Characteristic	Value
Age (years)	33 (28, 38)
Sex	
Female	17 (29%)
Male	42 (71%)
Median (IQR) duration between sampling and second dose (days)	61 (52, 79)

Table S6. Neutralization geometric mean titres of recipients of ChAdOx1 nCoV-19 against SARS-CoV-2 variants

Variant	N[1]	GMT by NT50 (95%CI)	p-value[2]	Fold reduction in GMT (%)
WT	49	599·4 (376·9, 953·2)	Ref.	1
alpha	49	244·7 (151·8, 394·4)	0·04	2·5
beta	49	97·6 (61·2, 155·8)	< 0·0001	6·1
kappa	47	112·8 (72·7, 175)	< 0·0001	5·3
delta	49	88·4 (61·2, 127·8)	< 0·0001	6·8

[1]Number of participants whose plasma 50% virus neutralization titres (NT50) were estimated by focus reduction neutralization test against specific variants
[2]Statistical test – Analysis of variance followed by Tukey post hoc comparison with the titre against WT as the reference
GMT - Geometric mean titre; NT50 – 50% neutralization in Focus Reduction Neutralization Test

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