

1 Isolation and Characterization of SARS-CoV-2 strains circulating in Eastern India.

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11

12 **Abstract:**

13 Emergence of SARS-CoV-2 as a serious pandemic has altered the global socioeconomic
14 dynamics. The wide prevalence, high death counts and rapid emergence of new variants urge
15 for establishment of research infrastructure to facilitate rapid development of efficient
16 therapeutic modalities and preventive measures. In agreement with this, five SARS-CoV2
17 strains (ILS01, ILS02, ILS03, ILS15 and ILS24) of four different clades (19A, 19B, 20A and
18 20B) were isolated from patient swab samples collected during the 1st COVID-19 wave in
19 Odisha, India. The viral isolates were adapted to *in-vitro* cultures and further characterized to
20 identify strain specific variations in viral growth characteristics. All the five isolates showed
21 substantial amount of virus induced CPE however ILS03 belonging to 20A clade displayed
22 highest level of CPE. Time kinetics experiment revealed spike protein expression was evident
23 after 16th hours post infection in all five isolates. ILS03 induced around 90% of cytotoxicity.
24 Further, the susceptibility of various cell lines (human hepatoma cell line (Huh-7), CaCo2 cell
25 line, HEK-293T cells, Vero, Vero-E6, BHK-21, THP-1 cell line and RAW 264.7 cells) were
26 assessed. Surprisingly, it was found that the human monocyte cells THP-1 and murine
27 macrophage cell line RAW 264.7 were permissive to all the SARS-CoV-2 isolates. The
28 neutralization susceptibility of viral isolates to vaccine-induced antibodies was determined
29 using sera from individuals vaccinated in the Government run vaccine drive in India. The
30 micro-neutralization assay suggested that both Covaxin and Covishield vaccines were equally
31 effective (100% neutralization) against all of the isolates. The whole genome sequencing of
32 culture adapted viral isolates and viral genome from patient oropharyngeal swab sample
33 suggested that repetitive passaging of SARS-CoV2 virus in Vero-E6 cells did not lead to
34 emergence of many mutations during the adaptation in cell culture. Phylogenetic analyses
35 revealed that the five isolates clustered to respective clades. The major goal was to isolate and
36 adapt SARS-CoV-2 viruses in *in-vitro* cell culture with minimal modification to facilitate
37 research activities involved in understanding the molecular virology, host-virus interactions,
38 application of these strains for drug discovery and animal challenge models development which
39 eventually will contribute towards the development of effective and reliable therapeutics.

40

41 **Introduction**

42 Since its emergence in December 2019, in Wuhan, China, the Severe Acute Respiratory
43 Syndrome Coronavirus 2 (SARS-CoV-2) has had an unprecedented effect on human health
44 and well-being world over ¹⁻³. According to WHO data, the virus has infected 240 million
45 individuals worldwide and has so far caused 4.8 million fatalities⁴. SARS-CoV-2 is a single-
46 stranded, positive-sense RNA virus of the *Coronavirus* genus, family *Coronaviridae* and order
47 *Nidovirales* ³. SARS-CoV-2 genome is around 30 kb in size and shares 79% and 50%
48 homology with the genome of SARS-CoV and MERS-CoV, the causative agents of two earlier
49 coronavirus epidemics in 2002-03 and 2012. Based on the reproductive number (R_0) SARS-
50 CoV-2 (2- 2.2) is highly infectious then SARS-CoV (1.7-1.9) & MERS-CoV (<1) ⁵.

51 The SARS-CoV-2 genome ORF1a/ORF1ab encodes for two polyproteins, pp1a/pp1ab which
52 account for 2/3rd of the viral genome, and the remaining 1/3rd near the 3'-end encodes for four
53 structural proteins Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N) ¹. The
54 overlapping pp1a and pp1ab, are proteolytically cleaved by papain-like and chymotrypsin-like
55 viral proteases (PL^{pro} & CL^{pro}) to yield 16 non-structural proteins, which play an important role
56 in virus life cycle with cooperation from other accessory viral proteins ⁶. The transmission of
57 this virus occurs mainly through aerosols/liquid droplets that emanate from the cough/sneeze
58 from infected patients ⁷. Majority of the infected individuals are either asymptomatic or exhibit
59 mild flu like symptoms, whereas few patients exhibit severe clinical manifestation leading to
60 the severe Acute Respiratory Distress Syndrome (ARDS) ⁸.

61 The global prevalence of SARS-CoV-2 & rampant growth in the human host lead to emergence
62 of mutational variability among circulating viruses. Presence of multiple variants with
63 variability in infection/transmission and disease manifestation urge for isolation of the
64 circulating SARS-CoV-2 variants to enhance our understanding in variant specific differences
65 in viral growth characteristics, host interactions and disease pathogenesis. In this study, five
66 circulating strains of SARS-CoV-2 belonging to early clades have been isolated from
67 laboratory confirmed COVID-19 patients swab samples collected during the 1st COVID-19
68 wave in Odisha, India. The isolated strains have been further characterized and sequenced to
69 enable utilization of these isolates as resources in research and development towards prevention
70 and effective therapeutic intervention against COVID-19.

71

72 **Materials and method:**

73 **Cells and Viruses:** Vero E6, Vero, BHK-21, HEK293T and Huh7 cells were maintained in
74 high glucose DMEM supplemented with 10% fetal bovine serum and 1X
75 Pencillin/Streptomycin. CaCo2 cells were maintained in DMEM supplemented with 20% fetal
76 bovine serum and 1X Pencillin/Streptomycin. THP-1 and RAW 264.7 were maintained in
77 RPMI supplemented with 10% fetal bovine serum, 1X Pencillin/Streptomycin, 10 mM sodium
78 pyruvate, 1M HEPES, and glucose. The details about all the eight cell lines are provided in
79 Table 1. All the cell cultures were maintained in humidified environment with 5% CO₂ at 37°C.

80

81

82

83 **Table 1:** Details of the various cell lines used in this study.

| S No | Cell line | Source |
|------|-----------|-------------------------------------|
| 1 | Vero | Monkey kidney epithelial cell line |
| 2 | Vero-E6 | Monkey kidney epithelial cell line |
| 3 | HEK 293T | Human embryonic kidney cell line |
| 4 | Huh-7 | Human hepatoma cell line |
| 5 | CaCo2 | Human colon epithelial cell line |
| 6 | BHK-21 | Hamster kidney epithelial cell line |
| 7 | THP | Human monocytes cells |
| 8 | RAW 264.7 | Mouse monocytes cells |

84

85 The cells were seeded a day before infection such that they attain confluence on the day of
 86 infection. On the day of infection complete media was removed and respective virus infection
 87 was given at MOI of 0.1 in serum free media for 1.5 hr at 37°C with gentle rocking at every 15
 88 mins. After 1.5 hr the inoculum was removed and cells were washed twice with PBS and
 89 supplemented with complete media. Five different viral strains were isolated and characterized
 90 in the current study. The details regarding these viral strains are mentioned in Table 2.

91 **Table 2:** Accession numbers of the genome sequence and clade information of the viral RNA
 92 from source swab samples (S) and isolated & culture adapted viruses (A) used in this study.

| Name | Accession no | Clade |
|-------|---------------------|-------|
| ILS01 | EPI_ISL_463010 (S) | 19A |
| | MW559533.2 (A) | 19A |
| ILS02 | EP_ISL_3039724 (S) | 20A |
| | EPI_ISL_1190402 (A) | 19B |
| ILS03 | EPI_ISL_463032 (S) | 20A |
| | EPI_ISL_1196305 (A) | 20A |
| ILS15 | EPI_ISL_463054 (S) | 20B |
| | MW828325.1 (A) | 20A |
| ILS24 | EPI_ISL_463058 (S) | 19B |
| | MW828330.1 (A) | 19B |

93

94 **Specimen collection:** Oropharyngeal swab samples collected in VTM from suspected
 95 symptomatic and asymptomatic patients by the various sample collection centres in the state
 96 of Odisha, India during April-June 2020 were used in this study. The samples were tested for
 97 presence of virus by qRT-PCR and samples with Ct (Cycle threshold) values below 15 were
 98 subsequently used for virus isolation. Upon confirmation of infection, the samples were
 99 aliquoted and kept in deep freezers until further use.

