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

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

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

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

The intratracheal inoculated-macaque and one of the conjunctival inoculated-animal were euthanized and necropsied on 7 dpi. For conjunctival route, viral load was primarily distributed in the nasolacrimal system and ocular, including the lacrimal gland, optic nerve, and conjunctiva nasal cavity; in the nose, including the nasal
mucosa, nasal turbinate, and nostril; in the pharynx including epiglottis, soft palate, and trachea; in the oral cavity including check pouch and parotid gland; as well as in other tissues including lower left lobe of the lung, inguinal and para (peri) rectal lymph node, stomach, duodenum, caecum, and ileum (Fig. 1D). By contrast, the distribution of viral might be somewhat different via intratracheal inoculation that viral replication was mainly in the lung, and viral load was also relative high in the nasal septum, tracheas, mandibular lymph node, tonsil, pulmonary lymph node, and some segments of the alimentary tract including caecum, colon, duodenum and jejunum (Fig. 1D). The diverse distributions of viruses by different inoculation routes were consistent with the anatomical structure. Notably, viruses were detectable in different segments of the alimentary canal revealing that the digestive system might be vulnerable and susceptible to SARS-CoV-2. Furthermore, the specific IgG antibody against SARS-CoV-2 was detectable in the conjunctival inoculated-macaque at 14 and 21 dpi proofing that the animal was infected with SARS-CoV-2 (Fig. 1E).

These data suggested that macaques can be infected with SARS-CoV-2 via the conjunctival route. By comparison, viral load and distribution in the conjunctival infected-macaque represented comparatively high in the nasolacrimal system but relatively mild and local in the lung compared with that in the macaque that inoculated via intratracheal routes. Similarly, both the two routes can cause alimentary canal infection.
We inoculated rhesus monkeys via a single route via conjunctiva to avoid multiple routes of co-inoculation for confirming the exact pathway of inoculation. These results suggest that conjunctiva is a portal for viral transmission. In our results, viral load can be detectable in several nasolacrimal system associated-tissues, especially in the conjunctiva, lacrimal gland, nasal cavity and throat, which outlined the anatomical bridge between ocular and respiratory tissues. Particularly, the lacrimal duct functions as a conduit to collect and transport tear fluid from the ocular surface to the nasal-inferior meatus, being convenient for the drainage of the virus from ocular to respiratory tract tissues. Actually, the previous report had demonstrated that although virus-containing fluid can be taken up through the conjunctiva, sclera, or cornea, the majority of liquid including tear and secretions is drained into the nasopharyngeal space or swallowed; the lacrimal duct epithelia are also possible to contribute to the absorption of tear fluid. Our results were highly consistent with the anatomical features that viruses enter the host via the conjunctival route. At present, people mainly wear masks to protect against SARS-CoV-2. This method mainly protects the nasal and oral mucosa. Conjunctiva exposed to the environment is easily overlooked. Respiratory viruses can stimulate ocular complications in infected patients, which then leads to respiratory infection [5]. The fact that exposed mucous membranes and unprotected eyes increased the risk of SARS-CoV[1] or SARS-CoV-2[2] transmission suggests that increase the awareness of eye protection is necessary, through regular hand-washing in daily life and wearing protective eyewear in close contact with the
patients or crowded places, especially for the clinicians. Only cutting off the
transmission of SARS-CoV-2 we can effectively prevent the spread of COVID-19.

Materials and methods

Ethics statement

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

Cells and Viruses

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

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

Animal experiments

Two male rhesus macaques (3–5 years old) were inoculated with 10^6 TCID_{50}/ml SARS-CoV-2 via ocular conjunctival routes and one was inoculated via intratracheal routes in sequence, respectively. On 0, 1, 3, 5, and 7 dpi, the conjunctival, nasal, throat and anal swabs were collected and incubated in 1 ml DMEM with 50 µg/ml streptomycin and 50 U/ml penicillin. The IPTT-300 temperature probes, which were injected interscapular into the macaques before the start of the experiment, were applied to do temperature monitor every day. Tissues were collected as followed, conjunctiva, lacrimal gland, optic nerve, cerebellum, cerebrum, different segments of the spinal cord, nostril, nasal turbinate, nasal mucosa, soft palate, cheek pouch, parotid gland, epiglottis, lingual tonsil, pharyngeal tonsil, different lobes of lung, trachea, different lymph nodes, heart, liver, spleen, pancreas, alimentary canal, kidney, bladder, and brown adipose tissues samples for detecting the viral loads. All sera were
collected on 0, 7, 14 and 21 dpi for serologic detection to exam whether there presence the IgG antibodies reactive with SARS-CoV-2 antigens.

**Preparation of Homogenate Supernatant**

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

**ELISA antibody assay**

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

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

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Competing interests

The authors have no competing interests to declare.

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Figure 1. Clinical features, viral distribution and antibody detection in the sera of the SARS-CoV-2-inoculated-rhesus macaques via conjunctival route and intratracheal route, respectively. A and B. Clinical signs including body weight and temperature were observed. C. Viral load of the conjunctival, nasal, throat, and anal swabs specimens from the three inoculated-rhesus macaques 0, 1, 3, 5, and 7 dpi. D. The comparison of viral distributions in the majority organs and tissues from the
euthanized and autopsied rhesus macaques inoculated via conjunctival route and intratracheal route on day 7 post-infection. The darker the blue color, the higher the viral load. The specific IgG antibody against SARS-CoV-2 in the sera of the inoculated-rhesus macaques was tested on 0, 7, 14, and 21 dpi. All data are presented as mean ± SEM in triplicate experiments. C-1 and C-2 were the two macaques that inoculated with the conjunctival route, IT-1 was the macaque that inoculated with the intratracheal route.