Infection of SARS-CoV on juvenile and adult Brandt's vole Microtus brandti

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Abstract We studied the infectious effect of SARS-CoV virus on juvenile and adult Brandt’s Vole (Microtus brandtii) by nasal cavity spraying method (CCID50 is 10^5). SARS virus caused serious deaths in adults. The death adults demonstrated hemorrhage from mouth, nasal cavity and intestine, hemorrhagic interstitial pneumonia and goren in liver, spleen and kidney. The survival adults demonstrated local hemorrhagic spot in lung and emphysema, but the other organs showed no pathological abnormality. SARS virus caused no deaths in juveniles, but locomotion of infected juveniles became slower. In the early stage, there was local pneumonia in lung and SARS viruses were isolated from the pathological tissue. Only one control juvenile lived and the infected juvenile showed local pneumonia in lung. The results demonstrated that SARS-CoV infected Brandt’s vole seriously and adults were more susceptible to SARS-CoV than juveniles. The Brandt’s vole may be a potential animal model for SARS research.

Keywords: SARS-CoV, animal model, pneumonia, Brandt’s vole.

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In the spring of 2003, severe acute respiratory syndrome (SARS) suddenly broke out in many countries of the world, especially China. About 8400 probable cases of SARS were identified worldwide and about 800 patients died. SARS not only caused severe panic all over the world, but also brought huge losses to global economy. SARS is recognized as a new kind of respiratory disease, caused by a novel coronavirus, SARS-CoV. It is characterized by pneumonic infection and pathological change. The typical pathological change of lung based on the corpus check, is diffuse hemorrhage on surface, interstitial pneumonia and thicker pulmonary alveoli[1-3]. The latent period of the disease is 2–7 d, its basic reproductive number is 2–4, and the mortality is about 3%–10%[4–6]. SARS mainly infects adults. But the mortality of old people is high. This disease is still a severe threat to human health because it spreads quickly with a high mortality, its host is still unknown and no effective therapy is available. Researchers in many countries are struggling to develop SARS vaccines or medicines. But the efforts are badly restricted by the lack of an ideal SARS animal for research. At present, Macaca fascicularis[7] and rhesus macaque[8] are popularly used SARS models. Recently in China, Rhesus monkeys have been applied for pre-clinical assessment of inactive SARS vaccine and anti-SARS drugs. To reduce expense and shorten reproduction duration, it is also necessary to target small animal models for SARS studies.

Microtus brandtii belongs to Microtinae of Cricetidae, Rodentia. It is widely distributed in grassland of Inner Mongolia, China and Mongolia. During outbreak years, the Brandt’s voles often cause tremendous damages to grassland, including grassland degeneration and decrease in carrying capacity for livestock[9,10]. In this work, in order to find suitable animal model for SARS research, we studied the infectious effect of SARS virus on Microtus brandtii.

1 Materials and methods

1.1 Animals

Wild Brandt’s voles were from the Institute of Zoology, the Chinese Academy of Sciences and the Chinese Agricultural University. Animals were housed in a temperature-controlled (22±1°C) laboratory with biosafety level III (BSL III); they were given free access to food and water. All animals were acclimated for at least one week prior to use. Normal, healthy male or female (not pregnant) were selected as experimental animals, including 6 adults (weighing 45–60 g) and 27 juveniles (weighing 14.3–19.0 g).

1.2 Virus strain

PUMC01 strain was provided by the Institute of Laboratory Animals Science, the Chinese Academy of Medical Sciences. Titers of virus was 7.08 LogCCID50/mL and stored at –80°C.

1.3 Instrument

Roche real-time PCR lightcycler.