100

101 **Ethics statement:** The current studies involving swab samples from the human participants
102 were reviewed and approved by the Institutional Human Ethics Committee, Institute of Life
103 Sciences. The Institutional Ethics Committee (IEC)/ Institutional Review Board (IRB)
104 reference number is 96/HEC/2020. The written consent form duly signed by the participants/
105 legal guardian was taken into consideration for the concerned study

106

107 **Virus Isolation:** Oropharyngeal swab samples of confirmed COVID-19 patients were used for
108 isolation of the virus. The oropharyngeal swab sample was diluted 1:1 with DMEM
109 supplemented with antibiotics and antifungal agents and filtered through 0.22-micron filter.
110 Vero E6 cells were infected with the filtered swab sample for 1.5 hr at 37°C with gentle rocking
111 every 15 mins. The inoculum was aspirated and cells washed with PBS and supplemented
112 with fresh media containing 2% FBS. The infected cells were regularly monitored for
113 cytopathic effect ⁹. 72 hr post infection the culture supernatants were collected and the clarified
114 supernatant (at 3000 rpm for 5 mins) were used as inoculum for subsequent (2nd) passage of
115 virus in naïve Vero E6 cells. This process was repeated every 48 hrs up to the 10th passage.
116 RNA isolated from the culture supernatants was used for confirmation of SARS-CoV-2 virus
117 isolation by qRT-PCR ¹⁰. Virus titres in the culture supernatants was estimated by TCID₅₀
118 assay. RNA isolated from 10th passage virus was used for determining the whole genome
119 sequence. SARS-CoV-2 virus isolation and culture was conducted in the biosafety level-3
120 containment facility according to the guidelines issued by the Department of Biotechnology,
121 Government of India. This study has been approved by the Institutional biosafety committee
122 (IBSC) (IBSC file no. V-122-MISC/2007-08/01).

123

124 **Viral RNA extraction & estimation:** RNA isolation from culture supernatant was performed
125 using QIAamp Viral RNA Kit (Qiagen, cat. no. 52906) according to the manufacturer's
126 instructions. The isolated RNA was subjected to qRT-PCR for determining the viral load by
127 absolute quantification by real-time RT-PCR using Takara PrimeScript™ one-step RT-PCR
128 Kit (RR055A) with forward (5'-GTGAAATGGTCATGTGTGGCGG-3') and reverse (5'-
129 CAGATGTAAAGACACTATTAGCATA-3') primers and probe (5'-FAM-
130 CAGGTGGAACCTCATCAG GAGATGC-BHQ-3') targeting the SARS-CoV2 RdRp gene.
131 Standard curve was generated using known quantities of SARS-CoV2 viral RNA purified from
132 the viral stock supernatants.

133

134 **Plaque Assay:** To determine the viral titre plaque assay was performed as described by Mishra
135 et.al (2016) ¹¹. In brief, 80% confluent VeroE6 cells were infected with serially diluted viral
136 culture supernatant. Subsequently the cells were overlaid with complete methyl cellulose and
137 maintained in the incubator at 37°C with 5% CO₂. After the development of the visible plaques
138 (6-7 days), the plaques were fixed by adding 8% formaldehyde. Later on, the cells were stained
139 using crystal violet. The number of plaques were counted as plaque forming unit/mL
140 (PFU/mL).

141

142 **TCID₅₀ Assay:** Vero E6 cells seeded at 90% confluency in 96-well plates were infected for 1
143 hr at 37°C with 100 uL of serially diluted (10-fold) virus inoculum in DMEM with 2% FBS. 1
144 hr post infection the inoculum was aspirated and cells were replenished with fresh media. 3
145 days post infection the cells were fixed in 4% paraformaldehyde and stained with 1% crystal
146 violet to determine the cytopathic effect. Median tissue culture infectious dose (TCID₅₀) was
147 determined by the Reed and Muench method ¹².

148

149 **Immunofluorescence Assay:** The immunofluorescence assay was performed according to the
150 method described by Kim et.al (2013) for the detection of infected cells ¹³. The Vero E6 cells
151 grown on glass cover slips were infected with 0.1 MOI of respective isolates and 48 hr post
152 infection fixed in 4% paraformaldehyde. Subsequently the cells were permeabilized and
153 blocked for 1 hr with PBS containing 0.1% TritonX-100 and 3% BSA, followed by incubation
154 with antibody targeting the SARS-CoV-2 nucleocapsid (Abgenex, cat. No. 11-2003) overnight
155 at 4°C. After 3x washes with PBS, the cells were stained with the respective Alexa Fluor
156 conjugated secondary antibody (Invitrogen, Carlsbad, CA), for 1 hr at room temperature
157 followed by 3x washes with PBS. After the final wash, the coverslips were mounted onto
158 ProLong Gold Antifade (Invitrogen, Carlsbad, CA). Images were captured under a 100×oil
159 immersion objective lens using a Leica TCS SP5 Confocal microscope for detection of virus-
160 infected cells protein.

161 **Western blot analysis:** Immunoblot analysis was carried out as mentioned before¹³. In brief,
162 cells were lysed in RIPA buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM NaF, 1 mM
163 Na₃VO₄, 0.1% SDS, and 0.5% TritonX-100) containing the protease inhibitor cocktail
164 (Thermo Scientific). The whole cell lysates (WCL) were subjected to SDS-PAGE and
165 transferred to nitrocellulose membrane (Thermo Scientific) followed by blocking and
166 immunoblotting with antibodies specific for SARS-CoV-2 spike (Abgenex, cat. No. 10-1007)
167 and nucleocapsid (Abgenex, cat. No. 11-2003).

168

169 **Micro-neutralization Assay:** Micro neutralization assay was performed as mentioned before
170 ¹⁴. Briefly, serum samples were heat-inactivated for 60 minutes at 56°C; and syringe filtered
171 through 0.22 μm. These samples were then two-fold serially diluted in a 96-well plate starting
172 from 1:10 and then mixed with equal volume of virus solution containing 1000 TCID₅₀ of
173 SARS-CoV-2. This serum-virus complex was incubated for 1 hour at 37°C followed by
174 addition in duplicate to a 96 well plate containing 90% confluent Vero E6 monolayer. The
175 plates were incubated for 36 hours at 37°C in a humidified atmosphere with 5% CO₂ (Ref 6).
176 Afterwards, the cells were washed and fixed with 4% paraformaldehyde followed by blocking
177 with 2% BSA for 1 hr at room temperature. Cells were then incubated with SARS-CoV-2 rabbit
178 anti-nucleocapsid (Abgenex, cat. No. 11-2003) antibody for 1-2 hour followed by 3x wash with
179 PBS and 1 hr incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG. After 3x
180 wash, equal volume of 3,3',5,5'-tetramethylbenzidine substrate was added to each well for 15
181 minutes with termination of reaction by addition of 2N H₂SO₄. The plates were read at 450/620
182 nm using a microplate reader. The neutralization percentage was determined by following the
183 formula: each well is 100 – [(X-average of 'no virus' wells)/ (average of 'virus only' wells -
184 average of 'no virus' wells) *100], where X is the read for each well. Non-linear regression

185 curve fit analysis over the dilution curve was performed in the Graphpad Prism 5 software
186 while setting the top and bottom constraints at 100% and 0% ^{15,16}.

