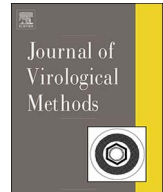




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Research paper

Characterization of novel monoclonal antibodies against the MERS-coronavirus spike protein and their application in species-independent antibody detection by competitive ELISA



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ABSTRACT

Since discovering the Middle East respiratory syndrome coronavirus (MERS-CoV) as a causative agent of severe respiratory illness in the Middle East in 2012, serological testing has been conducted to assess antibody responses in patients and to investigate the zoonotic reservoir of the virus. Although the virus neutralization test is the gold standard assay for MERS diagnosis and for investigating the zoonotic reservoir, it uses live virus and so must be performed in high containment laboratories. Competitive ELISA (cELISA), in which a labeled monoclonal antibody (MAb) competes with test serum antibodies for target epitopes, may be a suitable alternative because it detects antibodies in a species-independent manner. In this study, novel MAbs against the spike protein of MERS-CoV were produced and characterized. One of these MAbs was used to develop a cELISA. The cELISA detected MERS-CoV-specific antibodies in sera from MERS-CoV-infected rats and rabbits immunized with the spike protein of MERS-CoV. The MAb-based cELISA was validated using sera from Ethiopian dromedary camels. Relative to the neutralization test, the cELISA detected MERS-CoV-specific antibodies in 66 Ethiopian dromedary camels with a sensitivity and specificity of 98% and 100%, respectively. The cELISA and neutralization test results correlated well (Pearson's correlation coefficients = 0.71–0.76, depending on the cELISA serum dilution). This cELISA may be useful for MERS epidemiological investigations on MERS-CoV infection.

1. Introduction

Middle East respiratory syndrome (MERS) is a respiratory disease in humans that is caused by a lineage C Betacoronavirus, namely, MERS-coronavirus (MERS-CoV). It was first identified in the Middle East in 2012 (Zaki et al., 2012). Patients with MERS who live outside of the endemic region but have a history of travel to or a temporary residence in the Middle East have been also identified (de Groot et al., 2013). To date, at least 2000 laboratory-confirmed cases of MERS have been reported: the case fatality rate (CFR) is more than 30% (WHO, 2017). While most patients show severe symptoms with a high CFR, some mild or asymptomatic cases are reported during MERS outbreaks, and human-to-human transmission of MERS-CoV is relatively limited when

compared with that during outbreaks of severe acute respiratory syndrome (SARS) in 2003 (Al-Gethamy et al., 2015; Drosten et al., 2014; Memish et al., 2014). These observations and reports are supported by a cross-sectional study in Saudi Arabia which showed that in December 2012–December 2013, 0.15% of the healthy population had anti-MERS-CoV antibodies (Muller et al., 2015).

Several studies show that, in the Middle East, humans are infected with MERS-CoV through direct or indirect contact with dromedary camels, indicating that dromedary camels are implicated as amplifying host of MERS-CoV and a strong potential source of zoonotic infection (Al Hammadi et al., 2015; Who Mers-Cov Research, 2013). Furthermore, MERS-CoV appears to be circulating outside the Middle East since the virus has been detected in dromedary camels in East, West,

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and North Africa (Reusken et al., 2014). Since bat-coronaviruses (BtCoV)-HKU4 and -HKU5, which are detected in *Tylonycteris* and *Pipistrellus* bats, respectively, are closely related to MERS-CoV, bats may be suspected to be a natural host of MERS-CoV (Lu and Liu, 2012). However, it is not clear whether MERS-CoV can be transmitted from bats to camels, or to other animal species.

To identify the zoonotic reservoirs of MERS-CoV and determine how cross-species transmission of MERS-CoV occurs, a serological assay that can detect MERS-CoV antibodies in the sera of various animal species is needed. This assay could also be useful for systematic epidemiological surveillance in Middle Eastern communities and for clarifying whether asymptomatic infection can occur via human-to-human transmission.

