

1 **Rhesus macaques can be effectively infected with SARS-CoV-2 via ocular**
2 **conjunctival route**

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19 **Abstract:** The outbreak of Corona Virus Disease 2019 (COVID-19) is highly
20 infectious and transmitted mainly through human-to-human transmission via
21 respiratory droplets and direct or close contact to the patients with SARS-CoV-2. The
22 other potential transmission routes remain to be further researched. In some clinical
23 cases, samples of tears and conjunctival secretions from both SARS-CoV[1] and
24 SARS-CoV-2 patients with conjunctivitis[2] displayed detectable viral RNA. A
25 previous study reported the case of a clinician who was infected with SARS-CoV-2
26 while working with patients under all safeguards except eye protection [2]. By contrast,
27 no SARS-CoV-2 could be detected by RT-PCR in 114 conjunctival swabs samples
28 from patients with COVID-19 pneumonia [4]. Anatomically, the linkage of the ocular
29 with respiratory tissues is primarily by the nasolacrimal system [5]. The potential
30 extra-respiratory transmissible routes of SARS-CoV-2 via ocular remained unclear.
31 Whether ocular conjunctiva is one of the portals that SARS-CoV-2 enters the host
32 needs to be further research by laboratory-confirmation for providing significant data
33 to oversight and prevention in people.

34

35 Three rhesus macaques between the ages of 3 and 5 years were inoculated with 1×10^6
36 50% tissue-culture infectious doses (TCID₅₀) of SARS-CoV-2. Two of them were
37 randomly selected to apply for ocular conjunctival inoculation, the remaining one
38 macaque was inoculated via intratracheal route as a comparison to compare the
39 distribution and pathogenesis of viruses in infected-host via different routes. Based on
40 the clinical symptoms, viral load detection, and serological examination, we tested

41 whether rhesus macaque can be infected with SARS-CoV-2 via ocular conjunctival
42 routes.

43

44 We daily observed the macaques for clinical signs. There was no significant clinical
45 change in the body weight (Fig1A) and the temperature (Fig1B) of the inoculated
46 macaques via both routes. Routine specimens, including nose swabs and throat swabs,
47 were collected on 0, 1, 3, 5, and 7-day post-inoculation (dpi). Additionally,
48 conjunctival swabs and anal swabs were also gathered to explore the potentially
49 excretory routes of SARS-CoV-2 in the host. Remarkably, viral load can be tested in
50 conjunctival swabs on 1 dpi via ocular conjunctival route and then became
51 undetectable implying that the inoculated-SARS-CoV-2 may be transferred from
52 conjunctiva to respiratory tract and other tissues. All three animals were able to detect
53 a continued viral load in their nose swabs and throat swabs from 1 to 7 dpi. For anal
54 swabs, although the viral load was undetectable in the conjunctival
55 inoculated-animals, it can be ongoingly examined in the macaque inoculated via the
56 intratracheal route (Fig1C).

57

58 The intratracheal inoculated-macaque and one of the conjunctival inoculated-animal
59 were euthanized and necropsied on 7 dpi. For conjunctival route, viral load was
60 primarily distributed in the nasolacrimal system and ocular, including the lacrimal
61 gland, optic nerve, and conjunctiva nasal cavity; in the nose, including the nasal

62 mucosa, nasal turbinate, and nostril; in the pharynx including epiglottis, soft palate,
63 and trachea; in the oral cavity including cheek pouch and parotid gland; as well as in
64 other tissues including lower left lobe of the lung, inguinal and para (peri) rectal
65 lymph node, stomach, duodenum, caecum, and ileum (Fig. 1D). By contrast, the
66 distribution of viral might be somewhat different via intratracheal inoculation that
67 viral replication was mainly in the lung, and viral load was also relative high in the
68 nasal septum, tracheas, mandibular lymph node, tonsil, pulmonary lymph node, and
69 some segments of the alimentary tract including caecum, colon, duodenum and
70 jejunum (Fig. 1D). The diverse distributions of viruses by different inoculation routes
71 were consistent with the anatomical structure. Notably, viruses were detectable in
72 different segments of the alimentary canal revealing that the digestive system might
73 be vulnerable and susceptible to SARS-CoV-2. Furthermore, the specific IgG
74 antibody against SARS-CoV-2 was detectable in the conjunctival inoculated-macaque
75 at 14 and 21 dpi proofing that the animal was infected with SARS-CoV-2 (Fig. 1E).

76

77 These data suggested that macaques can be infected with SARS-CoV-2 via the
78 conjunctival route. By comparison, viral load and distribution in the conjunctival
79 infected-macaque represented comparatively high in the nasolacrimal system but
80 relatively mild and local in the lung compared with that in the macaque that
81 inoculated via intratracheal routes. Similarly, both the two routes can cause alimentary
82 canal infection.