187

188 **Viral Genome sequencing and analysis:** For the whole genome sequencing of the isolated
189 viruses, the viral RNA amplicon libraries were prepared using the QIAseq FX DNA Library
190 Kit and the QIAseq SARS-Co V-2 Primer Panel (Qiagen, cat. no. 180475, cat. no. 333896) as
191 instructed by the manufacturer's manual. The library was sequenced using the Illumina
192 platform. The adapter sequence used for each sample was compatible with the Illumina
193 NextSeq 550 instrument with 96-sample configurations (Qiaseq unique dual Y-adapter kit).
194 The average insert length was in the 250–650 bp range. The raw data pre-processing, alignment
195 with viral genome, consensus sequence generation, variant calling and phylogenetic analysis
196 was performed as described by Raghav et al., 2020 ¹⁷.

197

198 **Statistical Analysis:** Statistical analysis was performed using the GraphPad Prism software
199 version 5. Data were presented as mean \pm standard deviation (SD). The Non-Linear fit log
200 (inhibitor) vs. response - Variable slope was used to determine the percentage inhibition of
201 virus infection due to vaccine-induced antibody-mediated neutralization.

202

203 **Results**

204 There is an urgent need to isolate and establish culture of the SARS-CoV-2 circulating viral
205 strains to aide in research and development towards finding efficient therapeutic modalities
206 and vaccine development. Hence attempts were made to isolate SARS-CoV-2 virus from
207 COVID-19 patients oropharyngeal swab samples collected during April-June 2020 at various
208 location in the state of Odisha, India. The viral RNA obtained from the swab samples was
209 subjected to whole genome sequencing to identify the viral strain and emerging mutations.
210 Based on the whole genome sequencing result and cycle threshold values of qRT-PCR swab
211 samples were chosen, which were expected to have high viral load and of respective clades
212 19A, 19B, 20A & 20B for virus isolation and propagation.

213 Virus isolation was carried out using the protocol adapted by Harcourt et al, 2020 with minor
214 modification. Based on previous reports Vero-E6 cells were used for virus propagation ⁹. All
215 the five isolates were passaged for ten times on Vero-E6 cells and the culture supernatants were
216 collected during every passage and a portion of clarified supernatant was used as inoculum for
217 subsequent passage. The 10th passage clarified supernatant was used as viral stock for the whole
218 genome sequencing, virus characterization, and further experiments. During the passages,
219 RNA isolated from the collected supernatants was subjected to qRT-PCR to confirm the
220 presence of SARS-CoV-2. Viral titres in the 10th passage supernatant were determined by
221 standard plaque (**Figure 1A**) and TCID₅₀ assays (**Figure 1B**). The viral titres of the respective
222 isolates ranged from the 10⁶ to 10⁸/mL. Based on the titres obtained, the Vero-E6 cells were
223 infected with 0.1 MOI of all isolates for subsequent experiments. To visualize the cytopathic
224 effect (CPE) bright field images were captured at 48 hr post infection. All the five isolates
225 displayed significant amount of virus induced CPE however ILS03 belonging to 20A clade
226 displayed highest level of CPE among the five and the other four displayed nearly similar levels

227 of CPE (**Figure 1C**). Absolute quantification of viral genome copies in the culture supernatant
228 collected at 48 hr post infection was determined by qRT-PCR using gene specific primers and
229 probes for nucleocapsid and ORF1 (**Figure 1D**). Viral gene expression was also confirmed by
230 Western blot analysis of the cell lysates using antibodies targeting SARS-CoV-2 spike and
231 nucleocapsid (Figure 1E). Cells infected with all 5 isolates showed profound level of viral gene
232 expression as adjudged by Western blot analysis. To determine the level of infectivity or any
233 isolate specific variation in subcellular infection pattern immunofluorescence assay was
234 performed in Vero-E6 cells infected with the respective isolates at 0.1 MOI for 48 hours. No
235 significant variation was observed in the subcellular distribution of the SARS-CoV2
236 nucleocapsid protein and all the isolates displayed reticular cytoplasmic staining across the
237 entire cytoplasm (**Figure 1 F**). Quantification of the percentage of infected cells showed that
238 around 70-90% of cells were infected at 48 hours post infection with the respective isolates
239 using 0.1 MOI (**Figure 1 G**).

240 To access the relative differences in the kinetics of viral gene expression, time kinetics
241 experiment was conducted by infecting Vero-E6 cells at MOI of 0.1 and collecting cells at
242 every 4 hours interval for 24 hrs. Western blot analysis of the cell lysates for spike and
243 nucleocapsid proteins of SARS-CoV-2 showed that in isolates ILS01, ILS02, & ILS03,
244 nucleocapsid expression is noticeable from the 16th hour post infection, whereas in isolates
245 ILS15 and ILS24, it appears from 12th hour onwards (**Figure 2**). Interestingly, spike protein
246 expression was evident only after 16th hours post infection in all five isolates (**Figure 2**). To
247 further access the specific variations between the isolates in virus mediated cytotoxicity and
248 viral replication kinetics, Vero E6 cells were infected at 0.1 MOI with respective isolates and
249 the cell culture supernatants collected every 12 hours upto 60 hours post infection to estimate
250 cytotoxicity and viral release. Based on the LDH levels in the supernatants, it appears that
251 isolates ILS01, ILS02, ILS15 & ILS24 induce around 30% of cytotoxicity with respect to mock
252 at 48 hr post infection, whereas isolate ILS03 induces around 90% of cytotoxicity (**Figure 3A-**
253 **3E**). Quantification of viral genome copies in the culture supernatants suggest a steady increase
254 in the genome copies from 12 to 36 hr post infection indicating that there is an exponential
255 increase in the release of viral particle upto 36 hrs post infection followed by plateau (**Figure**
256 **3F**).

257 Further the susceptibility of various cell lines was assessed towards isolates to decipher isolate
258 specific variations in cell susceptibility. Various cell lines were infected with the respective
259 isolates at 0.1 MOI and culture supernatants were collected at 24 hrs post infection (hpi) to
260 quantify the viral genome copies. The human hepatoma cell line (Huh-7), which is highly
261 susceptible to Dengue, Chikungunya, and Hepatitis C viruses (HCV), was found to be more or
262 less equally susceptible to all the five isolates (**Figure 4A**). Similarly, CaCo2 cell line, which
263 is a human intestinal epithelial cell line that has been shown by various groups to be permissive
264 to SARS-CoV-2 was also found to be susceptible to all the five isolates (**Figure 4B**). However,
265 the isolate ILS01 was found to be less infectious compared to the other isolates. Similarly,
266 HEK-293T cells (a human kidney cell line) was found to be more permissive to isolate ILS01,
267 ILS02, ILS15 & ILS 24 as compared to isolate ILS03 (**Figure 4C**). Immune cells
268 predominantly show selective susceptibility to the viruses. Surprisingly, in our study, we found
269 that the human monocyte cells THP-1 and murine macrophage cell line RAW 264.7 were
270 permissive to all the SARS-CoV-2 isolates (**Figure 4D and 4E**).

271 To decipher any clade specific variations towards neutralization, the neutralization capacity
272 and protection of the vaccine-induced antibodies against the respective isolates was
273 determined. Neutralizing antibody levels predict vaccine efficacy and immune protection. In
274 India, initially only two vaccines, Covaxin and Covishield were given emergency approval and
275 used in Government run COVID-19 vaccination drive. We used vaccinated sera from Covaxin
276 and Covishield vaccinated healthy individuals with no history of SARS-CoV-2 infection. The
277 sera were collected after completion of the 2nd vaccine dose fifteen days post 2nd vaccine dose.
278 Horse sera was used as negative control as it was difficult to obtain age-matched healthy control
279 sera from individuals who had not been vaccinated or exposed to COVID-19. The micro-
280 neutralization assay suggested that both the vaccine was equally effective against all of the
281 isolates. Nearly 100% neutralization was observed at 1:10 dilution, which declined to ~50% at
282 dilutions 1:160 or higher (**Figure 5**).