The most preferred screening tools for detecting serum antibodies against pathogens are immunofluorescence assays (IFA) and conventional enzyme-linked immunosorbent assays (ELISA). However, the usefulness of these assays in terms of detecting anti-MERS-CoV antibodies is limited by the fact that antibodies against the conserved proteins of coronaviruses are often cross-reactive; as a result, these assays often yield false-positive reactions (Chen et al., 2015; Corman et al., 2012; Meyer et al., 2014). Neutralization assays such as the plaque reduction neutralization test or micro-plate neutralization test are set up using susceptible cell lines and live MERS-CoV. These neutralization assays are considered to be the gold standard for detecting and measuring serum antibody responses to MERS-CoV because they are highly specific and sensitive (Hemida et al., 2014; Perera et al., 2013; Reusken et al., 2013b). However, these assays require high containment laboratories due to the use of highly pathogenic live MERS-CoV. Alternative neutralization assays based on replication-incompetent pseudoparticles, which are generated using vesicular stomatitis virus (VSV) or human immunodeficiency virus type 1 (HIV-1), have been developed as safe and high-throughput neutralization tests (Fukuma et al., 2015; Hemida et al., 2014; Perera et al., 2013). In addition, a cell-free protein microarray that uses the S1 fragment of the MERS-CoV S protein as the antigen has been developed (Reusken et al., 2013a).

A possible type of test that has not yet been reported in the MERS-CoV field is the competitive ELISA (cELISA). cELISAs employ a labeled monoclonal antibody (MAB) that competes with the test antibodies for the target antigen. It has been used widely for serological surveillance in human and veterinary medicine (Blomstrom et al., 2016; Chand et al., 2017; Houlihan et al., 2017; Moreno et al., 2013) and it has a significant advantage over conventional ELISA and IFA, namely, it can detect antibodies that are specific for the target antigen in any animal, including humans.

In the present study, we developed a cELISA for detecting anti-MERS-CoV antibodies. Our main idea is that neutralizing responses in the test serum can be determined by cELISA when a labeled MAB used in cELISA recognizes neutralizing epitopes in the MERS-CoV antigen. Thus, novel MABs with neutralizing activity against MERS-CoV were produced and characterized, and a cELISA using one of these MABs was developed. The cELISA was then validated by using sera taken from dromedary camels, and was also compared to the neutralization test.

2. Materials and methods

2.1. Cells

HeLa 229 and Vero cells obtained from the American Type Culture Collection and Vero cells expressing TMRSS2 and Vero-TMRSS2 (Shirato et al., 2015) were maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% heat-inactivated fetal bovine serum (FBS). The murine myeloma cell line Sp2/O-Ag14 was maintained in RPMI1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS. Hybridoma cells were maintained in HAT medium (Gibco, ThermoFisher Scientific, Rockford, IL).

2.2. Serum samples

All sera were heat inactivated at 56 °C for 30 min. When generating and validating the cELISA, three positive control sera and 42 negative control sera were used. The three positive controls were from two rabbits that had been immunized with recombinant MERS-CoV S and a rat that had been infected with MERS-CoV (Fukuma et al., 2015; Iwata-Yoshikawa et al., 2016). The 42 negative controls were sera from ten mice that had been immunized with recombinant SARS-CoV S protein (Ishii et al., 2009), two rabbits immunized with recombinant MERS-CoV N protein (Fukuma et al., 2015), a rabbit immunized with recombinant SARS-CoV S protein (Fukuma et al., 2015), a rabbit immunized with UV-inactivated SARS-CoV particles (Fukushi et al., 2005), four mock-infected rats, 12 non-immunized mice, and 12 healthy human donors. Since we expected that sera from multiple animal species could be applied to our cELISA, these 42 negative control serum samples were used to determine the cut-off value for the cELISA.

Sera collected from 66 dromedary camels in Awash River basins in Ethiopia in 2013 were used to further validate the cELISA. The transportation 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.

2.3. Preparation of MERS-CoV antigen for the cELISA

MERS-CoV was cultured in BSL-3 laboratory of National Institute of Infectious Diseases as described previously (Iwata-Yoshikawa et al., 2016). Vero cells were inoculated with MERS-CoV at a multiplicity of infection (MOI) of 1.0 per cell. After 26 h, the cells were lysed with PBS containing 1% NP40 to extract the viral antigens from infected cells (Iwata-Yoshikawa et al., 2016). After centrifugation at 8000g for 10 min, the supernatant was collected and used as the MERS-CoV antigen in the cELISA. The antigen was inactivated by UV irradiation (312 nm, 2.5 mW/cm²) on a trans-illuminator for 10 min before use. The viral inactivation was confirmed to be complete before use by inoculation of the antigen to Vero cells followed by cultivation of the cells for at least 3 weeks.