1.4 Virus attack experiment

Juveniles: 27 juveniles were randomly divided into 9 groups (three animals per group). Two animals of each group were treated with nasal spray of (CCID50 is 10^5.1) PUMC01 strain and one control animal without SARS attack was caged together with the two treatment animals. After treatment, animals of group 1 were killed every 2 d and their blood, hearts, spleens, lungs and kidneys were collected for virus isolation and real-time PCR, and also used for pathological examination.
Adults: 7 adults were treated with nasal spray of (CCID_{so} is 10^{3.7}) PUMCO1 strain. One treatment animal died of anesthesia; thus there are only 6 treatment adults. The heart, spleen, lung and kidney of dead animals were collected for pathological detection. All survival animals were killed 14 d after treatment with SARS virus, and their blood, heart, spleen, lung and kidney were collected for pathological examination.

1.5 Virus isolation

0.5 mm² tissue was homogenized in DMEM media using a disposable tissue grinder. The ground tissue supernatant was collected after centrifuging at 4°C 6000 g for 10 min. The confluent monolayers of Vero cells per well were inoculated with 100 μL of the clarified homogenate and 400 μL maintenance medium. After adsorption at 37°C for 1 h, the inoculum was removed and the monolayers were washed three times with PBS. The cell cultures were fed with 2 mL per well DMEM for 3 to 5 d in a humidified CO₂ incubator at 37°C. Vero cells were collected into 10⁶ cell/mL suspension after proliferating three times. Multiple cell smears were prepared on slides and air-dried. After fixing in cold acetone for 10 min, the slides were stained by immunohistochemistry using mouse anti-SARS serum and mouse ABC kits (VECTOR, America).

1.6 Histopathological examination

Each sample was fixed in 10% formalin and was then gradually dehydrated in ethanol and embedded in paraffin. The sections (8–10 μm thick) were stained with hematoxylin and eosin and were observed under a microscope. Lung sections of animals with pathological changes were stained by immunohistochemistry using anti-SARS monoclonal antibody (presented by Starvax, Inc.) and the positive staining cells were observed.

1.7 Real-time PCR

The SARS-CoV RNA was extracted from 200 μL sample by MagNA Pure LC Total Nucleic Acid Isolation Kit (Cat. No. 03604438). PCR master mixture contained the following ingredients: 5 μL RNA, 7.5 μL lightcycler SARS-CoV Reaction Mix, 21.1 μL manganese (OAc), 2 μL lightcycler SARS-CoV Detection Mix, 0.5 μL lightcycler SARS-CoV Internal Control RNA, 3.9 μL H₂O. The samples were run with the following cycle profile: 1 cycle of 61°C 20 min, 95°C 30 s; 45 cycles of 95°C for 5 s, 55°C for 15 s and 72°C for 10 s. Positive control, negative control and five standard samples were set. Correct results of positive and negative control were precondition of the experiment. Copy numbers of samples were gained based on standard samples.

2 Results

2.1 Juveniles

Within the first two days after treatment with virus, locomotion of juveniles with treatment of virus was obviously slower than control ones. No juvenile died of virus treatment. All control animals caged together with treatment animals behaved normally. At day 2, 4 and 8, the lungs of one treatment animal of each group were detected to be positive by real-time PCR (Table 1), and viruses were isolated by immunohistochemistry with supernate of the cell culture (Fig. 1(a)). The other animals as well as the other tissues were detected to be negative (Figs. 2(d)–(f), 3).

The lung tissues of treatment animals showed local pneumonia (Fig. 4(a), (b)), but no positive cells were detected by immunohistochemistry, indicating that infection of SRAS-CoV did not cause significant pathological changes in lungs of juveniles. One control animal (day 8 C#4) caged with treatment animals was found to have local pneumonia in lung, suggesting that the infection might be caused through respiratory tract.

2.2 Adults

Two adults of the six treatment adults died of virus attack within 1 d. Within the first two days, treatment adults showed slower locomotion than control ones. The two dead animals demonstrated hemorrhage from mouth, nasal cavity and intestines, and their lungs showed clear hemorrhagic interstitial pneumonia (Figs. 3(d), 4(e)). There were gores in their livers, spleens, kidneys and pancreases. Positive cells in the location of pathological change were detected by immunohistochemistry (Fig. 1). The lungs of two survival animals suffered from interstitial pneumonia. One of them had a very serious pneumonia in lungs and the other demonstrated local hemorrhagic spot and emphysema. There was no obvious abnormality in other organs of the survived treatment animals except for lungs (Fig. 5(a)–(g)).