283 The whole genome sequencing of culture adapted viral isolates and viral genome from patient
284 oropharyngeal swab sample suggested that repetitive passaging of SARS-CoV2 virus in Vero-
285 E6 cells did not lead to emergence of many mutations during the adaptation in cell culture. The
286 number of viral gene mutations found in the source swab samples and isolated viruses in
287 comparison to the Wuhan reference strain is shown in **Table 3**. Comparative analysis of
288 common and unique sequence mutation between the source sample and isolate (**Table 4**) and
289 mutational plot analysis of non-synonymous mutations (**Figure 6**) suggests that during the
290 culture adaptation very minimal changes occurred. ILS01 isolated from source sample of
291 clade19A gained only one mutation (A23014C) in spike gene during cell culture adaptation,
292 while it retained all other ten mutations found in source swab samples. Isolate ILS24 obtained
293 from source samples of clade 19B gained three mutations (C2143T, C10138T, C10702T) in
294 the ORF1ab and one mutation (G28326T) in the N genes during adaptation. It retained 5
295 mutations found in the source swab sample material, and one reversion (G26730T) to Wuhan
296 reference strain in the M gene. ILS03 isolated from swab sample of clade 20A retained 9
297 mutations found in swab sample and gained one mutation each in ORF1ab (G19514T) and S
298 (A24538C) genes during adaptation. Interestingly during isolation and adaptation of ILS15
299 from swab sample of clade 20B, five reversions occurred, which included two (C8917T,
300 G9389A) in ORF1ab and three (G28882A, G28881A, G28883C) in N gene resulting in the
301 reclassification of the cell culture adapted strain ILS15 in clade 20A. To understand the
302 evolution of the virus and trace lineage phylogenetic network analysis was performed using
303 the genome sequence of the four isolates and 33 other largely complete sequences of SARS-
304 CoV-2 genome from different regions of the world. Phylogenetic analysis indicated that the
305 genome sequence of the swab sample and culture adapted viruses remain identical as they
306 cluster close together in the respective clades (**Figure 7**), which was also in agreement with the
307 mutational plot analysis evident by the presence of similar nonsynonymous mutation
308 throughout respective genomes (**Figure 6**). Both the swab sample and adapted virus of isolates
309 ILS01& ILS24 closely clustered together with the Wuhan reference strain as they belong to
310 very early clade 19A & 19B respectively. Swab sample in case of isolate ILS15 cluster together
311 with viral genome from India & Brazil belonging to clade 20B whereas the adapted virus strain
312 cluster together with the genome sequences from Australia and South Korea of clade 20A
313 which may be due the 5 reversions found in the adapted virus. Interestingly, in case of isolate
314 ILS03 both the swab sample and adapted virus strain extended out and clustered separately
315 from the other viral genome used in this analysis.

316

317 **Discussion:**

318 In the prevailing pandemic state, it is important to isolate and characterize the disease-causing
319 pathogen to facilitate development of therapeutic strategies and vaccine candidates. Therefore,
320 in this study we have isolated and characterized five circulating local strains of SARS-CoV-2
321 as limited COVID-19 resources were available in India to aide in research and development.

322 As done by other groups Vero-E6 cells were used for the isolation of SARS-CoV-2 viruses
323 ^{18,19}. We observed a robust virus-induced cytopathic effect from 5th passage onwards similar to
324 previous reports ²⁰. The viral titres were around 1×10^6 TCID₅₀ /ml in final passages for all the
325 isolates (**Figure 1A & 1B**) similar to the titres reported by other groups ^{21,22}. Subsequent
326 infection with the isolated viruses leads to robust infection in Vero-E6 cells, which was evident
327 by exponential increase in virus release from 12-36 hours post infection and detection of
328 infection in 80-100% of Vero-E6 cells, 48 hours post infection. In agreement with studies from
329 other labs the isolates of the current study also showed infectivity in various cell lines ranging
330 from primate to human epithelial & immune cells. The immune cells have been shown to
331 display selective susceptibility to some viruses. For example the THP-1 monocyte cells are not
332 permissive to HCV and Chikungunya viruses ^{23,24}, whereas they permissive to Dengue virus
333 ²⁵. In this study it was found that the viral replication levels of all the isolates were nearly
334 similarly in immune cells in comparison to the cells of epithelial lineage. Although the viral
335 growth kinetics was similar between ILS03 and other isolates, ILS03 displayed 2 fold higher
336 cytopathic effect compared to other isolates suggesting that the high CPE observed with ILS03
337 might be due to unique characteristics of ILS03 and not due to mere high viral load (**Figure 3**).
338 However, further studies are warranted to characterize mechanism specific to ILS03-mediated
339 CPE and decipher isolate-specific variations in host-virus interactions. Our current
340 observations suggest that all the five isolates belonging to the four different clades showed
341 almost similar virus growth characteristics despite the genomic variations between the clades
342 suggesting that the adaptive evolution occurring in the natural host may not be applicable to
343 growth *in-vitro* in cells highly permissive to viral infections.

344 In natural environment SARS-CoV-2 evolves at an estimated nucleotide substitution rate
345 ranging between 10^{-3} and 10^{-4} substitutions per site per year ²⁶ which is a very slow mutational
346 rate. However, the rapid emergence of SARS-CoV2 variants has been speculated to have
347 happened in chronically infected immunosuppressed patients with high levels of viral
348 replication for extended periods under conditions of challenge with treatment modalities like
349 transfusion of convalescent plasma or broadly neutralizing monoclonal ²⁷ driving the selection
350 of variants that evade antibody responses. However, the high prevalence of SARS-CoV2
351 during the past years and the rampant growth in the human host may have also contributed to
352 mutational variability among circulating viruses. In natural host due to higher barrier towards
353 infection, the viruses evolve and variants with higher replicative fitness get selected over time,
354 however in *in vitro* cell cultures using highly permissive cell lines the barrier against viral
355 replication is very low which may not favour rapid evolution of viral variants. In correlation,
356 minimal number of mutations were observed in the adapted viruses as compared to their source
357 swab sample even after 10th passage (**Table 4, Figure 6**) suggesting that *in-vitro* cultured
358 viruses are highly stable.

359 The five isolates used in this study belong to the four clades (19A, 19B, 20A, & 20B) with the
360 clades 20A & B harbouring the D614G mutation in spike protein which has been suggested to
361 promote higher infectivity and transmission¹⁷. The observations of the current study suggest
362 that the two vaccines, Covaxin and Covishield are equally effective and offer protection against
363 these viral isolates from samples collected during the 1st wave of COVID-19 in Odisha, India.
364 The Covaxin is a whole inactivated virus (strain NIV 2020-770) and Covishield (Chimpanzee
365 Adenovirus encoding the SARS-CoV-2 spike glycoprotein (ChAdOx1-S) based on the early
366 viral isolates closer to the Wuhan strain. However, during the 2nd wave many new variants were
367 emerged across the world and they escaped neutralization by antibodies induced by vaccines
368 based on early isolates. Majority of the neutralizing antibodies found in convalescent sera target
369 the spike and RBD domain of spike^{28,29}, therefore many organizations have adapted the
370 strategy of developing vaccine candidates based on Spike protein. However, further studies are
371 warranted to evaluate the efficacy of vaccines based on whole inactivated viruses and other the
372 antigenic motifs other than spike as they can induce a broad antibody response that may be
373 effective against the spike variants. Use of vaccine cocktails may also be an effective strategy
374 to overcome the burden of vaccine escaping viral variants. In agreement, recent evidence
375 suggests that heterologous prime-boost vaccination strategy is more effective alternative than
376 homologous prime-boost vaccination strategy against the emerging variants³⁰.

377 In summary, in the current investigation virus cultures of five SARS-CoV-2 strains belonging
378 to various clades were established from the laboratory-confirmed SARS-CoV-2-infected
379 patients and their growth kinetics and genome sequences were characterized. Further studies
380 are required to clearly elucidate the strain specific variation among the isolates. These isolates
381 will be highly useful resource to facilitate research and development in the field of coronavirus
382 biology and COVID-19.

