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# Serological survey of SARS-CoV-2 for experimental, domestic, companion and wild animals excludes intermediate hosts of 35 different species of animals

Junhua Deng[1] | Yipeng Jin[2] | Yuxiu Liu[3] | Jie Sun[3] | Liying Hao[3] | Jingjing Bai[3] | Tian Huang[3] | Degui Lin[2] | Yaping Jin[1] | Kegong Tian[3]

[1]College of Veterinary Medicine, Northwest A&F University, Yangling, China

[2]College of Veterinary Medicine, China Agricultural University, Beijing, China

[3]National Research Center for Veterinary Medicine, Luoyang, China

**Correspondence**
Yaping Jin, College of veterinary medicine, Northwest A&F University, Yangling 712100, China.
Email: yapingjin@163.com

Kegong Tian, National Research Center for Veterinary Medicine, Luoyang 471003, China.
Email: tiankg@263.net

## Abstract

The pandemic SARS-CoV-2 has been reported in 123 countries with more than 5,000 patients died from it. However, the original and intermediate hosts of the virus remain unknown. In this study, 1,914 serum samples from 35 animal species were used for detection of SARS-CoV-2-specific antibodies using double-antigen sandwich ELISA after validating its specificity and sensitivity. The results showed that no SARS-CoV-2-specific antibodies were detected in above samples which excluded the possibility of 35 animal species as intermediate host for SARS-CoV-2. More importantly, companion animals including pet dogs (including one dog the SARS-CoV-2 patient kept and two dogs which had close contact with it) and cats, street dogs and cats also showed serological negative to SARS-CoV-2, which relieved the public concerns for the pets as SARS-CoV-2 carriers.

**KEYWORDS**

intermediate hosts, SARS-CoV-2, wild animals

## 1 | INTRODUCTION

SARS-CoV-2, previously was named as COVID-2019 by the WHO, is now pandemic which has been reported 5,077 human death of 136,895 confirmed cases in 123 countries (updated on 14 March 2020 from WHO official website). The viruses have been successfully isolated, but the pathogenesis mechanisms and effective vaccines are undergoing extensively study. SARS-CoV-2 belongs to *Betacoronavirus* genera in the subfamily *Orthocoronavirinae* of family *Coronaviridae*, in which SARS-CoV and MERS-CoV are also in this group. The natural host of highly pathogenic SARS and MERS coronaviruses was confirmed as bats, and bats are also thought to be the natural hosts for SARS-CoV-2 based upon genomic sequence analysis (Wang, Horby, Hayden, & Gao, 2020). Coronaviruses needed intermediate hosts before being able to infect humans. Masked palm civets and dromedary camels were confirmed as intermediate hosts for SARS-CoV and MERS-CoV (Guarner, 2020), but the intermediate hosts remain unknown for SARS-CoV-2 (Ward, Li, & Tian, 2020).

In order to find the intermediate host of SARS-CoV-2, a commercial double-antigen sandwich ELISA, which could be applied for different species of animals, was used to detect SARS-CoV-2-specific antibodies in different species of animals. Before applied to clinical serum samples, the sensitivity and specificity of kit were initially confirmed using SARS-CoV-2-positive and SARS-CoV-2-negative sera from experimental animals including rabbit, mouse, pig and ferret. SARS-CoV-2-negative sera from other species of experimental animals were also used which included chicken, duck, rat, guinea pig, beagle dog and rhesus monkey. After that, the kit was used to detect SARS-CoV-2-specific antibodies in domestic livestock (pig, cow, sheep, horse), poultry (chicken, duck, goose), experimental animals (mice, rat, guinea pig, rabbit and monkey), companion animal (dog and cat) and wild animals (camel, fox, mink, alpaca, ferret, bamboo rat, peacock, eagle, tiger rhinoceros, pangolin, leopard cat, jackal,

  

giant panda, masked civet, porcupine, bear, yellow-throated marten, weasel, red pandas and wild boar). The results showed that no SARS-CoV-2-specific antibodies were detected in above species of animals including pangolin which has been reported as an intermediate host of SARS-CoV-2 (Kangpeng Xiao, 2020). More importantly, we found companion animals including dogs and cats were serologically negative to SARS-CoV-2 including one dog kept by the SARS-CoV-2 patient and two dogs with close contact with it during the quarantine.