2.4. Preparation of MERS-CoV particles for immunization

To generate hybridomas, BALB/c mice were immunized with MERS-CoV particles that were purified from culture supernatants as follows. Vero cells were inoculated with MERS-CoV at 0.1 MOI. After 2 days, the culture supernatant fraction was collected, mixed with 8% polyethylene glycol and 0.5 M NaCl, and centrifuged at 8000g for 30 min to precipitate the viral particles. The precipitate was dissolved with PBS, applied to a 20%/60% sucrose cushion, and subjected to ultracentrifugation at 30,000 rpm for 2 h using a SW41 rotor. The interphase fraction between the 20%/60% sucrose layers was collected. The ultracentrifugation step was repeated. The purified virus particles in the interphase fraction between the 20%/60% sucrose layers were then collected and used as the antigen for immunization. The MERS-CoV particles were inactivated by UV irradiation and the viral inactivation was confirmed to be complete as described above.

2.5. Production and isotyping of MABs

BALB/c mice were first immunized subcutaneously with 20 µg of the purified MERS-CoV particles emulsified with complete Freund's adjuvant (Sigma-Aldrich). For the second and third immunization, 7 µg of the purified MERS-CoV dissolved with PBS was administered intravenously. Hybridomas were produced by fusing Sp2/O-Ag14 cells with the splenic cells from the mice that were obtained 3 days after the last immunization. The culture supernatants of the hybridoma cells were screened for the presence of antibodies against MERS-CoV antigen

by ELISA as described previously (Iwata-Yoshikawa et al., 2016). To exclude hybridomas that produced antibodies that were cross-reactive with other coronaviruses, the culture supernatants were also subjected to ELISA using antigens prepared from cells infected with human coronavirus 229E, NL63, and OC43 (kindly provided by Dr. Shutoku Matsuyama, NIID). This ELISA was also performed as described previously (Iwata-Yoshikawa et al., 2016). The MAb-producing hybridoma cells that reacted specifically with MERS-CoV were selected and were further cloned by using the limiting dilution method. The isotypes of the MAbs were determined by using a Mouse Monoclonal Antibody Isotyping Kit (AbD Serotec, Kidlington, UK). The MAbs were purified from the culture supernatants by using protein G column chromatography (MabTrap Kit, GE Healthcare UK Ltd, Buckinghamshire, UK) according to the manufacturer's instructions. The concentration of each purified MAb was determined by using the Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific).

2.6. ELISA using the receptor binding domains (RBDs) of MERS-CoV and SARS-CoV

To verify the specificity of MAbs, the RBDs of the MERS-CoV and SARS-CoV S proteins were prepared using a baculovirus expression system, as described previously (Fukuma et al., 2015). Briefly, the nucleotide sequences encoding amino acids 358–588 of the S protein of MERS-CoV and amino acids 318–510 of the S protein of SARS-CoV were tagged at the C-terminus with histidine-tags and cloned into the pAcYM1 transfer vector. Insect Sf9 cells were transfected with mixtures of the transfer vector and BD BaculoGold Linearized Baculovirus DNA (BD Biosciences, San Jose, CA) according to the manufacturer's instructions, thus producing MERS-CoV RBD-expressing baculovirus (AcMERS-RBD-His) and SARS-CoV RBD-expressing baculovirus (AcSARS-RBD-His). Insect Tn5 cells were then infected with AcMERS-RBD-His or AcSARS-RBD-His, thus producing MERS-CoV RBD and SARS-CoV RBD, respectively. Three days post infection, the RBD proteins were purified from the culture supernatants by using by Ni²⁺-nitrilotriacetic acid affinity chromatography (Qiagen, Hilden, Germany) according to the manufacturer's instructions. These RBD proteins were used in conventional ELISAs. Briefly, 96-well ELISA plates (Thermo Fisher Scientific) were coated with 125 ng of the purified RBD proteins and an IgG-ELISA was performed as described previously (Iwata-Yoshikawa et al., 2016).