383

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389

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Figure 1

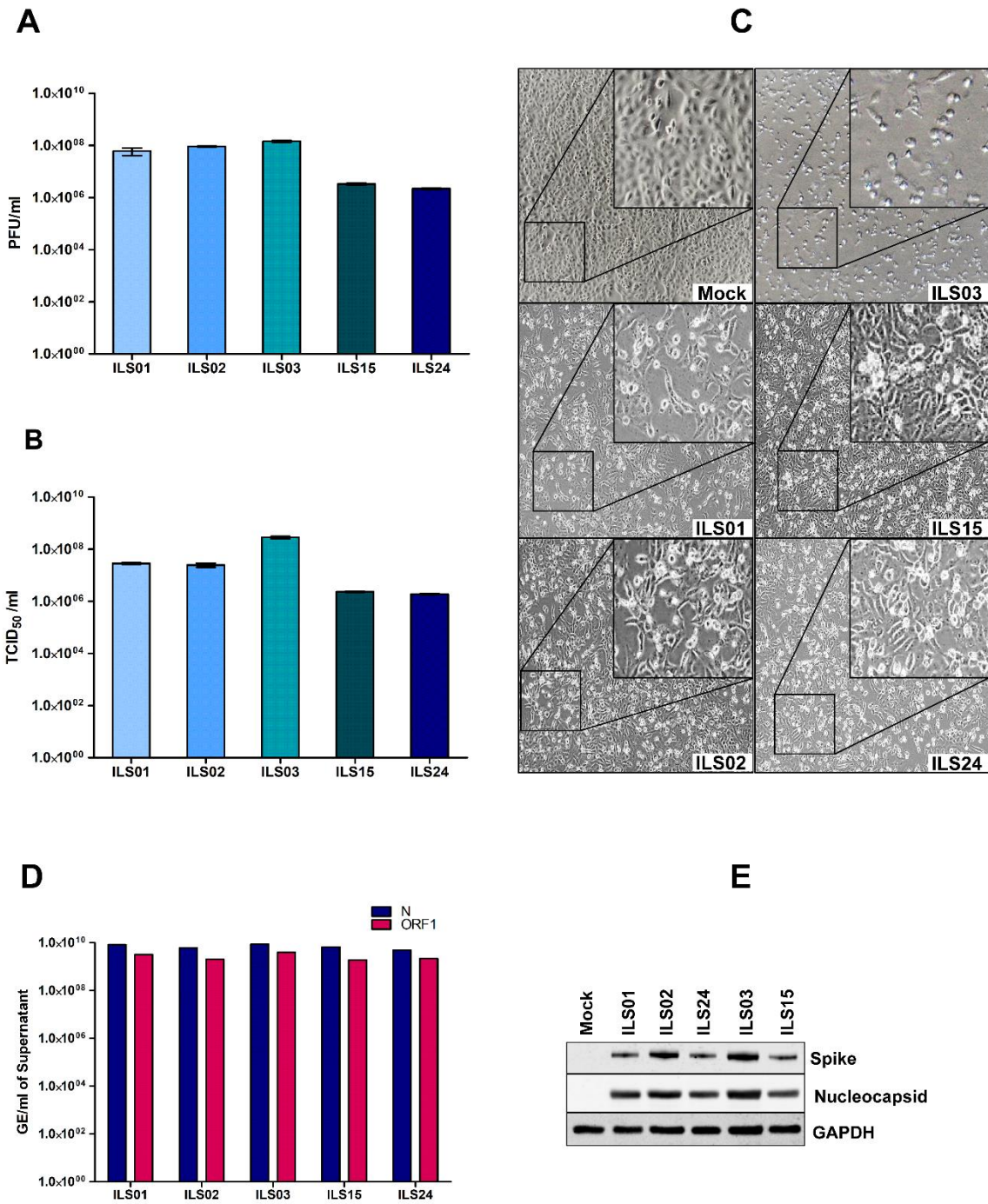
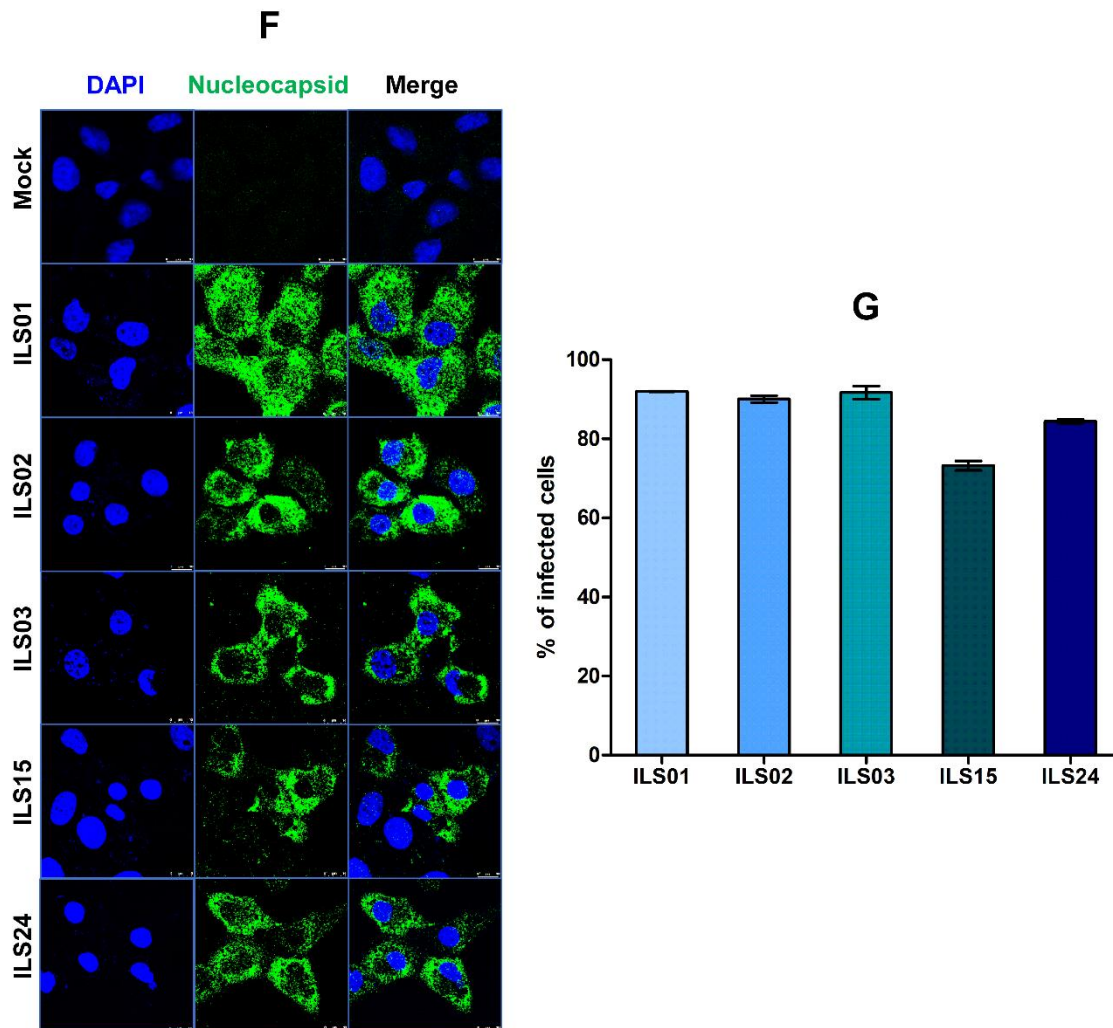