## 2 | MATERIALS AND METHODS

The SARS-CoV-2 double-antigen sandwich ELISA was purchased from Luoyang Putai Biotechnology Co., Ltd. The coating was based on S1 protein of SARS-CoV-2. The same antigen was linked to horseradish peroxidase (HRP) to function as conjugate. The serum samples were tested according to the manufacture manual instructions. Briefly, 100 μl serum sample was added into each well of ELISA plate and incubated at 37°C for 30 min. After washing the plate with washing buffer for five times, HRP-labelled antigen was added into the wells at 37°C for 30 min before 100 μl of the substrate solution was added to each well and incubated at 37°C for 10 min to stop the reaction. The optical density (OD) was measured at 450 nm. The final value of $OD_{450}$ of sample = the value of $OD_{450}$ readout of sample - the value of $OD_{450}$ of blank control. The cut-off was set as 0.26 + the mean value of $OD_{450}$ of negative controls.

The serum samples of chicken, duck, mouse, rat and pig were preserved in our laboratory. The ferret SARS-CoV-2-positive and SARS-CoV-2-negative serum samples were provided by Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The ferrets (3 months old) were infected with 1.2 ml SARS-CoV-2 ($1 \times 10^5$ $TCID_{50}$/ml) by intranasal infection in ABSL-3 facility. The infected ferrets were bled at 0, 7, 12, 17 and 22 dpi and euthanized at 22 dpi. The serum samples were collected and inactivated before use. The positive sera for other different coronaviruses were also used in this study. The positive serum samples for porcine epidemic diarrhoea virus (PEDV), porcine transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) were made by immunization of SPF pigs with the corresponding virus, respectively. The positive serum samples for infectious bronchitis virus (IBV) were immunization of SPF chickens with the virus. Positive sera for mouse hepatitis virus (MHV) and rat coronavirus (RCV) were made by infection of SPF mice and rats by MHV and RCV, respectively. The rest of serum samples from different species were collected from November 2019 to March 2020 and kept in our laboratory. All samples were collected in compliance with fundamental ethical principles. The numbers of different animal species used in this study were shown in the brackets below.

## 3 | RESULTS AND DISCUSSION

To test the specificity of ELISA kit, the serum samples of SPF chicken (28), duck (25), mouse (23), rat (20) and pig (20) were applied. The final value of $OD_{450}$ of samples ranged from 0.005 to 0.103 (median 0.007), 0.004 to 0.008 (median 0.006), 0.005 to 0.190 (median 0.007), 0.004 to 0.050 (median 0.007) and 0.005 to 0.134 (median 0.007) for chicken, duck, mouse, rat and pig, respectively. Serum samples from other species of experimental animals including guinea pig (30), rabbits (34), beagle dogs (130) and rhesus monkeys (38) were also tested. There were no SARS-CoV-2 antibodies detected in above animals (data not shown). Next, the potential cross-reaction with other coronavirus including IBV (26), PEDV (24), TGEV (20), PDCoV (20), MHV (20) and RCV (20) were tested with corresponding positive serum samples. The final value of $OD_{450}$ of samples ranged from 0.004 to 0.134 (median 0.007), 0.005 to 0.022 (median 0.007), 0.007 to 0.071 (median 0.018), 0.005 to 0.040 (median 0.010), 0.004 to 0.061 (median 0.006) and 0.005 to 0.064 (median 0.009) for IBV, PEDV, TGEV, PDCoV, MHV and RCV, respectively. The above results showed that the SARS-CoV-2 ELISA has good specificity without cross-reaction with other coronaviruses from different animal species.

