The Journal of Veterinary Medical Science

FULL PAPER

Virology

Evaluation of serological assays available in a biosafety level 2 laboratory and their application for survey of Middle East respiratory syndrome coronavirus among livestock in Ethiopia

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J. Vet. Med. Sci.
81(12): 1887–1891, 2019
doi: 10.1292/jvms.19-0436

Received: 7 August 2019
Accepted: 24 October 2019
Advanced Epub:
5 November 2019

ABSTRACT. A serological survey of Middle East respiratory syndrome coronavirus (MERS-CoV) was conducted among dromedary camels and herbivorous animals sharing the same pasturage in Ethiopia. The pseudotyped vesicular stomatitis virus coated with the spike protein of MERS-CoV was used in virus neutralization (VN) tests performed in a biosafety level (BSL)-2 laboratory. The results were similar to those obtained from the VN test using live MERS-CoV and were more sensitive than the ELISA performed using synthetic MERS S1 fragment as the antigen as well as the competitive ELISA performed using a monoclonal antibody against MERS-CoV. According to the comprehensive results of the four types of serodiagnosis methods, positive antibodies were detected only in dromedary camels and the remaining herbivorous animals were not infected with the virus. Moreover, using the present procedure, serological tests for MERS-CoV can be conducted even in BSL 2 laboratory.

KEY WORDS: camel, coronavirus, Ethiopia, Middle East respiratory syndrome, serological survey

Middle East respiratory syndrome (MERS), first confirmed in Saudi Arabia in 2012, is an emerging viral infection that causes severe respiratory symptoms in humans [1, 5, 19]. The pathogen, MERS-coronavirus (MERS-CoV), is identified as a novel coronavirus species [5], and the dromedary camel is considered a natural host [10, 11, 14, 15, 18]. Patients with MERS have mainly been identified in and near the Arabian Peninsula [2]. However, several cases have been detected in other countries among the travelers who have visited the Arabian Peninsula, and MERS-CoV has been reported to spread from patients to others via close contact [3, 4, 7, 12]. Therefore, MERS-CoV has been classified as a biosafety level (BSL)-3 pathogen and its handling requires a high-containment laboratory that ensures biosecurity. Therefore, developing a serological diagnostic method that can be used even in a BSL-2 laboratory and exhibits the same specificity and sensitivity as the conventional serological test is crucial. A neutralization test using a pseudotyped virus integrated with green fluorescent protein (GFP) gene and coated with the spike protein of MERS-CoV (VSV-MERS/GFP) has recently been developed [8]. In the present study, the specificity and sensitivity of the neutralization test using VSV-MERS/GFP were confirmed using serum obtained from livestock that share a grazing area with the dromedary camel in Ethiopia. The results were compared with three other antibody tests—neutralization test using live MERS-CoV [16], S1-ELISA [17], and competitive ELISA using a monoclonal antibody against MERS-CoV (cELISA) [9]. Based on these serological tests, the MERS-CoV susceptibility of other herbivores sharing pasture lands with the dromedary camel to infection and to become a source of infection to humans was examined.

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MATERIALS AND METHODS

Pseudovirus

VSV-MERS/GFP, a recombinant vesicular stomatitis virus pseudotype, integrated with the GFP gene and coated with the spike protein of MERS-CoV was used in the neutralization test [8]. The infectivity of VSV-MERS/GFP was detected based on GFP expression observed using fluorescence microscopy. GFP-expressing cells in photographic images were counted using VH analyzer. The VSV-MERS/GFP titers were then expressed as focus forming units (FFU) per m*l*.

Cell culture

293T and Vero cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), 100 U/m*l* of penicillin, and 100 μg/m*l* of streptomycin at 37°C [8]. 293T cells were used to prepare VSV-MERS/GFP and MERS-CoV recombinant receptor binding domain (RBD) for the cELISA antigen. Vero cells were used for neutralization tests by live MERS-CoV and VSV-MERS/GFP, respectively.

Serum samples

Serum samples were collected from dromedary camels (n=38; mean age, 4.3 years; range, 1–13 years), goats (n=25; mean age, 3.9 years; range, 1–8 years), sheep (n=25; mean age, 2.7 years; range, 1–4 years), and cattle (n=15; mean age, 6.7 years; range, 1–11 years) from two herds in Bati district, Amhara region, and one herd in Fafen district, Somali region, Ethiopia. All animals appeared healthy, shared the same pasturage during the day, and stayed in barns or small grounds specific for each animal species surrounded by fences at night.

Transportation of the serum samples to Japan was conducted with the permission of the Japanese government (Animal quarantine inspection number NFIB070602-011) and followed the rules and regulations of the OIE/FAO for biological sample transportation.

Serum from a rabbit immunized with recombinant MERS-CoV S protein was used as a positive control for neutralization [8]. Sera from animals (5 cattle, 5 sheep and 5 goats) reared on the attached farm of Nihon University were used as negative controls.