2.7. IFA using the RBDs of MERS-CoV, Bt-CoV-HKU4, and Bt-CoV-HKU5

To determine whether the MAbs that recognized the RBD of MERS-CoV S protein also recognized the RBDs of other coronaviruses, expression plasmids that encoded the mouse Fc-tagged RBDs of MERS-CoV, Bt-CoV-HKU4, and Bt-CoV-HKU5 (kindly supplied by Prof. George Gao, Institute of Microbiology, Chinese Academy of Sciences, Beijing, PR China) were used. HeLa 229 cells were transfected with each expression plasmid. After 32 h, the cells were fixed with methanol-acetone (1:1). The MAbs were labeled with FITC by using Fluorescein Labeling Kit-NH2 (Dojindo, Kumamoto, Japan) and the fluorescent signals on the fixed cells were observed under a fluorescence microscope. Alexa Fluor 488-labeled anti-mouse IgG antibody (Thermo Fisher Scientific) served as a positive control for detecting mouse Fc-tagged RBD proteins.

2.8. Neutralization assay

A neutralization assay based on live MERS-CoV was performed as described previously (Fukushi et al., 2006; Shirato et al., 2015). Briefly, Vero or Vero-TMPRSS2 cells (Shirato et al., 2015) in a 96-well culture plate were inoculated with MERS-CoV pre-mixed with serially-diluted serum samples. Cytopathic effects (CPE) on Vero or Vero-TMPRSS2 cells were observed after 3 days or 1 day post-infection, respectively.

The neutralization titer was determined as the reciprocal of the highest dilution that showed at least 50% CPE.

For the plaque-reduction assay, approximately 100 plaque-forming unit (pfu) of MERS-CoV was mixed with serially-diluted MAbs and incubated at 37 °C for 1 h. Vero cells in 12-well culture plates were then inoculated with each mixture. After 4 days, the cells were fixed with 10% formalin and stained with crystal violet. Plaques were counted and the reduction rate (%) compared with that induced by the MAb control. An unrelated MAb, MAb 9D3 (Fukuma et al., 2016), was used as a negative control.

2.9. Selection of escape mutants and identification of epitopes

Approximately 1,000 or 10,000 pfu of MERS-CoV was mixed with 10 µg/mL of each MAb. Vero cells grown in 96-well plates were then inoculated with each mixture. Culture supernatants were harvested 5–7 days post infection. Vero cells grown in 6-well plates were then inoculated with the viruses that had escaped MAb neutralization. The escape mutants were purified by using the agarose-overlaying plaque assay. Three to five escape mutant clones were obtained for each MAb. The viral RNAs were extracted from the culture supernatants of Vero cells infected with these escape mutants and the nucleotide sequences of the S gene were determined.

2.10. Competitive ELISA

The cELISA was performed as follows. Whole MERS-CoV antigen was coated on 96-well ELISA plates overnight at 4 °C at an optimal concentration that had been determined beforehand (data not shown). The wells were then washed and incubated with PBS containing 2% bovine serum albumin and 0.05% Tween-20 (blocking reagent) for 2 h at 37 °C. After removing the blocking reagent, the wells were incubated for 1 h at 37 °C with 100 µL biotin-labeled MAb (16 ng/100 µL) mixed with serially diluted serum samples. After washing, the wells were incubated for 1 h at 37 °C with streptavidin-HRP (Thermo Fisher Scientific). After washing again, the wells were incubated for 20 min at room temperature with 100 µL ABTS [2,2-azino-bis(3-ethylbenzthiazolinesulfonic acid)] substrate solution (Roche Applied Science, Penzberg, Germany). The optical density (O.D.) at 405 nm was measured against a reference of 490 nm by using a microplate reader (Model 680 Microplate Reader; Bio-Rad Laboratories Inc., Hercules, CA). Percent inhibition at each serum dilution was calculated as follows:

$$\text{Percent inhibition} = 100 - [\text{O.D.}(405\text{--}490 \text{ nm}) \text{ of test sample}] / [\text{O.D.}(405\text{--}490 \text{ nm}) \text{ of no serum control}] \times 100$$

The cut-off values for each serum dilution were calculated as the mean plus three standard deviations of the percent inhibition at each serum dilution of 42 negative control samples. Thus, the cut-off values at 8-, 16-, 32-, 64-, 128-, 256-, and 512-fold serum dilutions were 23.3%, 17.3%, 16.5%, 15.5%, 14.6%, 13.6%, and 12.7% inhibition, respectively.

2.11. Statistical analyses

Pearson's correlation coefficients were calculated using GraphPad Prism software.