We next tested the sensitivity of ELISA kit. The SARS-CoV-2 experimental-infected ferret positive sera were tested. As shown in Table 1, the neutralizing antibody titres of 5 infected ferret (F1-F5) were between 1:128 and 1:256 at 22 days post-infection (dpi). By contrast, the neutralizing antibody titres of 5 placebo ferrets (C1-C5) were all negative at 22 dpi. In the line with the results of neutralizing antibodies, the final $OD_{450}$ of 5 positive sera detected by ELISA was all above 3, which indicated strongly serological positive to SARS-CoV-2. To further test the dynamic changes of ELISA titre of infected ferret, serum samples from one ferret were collected from 0, 7, 12, 17 and 22 dpi, respectively. The positive ELISA results were shown at 7 dpi and lasted until 22 dpi when the ferrets were humanely euthanized (Table 1). The above results showed that the ELISA has good specificity and sensitivity and suitable for different species of animals.

After confirming the specificity, sensitivity and suitability of SARS-CoV-2 ELISA kit for different species of experimental animals, clinical serum samples from domestic livestock (pig, cow, sheep, horse), poultry (chicken, duck, goose), experimental animal (mice, rat and rhesus monkey), companion animal (dog and cat) and wild animals (camel, fox, mink, alpaca, ferret, bamboo rat, peacock, eagle, tiger rhinoceros, pangolin, leopard cat, jackal, giant panda, masked civet, porcupine, bear, yellow-throated marten, weasel, red pandas and wild boar) were used for antibody detection. As shown in Table 2, all serum samples had negative results which exclude the above animal species as intermediate host of SARS-CoV-2. Real-time PCR with specific primers and probe for SARS-CoV-2 recommended by Chinese Center for Disease Control and Prevention was also performed for parts of serum samples including the dog kept by confirmed SARS-CoV-2 patient and two dogs with close contact with it, and the results were negative (data not shown). Of note, no SARS-CoV-2-specific antibodies were detected in all dogs and cats, even for the street dogs and cats in Wuhan City and the pet dog raised by SARS-CoV-2 patient.

So far, seven coronaviruses were confirmed infection of human including SARS-CoV, MERS-CoV, HCoV NL63, HCoV 229E, HCoV

**TABLE 1** ELISA and neutralizing antibody titre results of ferret sera

| Animal species | Serum sample ID | Final $OD_{450}$ | Results | Neutralizing antibody titre* | Results |
|---|---|---|---|---|---|
| Ferret | F1 (0 dpi) | 0.007 | – | <2 | – |
| | F1 (7 dpi) | 0.841 | + | 16 | + |
| | F1 (12 dpi) | 1.301 | + | 32 | + |
| | F1 (17 dpi) | 3.477 | + | 128 | + |
| | F1 (22 dpi) | 3.234 | + | 128 | + |
| | F2 (22 dpi) | 3.023 | + | 256 | + |
| | F3 (22 dpi) | 3.444 | + | 256 | + |
| | F4 (22 dpi) | 3.5 | + | 256 | + |
| | F5 (22 dpi) | 3.332 | + | 256 | + |
| | C1 (0 dpi) | 0.022 | – | <2 | – |
| | C2 (0 dpi) | 0.014 | – | <2 | – |
| | C3 (0 dpi) | 0.009 | – | <2 | – |
| | C4 (0 dpi) | 0.013 | – | <2 | – |
| | C5 (0 dpi) | 0.025 | – | <2 | – |
| | C1 (22 dpi) | 0.021 | – | <2 | – |
| | C2 (22 dpi) | 0.027 | – | <2 | – |
| | C3 (22 dpi) | 0.018 | – | <2 | – |
| | C4 (22 dpi) | 0.005 | – | <2 | – |
| | C5 (22 dpi) | 0.011 | – | <2 | – |

Abbreviation: dpi, days post-infection.