Neutralization test

The neutralization test using VSV-MERS/GFP was performed as previously described [8]. A medium composed of Eagle's MEM supplemented with 5% FBS was used for virus and serum dilution. Serially diluted 0.05 m*l* of test sera were mixed with equal volumes of 3,000 FFU of VSV-MERS/GFP and incubated at 37°C for 1 hr. The mixture was inoculated to Vero cells seeded on a 96-well culture plate and incubated at 37°C for 24 hr in a CO_2 incubator. GFP-positive cells were then detected using a fluorescence microscope. The number of positive GFP cells was counted as described above. The neutralization titer was determined as the highest serum dilution showing ≤50% of the number of GFP-positive cells compared with the no serum control.

Neutralization test using live MERS-CoV was performed as previously described except using Vero cells instead of Vero-TMPRSS2 cells [16]. Briefly, 0.05 m*l* of serially diluted test sera was mixed with an equal volume of 100 $TCID_{50}$ of MERS-CoV (EMC isolate) in a 96-well culture plate and incubated at 37°C for 1 hr; thereafter,Vero cells were added in each well and cultivated at 37°C. Cytopathic effects (CPE) on the Vero cells were observed at 3 days after infection. The neutralization titer was determined as the highest serum dilution showing at least 50% CPE on the inoculated cells.

S1-ELISA

Synthetic S1 fragment of MERS-CoV was obtained from Sino Biolobical Inc. (Beijing, China) and used as the antigen [17]. ELISA microplates were coated with 50 μl of 50 *n*g/m*l* antigen per well at 4°C overnight, following which the wells were incubated with PBS containing 2% Block Ace and 0.05% Tween 20 for 2 hr at 37°C. Following the removal of blocking reagent, diluted serum samples were added and incubated for 1 hr at 37°C. After washing the wells thrice with 0.05% Tween 20 in PBS (PBS-T), a peroxidase-labeled protein AG (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was added and incubated for 1 hr at 37°C. Following further washing thrice with PBS-T, 100 μl of 2,2′-azinobis-3-ethylbenzthiazolinesulfonic acid (ABTS) substrate solution (Roche Applied Science, Penzberg, Germany) was added and incubated for 20 min at room temperature. The optical density (O.D.) of each well was measured at 450 nm using a microplate reader, and mean absorbance was determined for each serum sample. One of camel serum that showed a high antibody titer in the neutralization test by live MERS-CoV was treated as a positive control.

Competitive ELISA

The MERS-CoV RBD was used as the antigen of the cELISA. For the preparation of recombinant RBD, the mammalian expression plasmid pCAGGS-RBD, which encodes histidine-tagged MERS-CoV RBD (amino acid 358–588), was transfected to 293T cells. At 2 days after transfection, the recombinant RBD was purified from the supernatant of transfected cells using His-Bind Purification Kit (Merck, Damastadt, Germany). The cELISA was performed as described by Fukushi *et al*. [9]. Briefly, MERS-CoV recombinant RBD with pre-determined optimal quantity was coated on a 96-well ELISA plate at 4°C for overnight, following which the wells were incubated with PBS containing 2% bovine serum albumin and 0.05% tween 20 (blocking reagent) for 2 hr at 37°C. Following the removal of the blocking reagent, 100 μl of a biotin-labeled monoclonal antibody mixed with serially diluted serum samples was added and incubated for 1 hr at 37°C. One of camel serum that showed a high antibody titer in

Table 1. Distribution of antibody titers in the neutralization test using VSV-MERS/GFP [a] among animals sharing grazing area with camels

Antibody titer	Animals (test sample numbers)			
	Camel (38)	Cattle (15)	Goat (25)	Sheep (25)
<16	7	14	20	20
16	15	0	0	0
64	7	1	0	0
≥256	9	0	0	0

a) Pseudotyped vesicular stomatitis virus coated with the spike protein of Middle East respiratory syndrome coronavirus.

Table 2. Comparison of other serological tests with the neutralization test using VSV-MERS/GFP[a]

Animals (test sample numbers)	NT[b] (VSV-MERS/GFP)	NT (MERS-CoV)	S1-ELISA	cELISA[c]
Camel (38)	31[d]	31	16	26
Cattle (15)	1	0	0	1
Goat (25)	0	0	0	ND[e]
Sheep (25)	0	0	0	ND

a) Pseudotyped vesicular stomatitis virus coated with the spike protein of Middle East respiratory syndrome coronavirus. b) Neutralization test. c) Competitive ELISA. d) Positive sample numbers. e) Not done.

the neutralization test by live MERS-CoV was treated as a positive control. After washing the wells, a streptavidin-HRP (Thermo Fisher Scientific) was added and incubated for 1 hr at 37°C. Following further washing, 100 μl of ABTS substrate solution was added and incubated for 20 min at room temperature. The O.D. at 405 nm was measured against a reference of 490 nm using a microplate reader (Model 680 Microplate Reader; Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.).