83

84 We inoculated rhesus monkeys via a single route via conjunctiva to avoid multiple
85 routes of co-inoculation for confirming the exact pathway of inoculation. These
86 results suggest that conjunctiva is a portal for viral transmission. In our results, viral
87 load can be detectable in several nasolacrimal system associated-tissues , especially in
88 the conjunctiva, lacrimal gland, nasal cavity and throat, which outlined the anatomical
89 bridge between ocular and respiratory tissues. Particularly, the lacrimal duct functions
90 as a conduit to collect and transport tear fluid from the ocular surface to the
91 nasal-inferior meatus, being convenient for the drainage of the virus from ocular to
92 respiratory tract tissues. Actually, the previous report had demonstrated that although
93 virus-containing fluid can be taken up through the conjunctiva, sclera, or cornea, the
94 majority of liquid including tear and secretions is drained into the nasopharyngeal
95 space or swallowed; the lacrimal duct epithelia are also possible to contribute to the
96 absorption of tear fluid. Our results were highly consistent with the anatomical
97 features that viruses enter the host via the conjunctival route. At present, people
98 mainly wear masks to protect against SARS-CoV-2. This method mainly protects the
99 nasal and oral mucosa. Conjunctiva exposed to the environment is easily overlooked.
100 Respiratory viruses can stimulate ocular complications in infected patients, which
101 then leads to respiratory infection [5]. The fact that exposed mucous membranes and
102 unprotected eyes increased the risk of SARS-CoV[1] or SARS-CoV-2[2] transmission
103 suggests that increase the awareness of eye protection is necessary, through regular
104 hand-washing in daily life and wearing protective eyewear in close contact with the

105 patients or crowded places, especially for the clinicians. Only cutting off the
106 transmission of SARS-CoV-2 we can effectively prevent the spread of COVID-19.

107

108 **Materials and methods**

109

110 *Ethics statement*

111 The animal biosafety level 3 (ABSL3) facility in the Institute of Laboratory Animal
112 Science was used to complete all the experiments with rhesus macaques infection
113 doing with HEPA-filtered isolators. The Institutional Animal Care and Use
114 Committee of the Institute of Laboratory Animal Science, Peking Union Medical
115 College, examined and authorized all the programs in this research including animals
116 (BLL20001).

117

118 *Cells and Viruses*

119 The SARS-CoV-2 named SARS-CoV-2/WH-09/human/2020/CHN was isolated by
120 the Institute of Laboratory Animal Science, Peking Union Medical College. Vero
121 cells were applied to the reproduction of SARS-CoV-2 stocks. Dulbecco's modified
122 Eagle's medium (DMEM, Invitrogen, Carlsbad, USA) were applied to incubate this
123 cell line at 37°C, 5% CO₂, complemented with 10% fetal bovine serum (FBS), 100
124 µg/ml streptomycin, and 100 IU/ml penicillin, and maintained. Titers for
125 SARS-CoV-2 were resolved by TCID₅₀ assay.

126

127 ***RNA extraction and RT-PCR***

128 All the collected-organs were applied to extract Total RNA as the description in the
129 previous report. Briefly, the RNeasy Mini Kit from Qiagen, Hilden, Germany and the
130 PrimerScript RT Reagent Kit from TaKaRa, Japan were used following manufacturer
131 instructions. RT-PCR reactions were applied to the PowerUp SYBG Green Master
132 Mix Kit from Applied Biosystems, USA, following cycling protocol: 50°C for 30 min,
133 followed by 40 cycles at 95°C for 15 min, 94°C for 15 s, and 60°C for 45 s. The
134 primer sequences used for RT-PCR were targeted against the envelope (E) gene of
135 SARS-CoV-2. The forward primer is 5'-TCGTTTCGGAAGAGACAGGT-3', the
136 reverse primer is 5'-GCGCAGTAAGGATGGCTAGT-3'.

137

138 ***Animal experiments***

139 Two male rhesus macaques (3–5 years old) were inoculated with 10^6 TCID₅₀/ml
140 SARS-CoV-2 via ocular conjunctival routes and one was inoculated via intratracheal
141 routes in sequence, respectively. On 0, 1, 3, 5, and 7 dpi, the conjunctival, nasal,
142 throat and anal swabs were collected and incubated in 1 ml DMEM with 50 µg/ml
143 streptomycin and 50 U/ml penicillin. The IPTT-300 temperature probes, which were
144 injected interscapular into the macaques before the start of the experiment, were
145 applied to do temperature monitor every day. Tissues were collected as followed,
146 conjunctiva, lacrimal gland, optic nerve, cerebellum, cerebrum, different segments of
147 the spinal cord, nostril, nasal turbinate, nasal mucosa, soft palate, cheek pouch,
148 parotid gland, epiglottis, lingual tonsil, pharyngeal tonsil, different lobes of lung,
149 trachea, different lymph nodes, heart, liver, spleen, pancreas, alimentary canal, kidney,
150 bladder, and brown adipose tissues samples for detecting the viral loads. All sera were

151 collected on 0, 7, 14 and 21 dpi for serologic detection to exam whether there
152 presence the IgG antibodies reactive with SARS-CoV-2 antigens.

153

154 ***Preparation of Homogenate Supernatant***

155 An electric homogenizer was applied to prepare tissues homogenates by 2 min 30s
156 incubated in 1ml of DMEM. The homogenates were centrifuged at 3,000 rpm at 4°C
157 for 10 min. The supernatant was harvested and repositied for viral titer at -80°C.