2.12. Ethics statement

All animal studies were performed according to a protocol that was approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases, Japan (no. 112144). The serum samples from the healthy human donors were collected and used in the indicated experiments according to protocols and procedures that had been approved by the research ethics committee of the National

Institute of Infectious Diseases (no. 439).

3. Results

3.1. Generation of MABs

To obtain MABs specific for MERS-CoV, BALB/c mice were immunized with purified UV-inactivated MERS-CoV particles. Hybridomas were generated and cloned. ELISAs were used to determine whether the MABs secreted by the hybridomas recognized whole MERS-CoV antigen and other human coronaviruses, namely, 229E, OC43, and NL63. Of the 27 MABs, seven were identified as MERS-CoV specific (Supplementary Fig. S1). To further limit the possibility of cross-reaction with other coronaviruses, we assessed whether any of the seven MABs specifically recognized MERS-CoV S antigen: this was because the S proteins of coronaviruses are more divergent than the other coronavirus proteins (Meyer et al., 2014). Thus, we performed IFA with MERS-CoV S protein-expressing cells and found that five MABs reacted to the S protein of MERS-CoV but not to its N protein (Supplementary Fig. S2). In addition, to determine whether any of the seven MABs could neutralize MERS-CoV infection, we performed viral neutralization assays using a replication-incompetent VSV that expressed MERS-CoV S protein (Supplementary Fig. S3). Three S protein-specific MABs that were designated 45E11, 45C2, and 43A9 showed neutralizing activity.

3.2. Characterization of the three MABs

The three MABs reacted with the receptor-binding domain (RBD) of the MERS-CoV S protein but not the RBD of S proteins from other

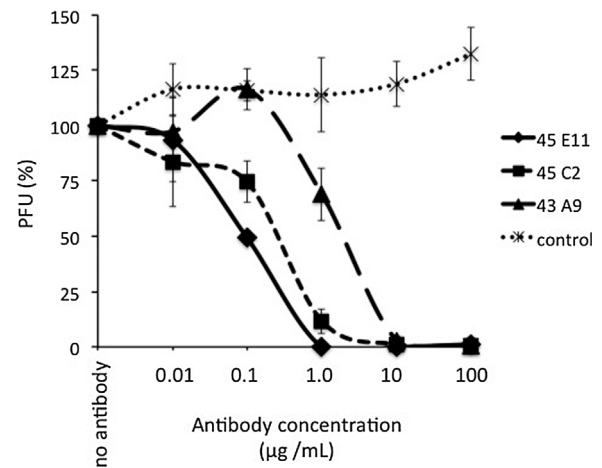
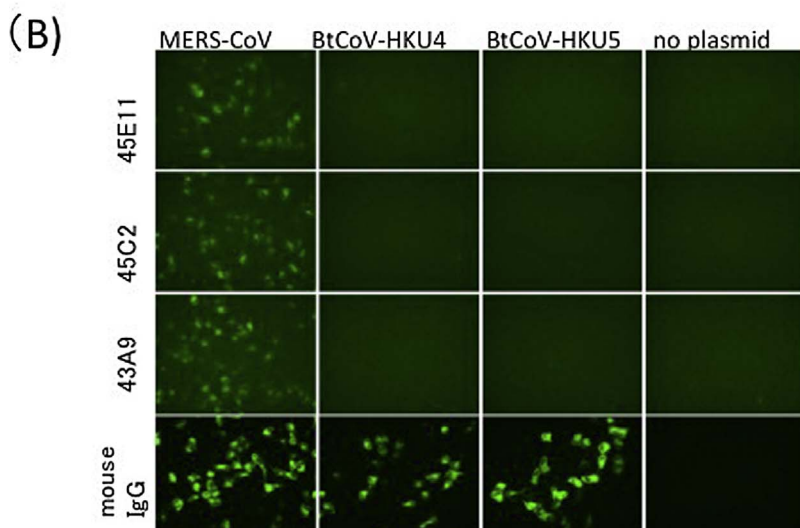
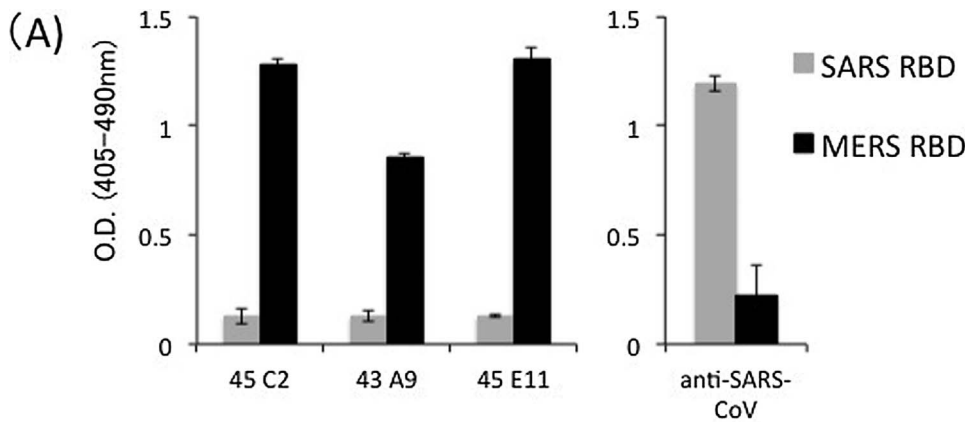