Figure1 cont

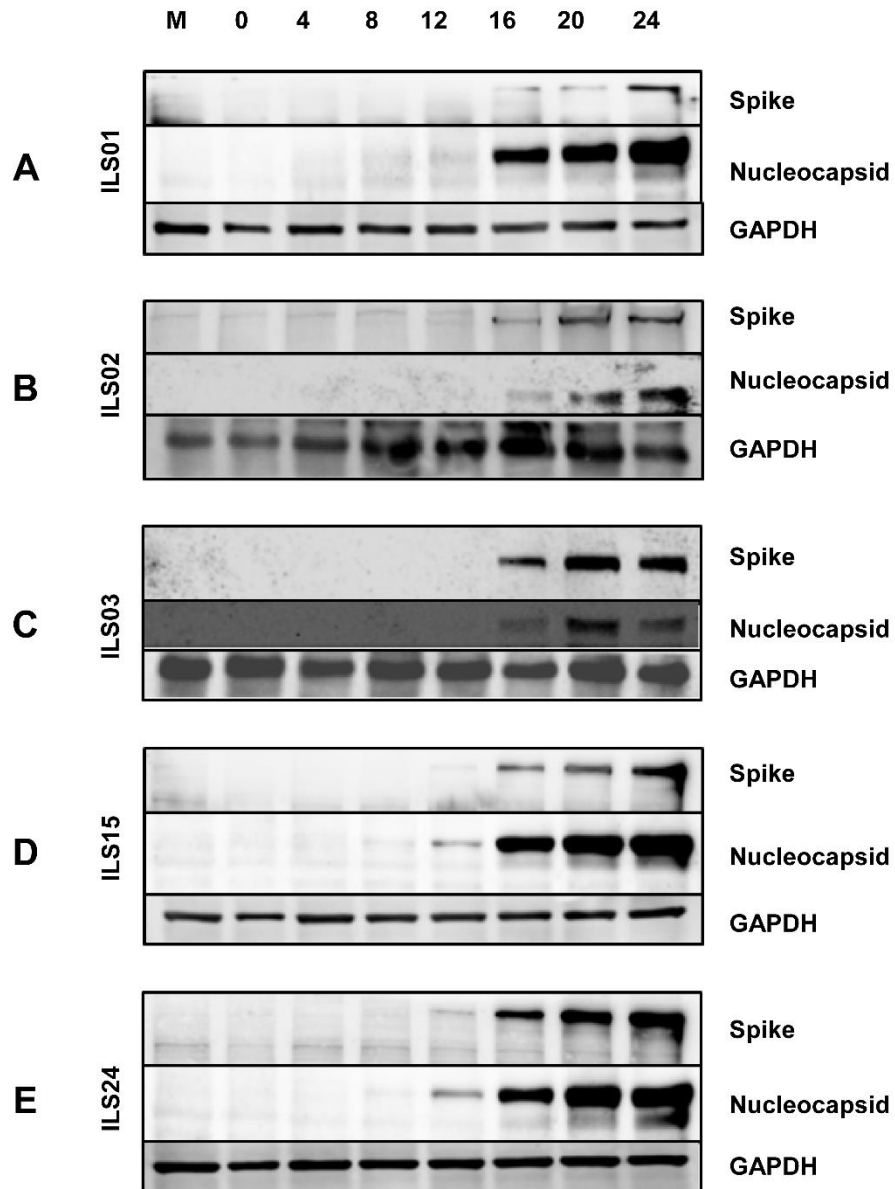


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472 **Figure 1: Characterization of isolated SARS-CoV2 circulating strains.** The SARS-CoV2
473 circulating strains were isolated from the COVID-19 patients swab samples through sequential
474 passage in Vero E6 cells as described in materials & methods. The viral titres, cytopathic effect
475 and gene expression was determined in the 10th passage viral stocks. Quantification of viral
476 titres of the five isolates by plaque forming unit (PFU) assay (A) and TCID₅₀ assay (B). Bright
477 field images depicting cytopathic effect in Vero E6 cells infected respectively with the five
478 isolates (C). Absolute quantification of viral genome copies in all five isolates using gene-
479 specific primer and probes targeting SARS-CoV2 nucleocapsid and ORF-1 gene (D). Western
480 blot analysis of infected Vero E6 cell lysates with antibodies against SARS-CoV2 spike and
481 nucleocapsid (E). GAPDH was used as protein loading control. Immunofluorescence detection

482 of SARS-CoV-2 infected cells using antibody against SARS-CoV2 nucleocapsid in Vero E6
483 cells infected with 0.1 MOI of respective isolates (F) and quantification of the percentage of
484 infection 48 hr post infection (G).

Figure 2

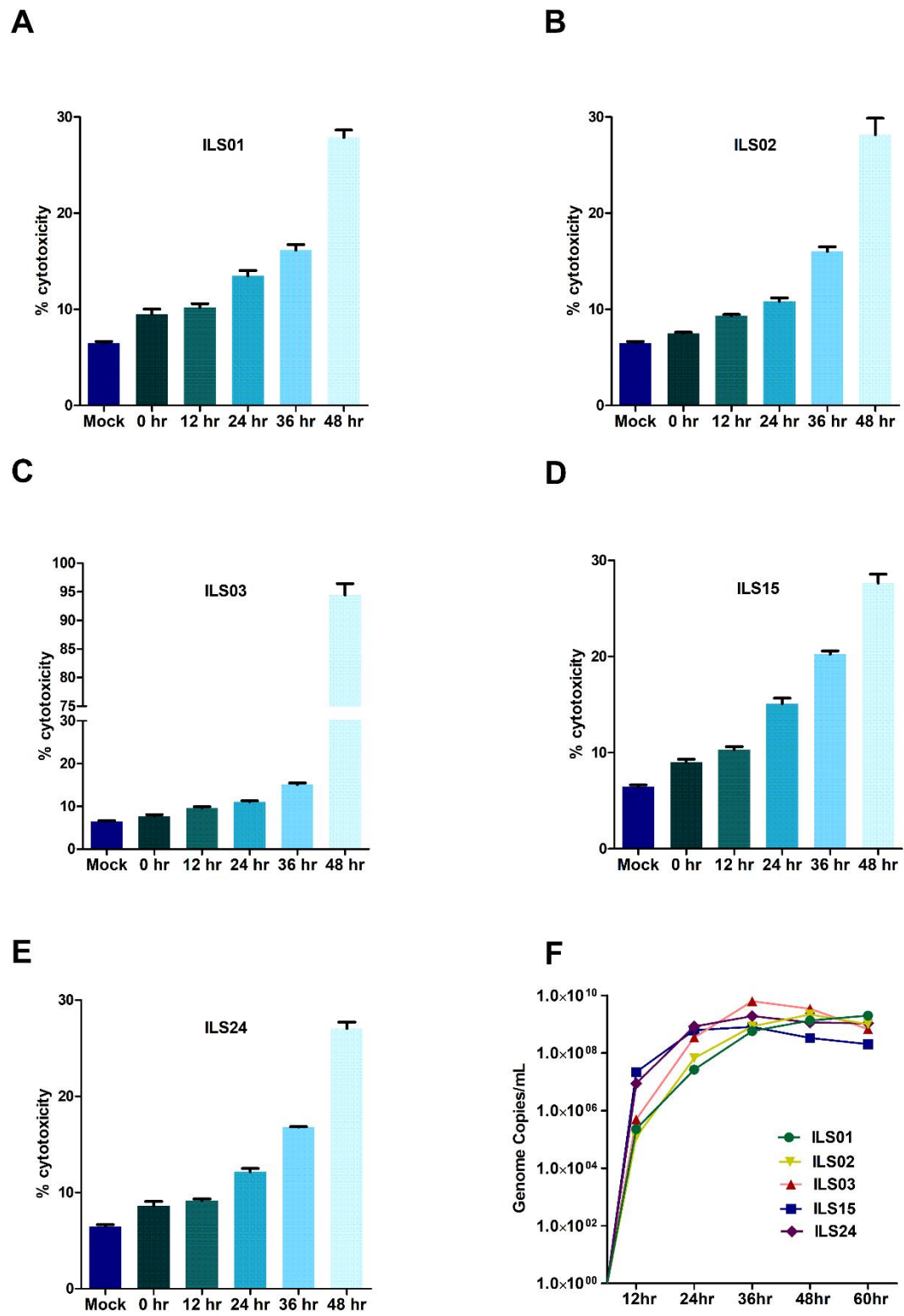


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487 **Figure 2: Time kinetics of viral gene(s) expression.** Vero E6 cells infected with respective
488 isolates of SARS-CoV2 were collected at indicated time points post infection. Cell lysates were
489 subjected to Western blot analysis with antibodies against SARS-CoV2 spike and nucleocapsid
490 proteins. GAPDH was used as an internal loading control.