3 Discussions

The early pathological changes of SARS patients are observed with serious diffusive and hemorrhagic inflammation in lungs. Local inflammatory reaction includes the accumulation of monocytes, and epithelium desquamation. The main pathological feature of lung lesions showed the thickened alveoli septum with mononuclear-like inflammatory cells infiltration in the late stage of interstitial pneumonia. SARS also caused 3%–10% death to people[1–3]. Adult Brandt's vole died of virus attack within 1 d (mortality rate = 2/6), and its symptom comprises snoot and intestine hemorrhage. All pathological changes including pulmonary inflammation, apparent thicker pulmonary alveoli, interstitial mononuclear inflammatory
### Table 1
Test on presence of virus in organs of voles using Real-Time PCR method

| Days after virus attack | Code of animal | Blood | Heart | Liver | Spleen | Lung (No. copies) | Kidney | Lymph |
|------------------------|----------------|-------|-------|-------|--------|-------------------|--------|-------|
|                        | T#1            | -     | -     | -     | -      | -                 | -      | -     |
| Day 2                  | T#2<sup>ab</sup> | -     | -     | -     | -      | 1.056×10<sup>5</sup> | -      | -     |
|                        | C#1            | -     | -     | -     | -      | -                 | -      | -     |
|                        | T#3            | -     | -     | -     | -      | -                 | -      | -     |
| Day 4                  | T#4            | -     | -     | -     | -      | 4.652×10<sup>5</sup> | -      | -     |
|                        | C#2            | -     | -     | -     | -      | -                 | -      | -     |
|                        | T#5            | -     | -     | -     | -      | -                 | -      | -     |
| Day 6                  | T#6            | -     | -     | -     | -      | -                 | -      | -     |
|                        | C#3            | -     | -     | -     | -      | -                 | -      | -     |
|                        | T#7            | -     | -     | -     | -      | -                 | -      | -     |
| Day 8                  | T#8            | -     | -     | -     | -      | 1.995×10<sup>4</sup> | -      | -     |
|                        | C#4<sup>b</sup> | -     | -     | -     | -      | -                 | -      | -     |
| Day 10, 12, 14, 16, 18 | T#9-T#18      | -     | -     | -     | -      | -                 | -      | -     |
|                        | C#5-C#9        | -     | -     | -     | -      | -                 | -      | -     |

<sup>a</sup>) "C" in code of animal means control juvenile which lived together with treatment ones, and "T" means treatment; "-" negative without presence of virus.
<sup>b</sup>) Positive of virus isolation. <sup>c</sup>) Interstitial pneumonia of control animal.

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Fig. 1. The positive results of Immunohistochemistry of SARS-CoV. (a) Virus isolated from juveniles showing the pathological tissue was positive (10×40); (b) anti-SARS virus dying from adults showing positive (10×10); (c) Anti-SARS virus dying from adults showing positive (10×40).

Infiltration in lung and gore in liver, spleen, kidney and pancreas were observed in adult voles. These symptoms of mortality and serious pathological change are very identical to that in SARS patients.

Real-time PCR showed that SARS virus could stay in lungs of juveniles for 8 d, more than 6 d reported in those of BALB/C mouse. Local pneumonia in lungs of juveniles was observed. No juvenile died of infection by SARS
Fig. 2. The negative results of Immunohistochemistry of SARS-CoV. (a) The negative immunohistochemical stain in the liver of adult animal after SARS virus attack (10x20); (b) the negative immunohistochemical stain in the kidney of adult animal after SARS virus attack (10x20); (c) the negative immunohistochemical stain in the heart of adult animal after SARS virus attack (10x20); (d) the negative immunohistochemical stain in the liver of juvenile animal after SARS virus attack (10x20); (e) the negative immunohistochemical stain in the kidney of juvenile animal after SARS virus attack (10x20); (f) the negative immunohistochemical stain in the heart of juvenile animal after SARS virus attack (10x20).