*The neutralizing antibody titre of positive samples was ≥4.

OC43, HKU1 and SARS-CoV-2. Bat was deemed to be the natural host for SARS-CoV, MERS-CoV, HCoV NL63 and HCoV 229E, and rodents for HCoV OC43 and HKU1 (Khan et al., 2020). The intermediate hosts for SARS-CoV, MERS-CoV, HCoV 229E and HCoV OC43 were found to be palm civets, dromedary camels, alpacas and cattle, respectively. However, the natural and intermediate hosts for SARS-CoV-2 remain unknown. Since SARS-CoV-2 is genetically close to SARS-CoV, it has been proposed that bat could be the natural host (Phan, 2020). Snake is also presumed as wildlife animal reservoir for SARS-CoV-2 based on the virus relative synonymous codon usage (RSCU) bias (Ji, Wang, Zhao, Zai, & Li, 2020). However, there is no report of SARS-CoV-2 isolation or molecular and serological confirmation of infection from snake samples. Pangolins recently was suggested to be direct animal source of SARS-CoV-2 for humans since the SARS-CoV-2-related coronaviruses were isolated from Malayan pangolins which shared 97.4% similarity with SARS-CoV-2 in virus receptor-binding domain in S gene (Kangpeng Xiao, 2020). In our study, we did not detect SARS-CoV-2 antibodies in 17 pangolin serum samples. Consistent with our results, Li et al., (2020) reported the coronavirus carried by pangolins did not have the RRAR motif, a unique peptide insertion in the human SARS-CoV-2 virus. The RRAR motif may be involved in the proteolytic cleavage of spike protein and host range and transmissibility which suggests human SARS-CoV-2 virus did not come directly from pangolins (Li et al., 2020). Masked civet and camel were confirmed to be natural hosts for SARS-CoV and MERS-CoV, and no specific SARS-CoV-2 antibodies were detected in 10 masked civets and 31 camels in this study.

The susceptibility of companion animals including cats and dogs to the SARS-CoV-2 has been major concern for the public. One pet dog was reported to be SARS-CoV-2-positive detected by RT-PCR in Hongkong (https://www.news.gov.hk/eng/2020/02/20200228/20200228_093205_796.html). Later, the serological result of the dog showed negative after quarantine of 14 days. In our study, 87 cats including 66 pet cats and 21 street cats showed serological negative to SARS-CoV-2 (Table 2). At the same time, 487 dogs including 90 beagle dogs, 147 pet dogs and 250 street dogs during the outbreak of SARS-CoV-2 were also tested serological negative. Among them, 15 pet dog and 99 street dog sera were collected from Wuhan City. It should be noted that one pet dog from confirmed SARS-CoV-2-infected patient showed serologically negative, and other two dogs which had close contact with this dog also tested to be negative. However, we cannot rule out of susceptibility of cats and dogs to SARS-CoV-2, which need to be tested by experimental infections.

Molecular techniques such as reverse-transcriptase PCR tests and viral genome sequencing are widely used for the confirmation of human infection. These techniques are also used to explore the potential hosts of SARS-CoV-2 (Pfefferle, Reucher, Norz, & Lutgehetmann, 2020). Compared to these molecular methods, serological test such as ELISA has several advantages. First, the host generates SARS-CoV-2-specific antibodies after infection which could last longer than the viraemia. It provides a wider detection window for ELISA than RT-PCR. Second, RNA extraction from susceptive infected samples has to be performed in a BSL-3 laboratory. By