Figure 3

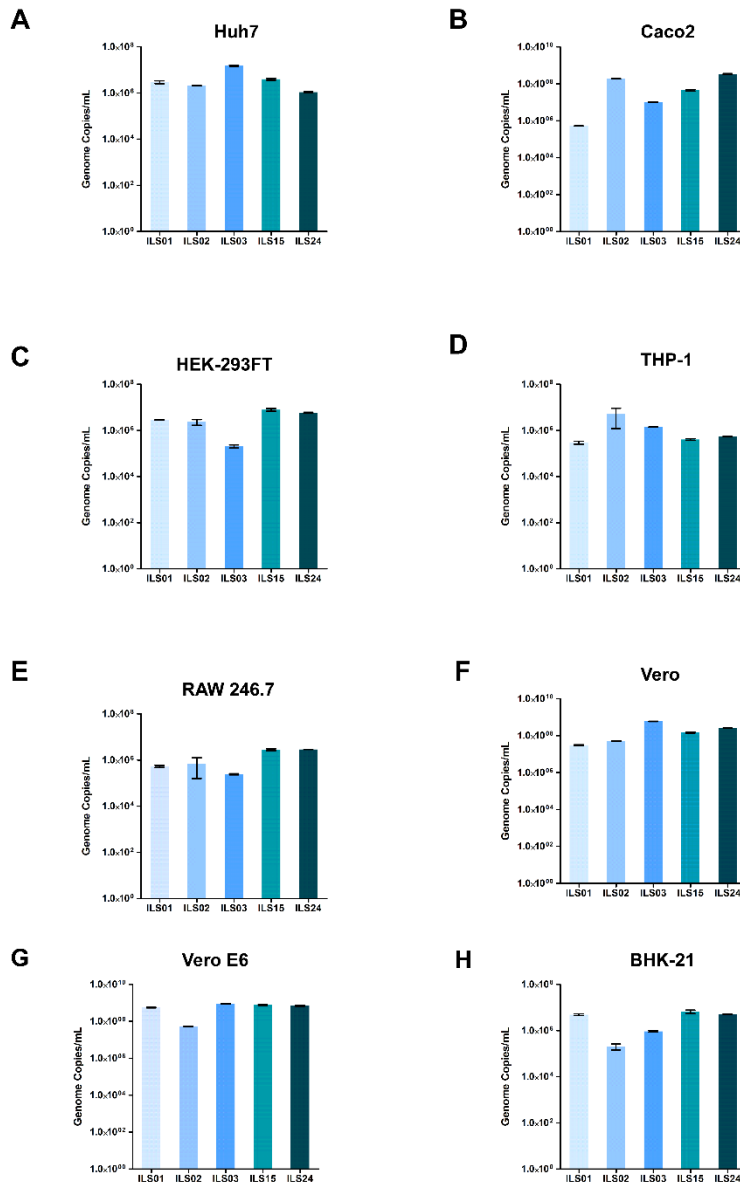


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493 **Figure 3: Viral cytopathy and growth kinetics.** Infection associated cytopathy was
494 determined by calculating LDH release as described in materials & methods. (A-E) Graph
495 depicting percentage of cytotoxicity in the infected Vero E6 cells at respective time points post
496 infection. (F) Line plot showing time-dependent increase in the viral genome copies in culture
497 supernatants determined by absolute quantification of viral genome.

Figure 4



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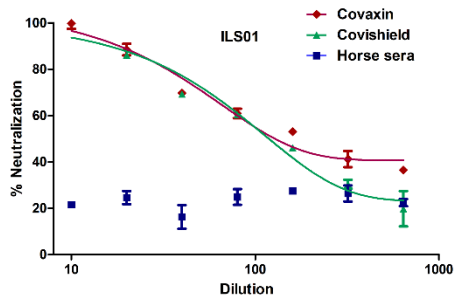
499

500 **Figure 4: Susceptibility of various cell lines to the SARS-CoV2 isolates.** Different cell lines
501 were subjected to infection with 0.1 MOI of respective isolates. 24h post infection the viral
502 load in the culture supernatants was determined by absolute quantification of viral genome
503 copies. Graphs depicting the viral copies per ml supernatant in Huh7 (A), Caco2 (B), HEK
504 293T (C), THP1 (D), RAW 264.7 (E), Vero (F), Vero E6 (G), and BHK-21 (H).

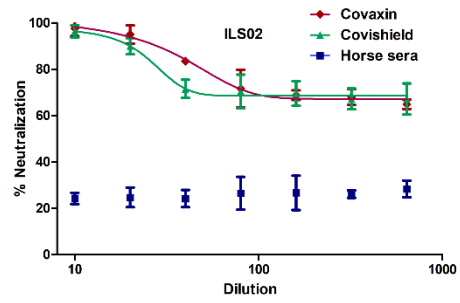
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Figure 5

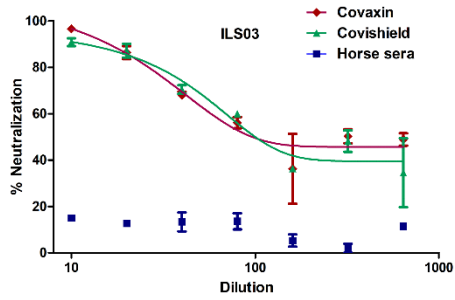
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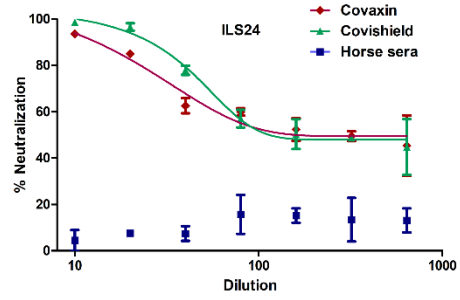
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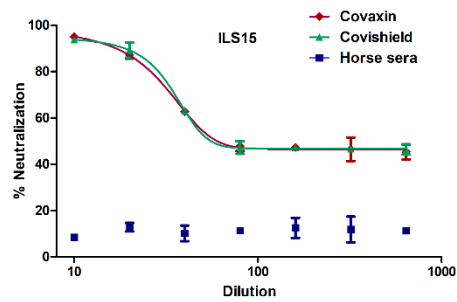
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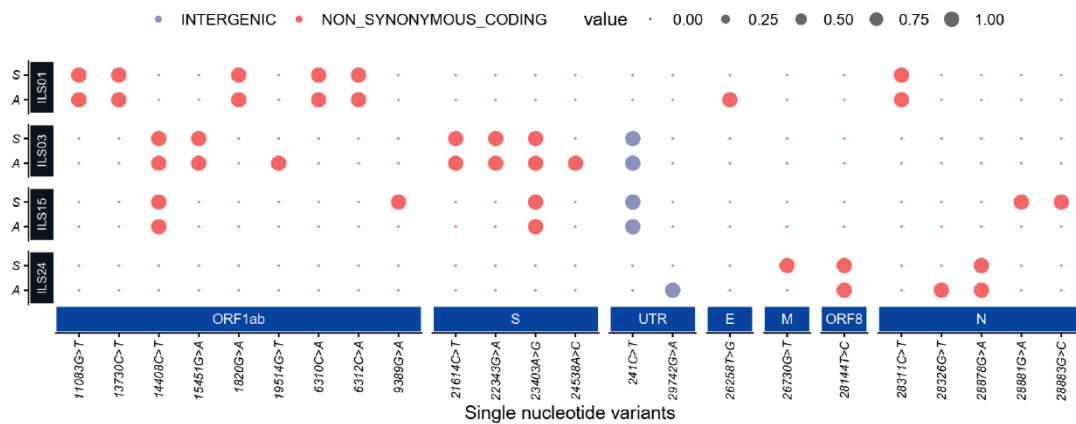