158

159 ***ELISA antibody assay***

160 Sera of Each animal were collected to detect the SARS-CoV-2 antibody through
161 enzyme-linked immunosorbent assay (ELISA) on 0, 3, 7, 11, and 14 dpi. The 96-well
162 plates coated with 0.1µg Spike protein of SARS-CoV-2 from Sino Biological (Product
163 code: 40591-V08H) at 4°C overnight were blocked by 2% BSA/PBST at room
164 temperature for 1 hour. Sera samples were diluted at 1:100, and then were added to
165 different wells and maintained at 37°C for 30 minutes, followed by the goat
166 anti-mouse antibody labeled with horseradish peroxidase (Beijing ZSGB
167 Biotechnology, ZB-2305) incubated at room temperature for 30 minutes. The OD450
168 value of sera from each of the animals was at least twice more than the negative
169 control was regarded as reacting with SARS-CoV-2 antigen and positive result.

170

171 ***Statistical analysis***

172 Between the two groups, the statistically significant differences were confirmed by
173 unpaired Student's *t*-tests. All data were analyzed with GraphPad Prism 8.0 software.

174

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179

180 **Competing interests**

181 The authors have no competing interests to declare.

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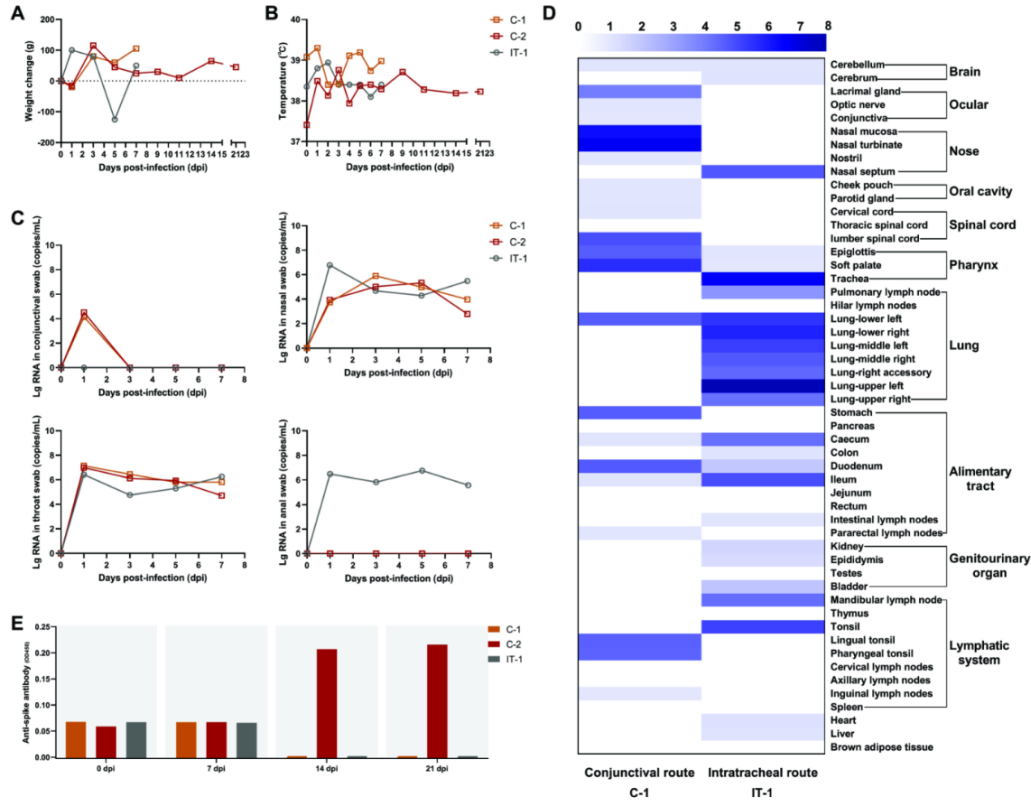
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203 **Figure 1. Clinical features, viral distribution and antibody detection in the sera**

204 **of the SARS-CoV-2-inoculated-rhesus macaques via conjunctival route and**

205 **intratracheal route, respectively.** A and B. Clinical signs including body weight and

206 temperature were observed. C. Viral load of the conjunctival, nasal, throat, and anal

207 swabs specimens from the three inoculated-rhesus macaques 0, 1, 3, 5, and 7 dpi. D.

208 The comparison of viral distributions in the majority organs and tissues from the

209 euthanized and autopsied rhesus macaques inoculated via conjunctival route and
210 intratracheal route on day 7 post-infection. The darker the blue color, the higher the
211 viral load. E. The specific IgG antibody against SARS-CoV-2 in the sera of the
212 inoculated-rhesus macaques was tested on 0, 7, 14, and 21 dpi. All data are presented
213 as mean \pm SEM in triplicate experiments. C-1 and C-2 were the two macaques that
214 inoculated with the conjunctival route, IT-1 was the macaque that inoculated with the
215 intratracheal route.