**TABLE 2** Summary of ELISA antibody titre results of 35 animal species

| | Animal species | Number | Minimum of ELISA readout | Maximum of ELISA readout | Median of ELISA readout |
|---|---|---|---|---|---|
| Domestic Animals (4) | Pig[1] | 187 | 0.005 | 0.134 | 0.007 |
| | Cow | 107 | 0.002 | 0.18 | 0.007 |
| | Sheep | 133 | 0.002 | 0.169 | 0.01 |
| | Horse | 18 | 0.002 | 0.189 | 0.011 |
| Poultry (3) | Chicken[2] | 153 | 0.005 | 0.134 | 0.006 |
| | Duck[3] | 153 | 0.004 | 0.189 | 0.007 |
| | Goose | 25 | 0.005 | 0.121 | 0.005 |
| Experimental and companion animals (7) | Mice[4] | 81 | 0.004 | 0.19 | 0.006 |
| | Rat[5] | 67 | 0.004 | 0.095 | 0.008 |
| | Guinea pig[6] | 30 | 0.005 | 0.031 | 0.008 |
| | Rabbit[7] | 34 | 0.005 | 0.029 | 0.006 |
| | Monkey[8] | 39 | 0.001 | 0.141 | 0.011 |
| | Dog[9] | 487 | 0.004 | 0.198 | 0.007 |
| | Cat[10] | 87 | 0.005 | 0.045 | 0.007 |
| Wild animals (21) | Camel | 31 | 0.005 | 0.178 | 0.008 |
| | Fox | 89 | 0.005 | 0.197 | 0.009 |
| | Mink | 91 | 0.001 | 0.195 | 0.008 |
| | Alpaca | 10 | 0.004 | 0.02 | 0.006 |
| | Ferret | 2 | 0.036 | 0.038 | 0.037 |
| | Bamboo rat | 8 | 0.005 | 0.008 | 0.006 |
| | Peacock | 4 | 0.006 | 0.009 | 0.007 |
| | Eagle | 1 | 0.0066 | 0.0066 | 0.0066 |
| | Tiger | 8 | 0.004 | 0.077 | 0.005 |
| | Rhinoceros | 4 | 0.005 | 0.006 | 0.005 |
| | Pangolin | 17 | 0.004 | 0.156 | 0.006 |
| | Leopard cat | 3 | 0.005 | 0.007 | 0.005 |
| | Jackal | 1 | 0.01 | 0.01 | 0.01 |
| | Giant panda | 14 | 0.005 | 0.05 | 0.007 |
| | Masked civet | 10 | 0.004 | 0.014 | 0.006 |
| | Porcupine | 2 | 0.007 | 0.007 | 0.007 |
| | Bear | 9 | 0.005 | 0.006 | 0.006 |
| | Yellow-throated marten | 4 | 0.005 | 0.095 | 0.008 |
| | Weasel | 1 | 0.006 | 0.006 | 0.006 |
| | Red pandas | 3 | 0.005 | 0.005 | 0.005 |
| | Wild boar | 1 | 0.005 | 0.005 | 0.005 |

*Note:* All above results were negative.

[1]Including 20 SPF pigs.

[2]Including 28 SPF chickens.

[3]Including 25 SPF ducks.

[4]Including 23 SPF mice.

[5]Including 20 SPF rats.

[6]Experimental animals.

[7]Experimental animals.

[8]Including 38 rhesus monkey and one wild Ruffed lemur.

[9]Including 90 beagle dogs, 250 street dogs and 147 pet dogs. Fifteen pet dog and 99 street dog sera were collected from Wuhan City.

[10]Including 66 pet cats and 21 street cats.

contrast, ELISA can be performed in a safety level 2 laboratory and does not require high containment facilities after the serum samples were inactivated at 56°C for 30 min. Third, double-antigen sandwich ELISA based on recombinant S1 protein could detect both IgM and IgG antibodies and is not limited to species. To find the host of SARS-CoV-2, the screening of other wild animals using ELISA is undergoing in our laboratory.

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## ETHICAL STATEMENT

We declare that ethical statement is not applicable.

## CONFLICT OF INTEREST

There was no conflict of interest with others.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

*Kegong Tian* https://orcid.org/0000-0001-5362-1415

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