Fig. 2. Neutralizing activity of MABs 45E11, 45C2, and 43A9 against MERS-CoV. Approximately 100 pfu of MERS-CoV was mixed with serially diluted purified MABs. The mixtures were then used to inoculate Vero cells in 12-well culture plates. After 4 days, the cells were fixed with 10% formalin and stained with crystal violet. The viral titers were expressed as% pfu relative to the viral titer in the absence of antibody. An unrelated MAB (MAB 9D3) served as a negative control.

coronaviruses, namely, SARS-CoV (Fig. 1A), Bt-CoV-HKU4, and Bt-CoV-HKU5 (Fig. 1B). The isotype of the 45E11 and 45C2 MABs was IgG1/kappa while the 43A9 isotype was IgG2a/kappa. In the plaque reduction assay, the concentrations of 45E11, 45C2, and 43A9 that inhibited MERS-CoV infection by 50% were 0.1, 0.25, and 1.94 µg/mL, respectively (Fig. 2).

Fig. 1. Monoclonal antibodies recognize the receptor-binding domain (RBD) of MERS-CoV but not the RBDs of SARS-CoV, Bt-CoV-HKU4, or Bt-CoV-HKU5. (A) ELISA: the baculovirus expression system was used to generate the RBDs of MERS-CoV (amino acids 358–588 of the MERS-CoV S protein) and SARS-CoV (amino acids 318–510 of the SARS-CoV S protein). The RBDs served as antigens in an IgG-ELISA. The ability of the supernatants of the indicated hybridoma clones to recognize the RBDs was examined (left histogram). Rabbit anti-SARS-CoV serum was used to confirm the antigenicity of the SARS-CoV RBD (right histogram). The results were expressed as optical density (O.D.) (405–490 nm). (B) Immunofluorescence assay: the indicated monoclonal antibodies were labeled with FITC and incubated with HeLa 229 cells that expressed the Fc-tagged RBDs of MERS-CoV or the bat CoVs Bt-CoV-HKU4 and Bt-CoV-HKU5. The fluorescent signals were detected under a fluorescence microscope. An Alexa Fluor 488-labeled anti-mouse IgG served as a positive control.