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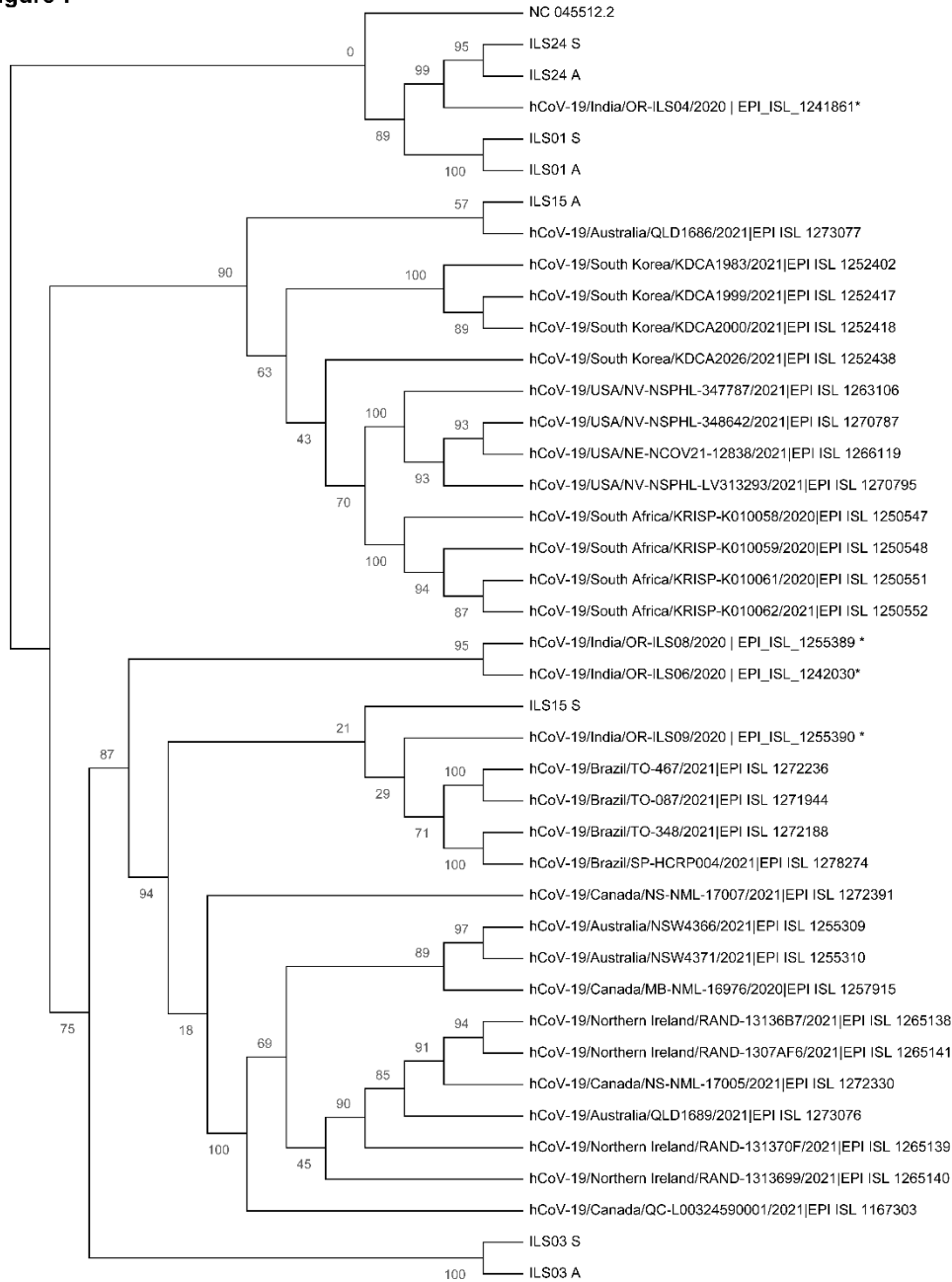
508 **Figure 5: Neutralization potential of sera obtained from vaccinated individuals:** The
 509 respective isolates were subjected to micro-neutralization assay using the sera obtained from
 510 Covaxin and Covishield vaccinated individuals to determine the neutralization potential of the
 511 post vaccination sera against the respective isolates. The dose-response curves were fitted using
 512 a nonlinear regression model using the GraphPad software Prism 5. (A-E) Neutralization
 513 efficiency of the respective vaccinated sera against the 5 isolates. Horse sera was used as
 514 negative control.

Figure 6



515 **Figure 6: Mutation plot of the isolates and source swab samples:** Dot plot representing high
 516 quality single nucleotide nonsynonymous and intergenic variant (SNV) present in the initial
 517 viral RNA isolated from patients swab samples (denoted as S) and viral RNA from culture
 518 adapted isolates (denoted as A). The large dot represents the presence of a SNV in the
 519 represented sample coloured by their functional annotations (grey for intergenic, red for non-
 520 synonymous SNVs).
 521

Figure 7



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524 **Figure 7: Phylogenetic network analysis of the isolated viruses:** Maximum likelihood (ML)

525 tree of studied viral sequences in combination with 33 SARS-CoV2 genome sequences

526 representing from different countries around the globe including four sequences from Odisha

527 India. Bootstrap (n =1000) values are represented as branch labels.

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532 **Table 3:** Tabular representation of genome sequences of all 5 isolates with reference to Wuhan
533 strain (NC_045512).

| Sample | Name | Clade | Total Coverage | Missing region | # of missing bases | # Of nonsynonymous mutation |
|-------------------|-------------|--------------|-----------------------|-----------------------|---------------------------|------------------------------------|
| Swab (S) | ILS01 | 19A | 29665 | 1-30 | 30 | 11 |
| | ILS02 | 20A | 29836 | 1-54,521-530 | 64 | 27 |
| | ILS03 | 20A | 29688 | 1-2 | 2 | 9 |
| | ILS15 | 20B | 29680 | 1-33 | 33 | 9 |
| | ILS24 | 19B | 29805 | 1-30 | 30 | 6 |
| Adapted Virus (A) | ILS01 | 19A | 29836 | 1-32 | 32 | 12 |
| | ILS02 | 19B | 29873 | 1-4 | 4 | 11 |
| | ILS03 | 20A | 29836 | 1-4 | 4 | 11 |
| | ILS15 | 20A | 29836 | 1-29 | 29 | 6 |
| | ILS24 | 19B | 29836 | 1-29 | 29 | 9 |

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549 **Table 4:** Tabular representation of SARS-CoV2 gene-specific non-synonymous mutations in
 550 both the swab samples and cell culture adapted strains.

| Name | # of mutation Swab sample | # of mutation Adapted virus | Common Mutations | Reversion of mutation | Gain of mutation |
|-------|---------------------------|-----------------------------|---|--|---|
| ILS01 | 11 | 12 | ORF1ab; (G11083T, C13730T, C19524T, G1820A, C6310A, C1498T, C6312A, C9451T) Spike; (C23929T), Membrane; (T26861C), Nucleocapsid; (C28311T) | None | Spike; (A23014C) |
| ILS03 | 9 | 11 | 5'-UTR; C241T, ORF1ab; (C3037T, C14408T, T20874A, C21297A), Spike; (C21614T, G22343A, A23403G) | None | ORF1ab; (G19514T), Spike; (A24538C) |
| ILS15 | 9 | 6 | 5'-UTR; C241T, ORF1ab; (C3037T, C14408T), Spike; (A23403G) | ORF1ab; (C8917T, G9389A), Nucleocapsid; (G28881A, G28882A, G28883C) | Spike; (T21703G, C22444T) |
| ILS24 | 6 | 9 | ORF1ab; (C8782T, G22468T), ORF8; (T28144C), Nucleocapsid; (G28878A, G29742A) | Membrane; (G26730T) | ORF1ab; (C2143T, C10138T, C10702T) Nucleocapsid; (G28326T) |

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