Fig. 3. No pathological changes in juvenile animals, H.E. (a) The liver of normal juvenile animal (HE 10x20); (b) the kidney of normal juvenile animal (HE 10x20); (c) the heart of normal juvenile animal (HE 10x20); (d) the liver of juvenile animal after SARS virus attack (HE 10x20); (e) the kidney of juvenile animal after SARS virus attack (HE 10x20); (f) the heart of juvenile animal after SARS virus attack (HE 10x20).
Fig. 4. Pathological changes of the lung, H.E. The pathological sections of lung tissue of control and treatment groups of adult or juvenile voles. (a) The local pneumonia after nasal spraying of SARS virus in lungs of juveniles. The arrow shows the common boundary between pneumonia and normal tissue (HE 10×20). (b) The local pneumonia after SARS virus treatment in juvenile voles. The arrow shows mesenchyme and syncretize in which there are tissue cells and lymph cell soakage (HE 10×40). (c) Control juvenile without pathological changes in lung (HE 10×20). (d) There are hemorrhage interstitial pneumonia in lung of adults after attack by SARS virus. The arrow shows that mesenchyme became thicker with hyperaemia, hemorrhage and monocyte soakage (HE 10×20). (e) Hemorrhage interstitial pneumonia in adults after SARS virus attack. The arrow shows that protein exudation in pulmonary alveolar, hyperaemia, hemorrhage and monocyte soakage in mesenchyme (HE 10×40). (f) Control adults without pathological changes in lung (HE 10×20).

Fig. 5. The result of the adult animals’ other organs, H.E. (a) The liver of normal adult animal (HE 10×20); (b) the kidney of normal adult animal (HE 10×20); (c) the heart of normal adult animal (HE 10×20); (d) the liver of adult animal after SARS virus attack (HE 10×20); (e) the liver congestion of dead adult animal after SARS virus attack (HE 10×20); (f) the spleen of adult animal after SARS virus attack (HE 10×20); (g) the kidney of adult animal after SARS virus attack (HE 10×20).
The results indicated juveniles are less susceptible to SARS virus, which is also similar to infection of SARS infection in human that children are more resistant to SARS infection. Robert reported that adult BALB/C mouse was more sensitive to SARS virus than the juvenile mouse, which was consistent with our results\[ll\]. One control (day 8) juvenile living together with the treatment juveniles was found to show local pneumonia in lungs, indicating that SARS virus might be transmitted among voles through air. This needs further investigation.

Previous studies show that SARS virus can infect many animal species including Macaca fascicularis\[7,12 \], rhesus monkey\[8J, Mustela furo and Felis domesticaus\[13 \], laboratory mouse\[14 \] and golden Syrian hamster\[15 \]. Recently WHO and Erasmus Medical Center jointly held a workshop on SARS animal models (http://www.who.int/vaccine_research/diseases/sars/events/2004/02/en/). It was concluded that many animal species such as rodents (laboratory mouse, laboratory rat, guinea pig, hamster), monkeys (Macaca fascicularis, rhesus monkey, African green monkey) cats and pole cat are susceptible to SARS virus; SARS virus can replicate in these animals and induce immunological reaction. Some animals (monkey, pole cat) show some slight clinical symptoms like transitory fever, decompensation and tetter. The pathological changes have been observed in all these animal species. However, there is no case report of death due to infection of SARS virus in these animal models.

SARS outbreak started in the Guangdong Province, southern China in November of 2002 and then spread over the whole country. The Inner Mongolia Autonomous Region was severely attacked by SARS. Since the Brandt’s vole is widely distributed in the grassland of Inner Mongolia, it is possible that virus had been escaped to wild vole populations. The SARS infection in wild populations of the Brandt’s vole is worth investigating.

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