Percent inhibition at each serum dilution was calculated as follows:

Percent inhibition=100−[O.D. (405–490 nm) of test sample]/[O.D. (405–490 nm) of no serum control] × 100

RESULTS

Neutralization test using VSV-MERS/GFP

According to the results of the previous study, antibody titers of ≥16 are treated as positive in neutralization test using VSV-MERS/GFP. In dromedary camels and cattle, 31 out of 38 and 1 out of 15, respectively, were MERS antibody positive. Goats and sheep were all MERS antibody negative (Table l). Moreover, in 15 camels, the antibody titer was 16 or 32; in 7, it was 64 or 128; and in 9 and control positive rabbit serum, it was ≥256. The antibody titer and the positive rate of antibody against MERS-CoV increased with the age of the camels. The antibody titer in one cow was 64. All sera collected from animals on the attached farm of Nihon University were negative.

Comparison of other serological tests with the neutralization test using VSV-MERS/GFP

Overall, 31 camels and control positive rabbit serum were found to be antibody positive in the neutralization test using MERS-CoV, and 16 camels were positive in S1-ELISA. In the cELISA, 26 camels and 1 cow were positive (Table 2). The sera collected as negative controls from animals on the attached farm of Nihon University were all negative in S1-ELISA and cELISA.

Each camel that was antibody positive in S1-ELISA or in neutralization tests using MERS-CoV was found to be positive in neutralization tests using VSV-MERS/GFP. Cows that were antibody positive in the neutralization test using VSV-MERS/GFP or cELISA were different animals and both were antibody negative in the neutralization test using MERS-CoV.

A comparison of the antibody titers detected in the neutralization tests using VSV-MERS/GFP with those detected using live MERS-CoV showed a high correlation between both antibody titers, with a correlation coefficient of 0.9753.

DISCUSSION

All camel sera that were positive in any one of the tests—the S1-ELISA, cELISA, or neutralization tests using live MERS-CoV—were positive in the neutralization test using VSV-MERS/GFP. One cow serum was positive for cELISA and another cow was positive in the neutralization test using VSV-MERS/GFP, but negative in all other tests. Because the neutralization test using live MERS-CoV is considered the most accurate serological test and because the two positive reactions were observed in only one serological test among the four, these two bovine sera should be considered as nonspecific reactors. The exact reason of nonspecific reaction of these sera remains unclear. The nonspecific reaction may result from differences in immunological conditions of animals or in the degree of hemolysis during the preparation of samples. For example, a nonspecific reaction sometimes appears in

cattle after injection with a certain inactivated vaccine in the ELISA kit for Johne's disease diagnosis in Japan. Some rhabdovirus cross react with VSV [6] and such a virus might be subclinically infected in Ethiopian cattle.

There are reports that MERS-CoV specific antibodies were not detected in sera from cattle, goat and sheep in Jordan and Saudi Arabia, the MERS-CoV prevalence region [11, 13]. However, they were kept indoor or did not share the pastureland. Present results indicate that MERS-CoV infects only dromedary camels and is unlikely to infect other domestic animals even sharing the pastureland. Since the antibody positive rate and antibody titer of MERS increased with age, it is considered that MERS-CoV establishes an infection cycle and inapparently present only among dromedary camels [11]. In present studies, the limited numbers of samples were tested because the import of animal sera from the foot-and-mouth disease endemic region is regulated in Japan. Further studies using additional samples from other countries would be required to clarify the role of other animal on the ecology and epidemiology of MERS-CoV.

A comparison of the two neutralization tests using VSV-MERS/GFP and MERS-CoV showed a high correlation between both antibody titers. The former showed an antibody titer of ≥256, whereas the latter showed an antibody titer of ≥512. S1-ELISA was not sensitive compared to other tests because only 16 serum samples were positive and they required an antibody titer of ≥64 in VSV-MERS/GFP. However, the sensitivity of S1-ELISA would be improved by examining the cut-off value and dilution of the test serum. In cELISA, five serum samples were negative out of the 31 positive samples in the neutralization tests, and an antibody titer of 16 was observed in both neutralization tests. Therefore, neutralization test using VSV-MERS/GFP exhibits sensitivity similar to the neutralization test using MERS-CoV and is more sensitive compared with the S1-ELISA as well as cELISA.

The present study shows that the neutralization test using VSV-MERS/GFP, S1-ELISA, and cELISA are as specific to MERS-CoV infection as the serological tests, although their sensitivities slightly differ. The detection of antibody and epidemiological survey of MERS-CoV could be possible by appropriately selecting these tests according to the desired purpose, without using a BSL-3 laboratory.

ACKNOWLEDGMENT. This work was partly supported by JSPS KAKENHI (grant number 17H04642), Japan Agency for Medical Research and Development (grant number JP18fk0108058) and the Academic Frontier Project for Private Universities (S1491007) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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