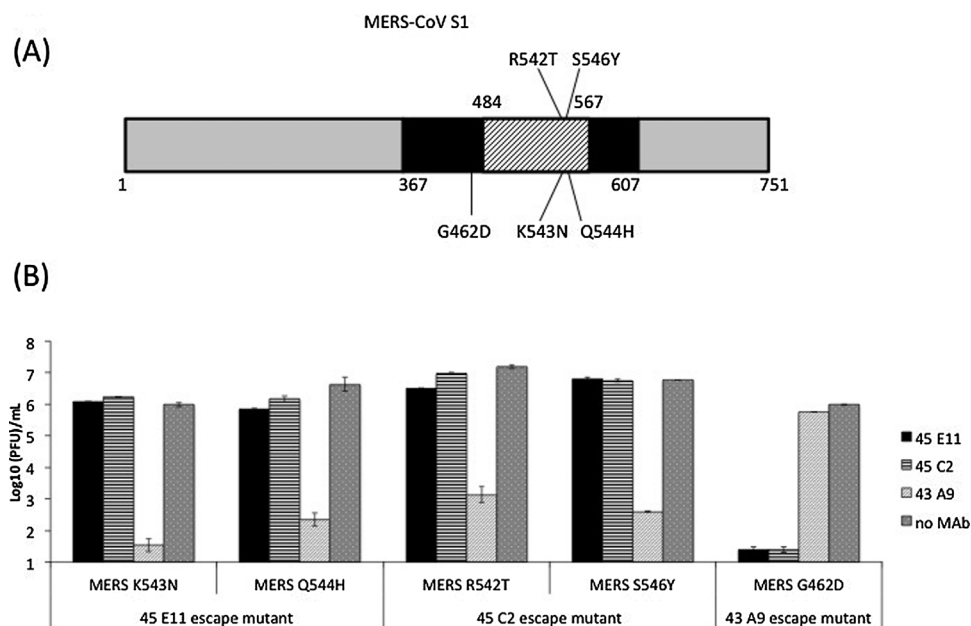


Fig. 3. Characterization of mutant viruses that escaped from neutralization by MABs. (A) Schematic depiction of the MERS-CoV receptor-binding domain (amino acids 367–607, black and hatched sections) and its receptor-binding motif (amino acids 484–567, hatched section). The amino acid substitutions detected in the S protein of the MERS-CoV mutants that escaped the 45E11, 45C2, or 43A9 MABs are indicated. (B) Ability of the escape mutants to grow in the presence of the three MABs, including the MAB from which they escaped originally. The MERS-CoV mutants that escaped 45E11 (MERS-K543N and MERS-Q544H), 45C2 (MERS-R542T and MERS-S546Y), and 43A9 (MERS-G462D) were mixed with 10 µg/mL of each MAB and incubated at 37 °C for 1 h. Vero cells were then inoculated with each mixture. After 2 days, the viral titer was determined by using the plaque forming assay. The viral titers are expressed as pfu/mL.

3.3. Identification of the epitopes of the three MABs

The target putative epitopes of the three MABs were determined by sequencing the S gene of variant viruses that escaped neutralization by each MAB. The critical amino acid residues required for binding MAB 45E11 (K543 and Q544), MAB 45C2 (R542 and S546), and MAB 43A9 (G462) were identified (Fig. 3A). According to the structure of the RBD of the MERS-CoV S protein complexed with the human receptor molecule DPP4 (Wang et al., 2013; Ying et al., 2015), all of these critical residues were located on the DPP4-binding site of the S protein (Fig. 3A). The fact that the putative epitopes of the three MABs were located on the DPP4-binding site of the RBD suggests that these MABs may inhibit the entry of MERS-CoV by blocking the interaction between the MERS-CoV RBD and DPP4. The 45E11 and 45C2 epitopes were likely to overlap with each other because the amino acid substitutions in the escape mutants were very close to each other (Fig. 3A). Indeed, the two MERS-CoV mutants that escaped neutralization by 45E11 (i.e., MERS-CoV-45E11-K543N and MERS-CoV-45E11-Q544H) grew efficiently in the presence of 45C2. Similarly, the two MERS-CoV mutants that escaped neutralization by 45C2 (i.e., MERS-R542T and MERS-S546Y) replicated well in the presence of 45E11. By contrast, the 43A9 MAB strongly neutralized all of these escape mutants. In addition, the 45E11 and 45C2 MABs effectively neutralized the G462D escape mutant of 43A9 (Fig. 3B). Thus, the mutant viruses that escaped from 45E11 also escaped from 45C2, and *vice versa*, which indicates that the epitopes of these two MABs overlap. Sequence analysis of the heavy and light chain genes of 45C2 and 45E11 revealed several amino acid differences in the variable region, including in the complementary determining region (Supplementary Fig. S4).

3.4. Development of the MERS-CoV cELISA

The cELISA was developed using the three MABs established in this study. The ability with which rabbit MERS-CoV S protein-specific antibodies competed with each MAB for MERS-CoV antigen was tested in cELISA. Increasing concentrations of the rabbit serum steadily decreased the binding of each MAB to the antigen (Supplementary Fig. S5A). By contrast, the serum of a rabbit immunized with MERS-CoV N protein only slightly inhibited the binding of each MAB (Supplementary Fig. S5B). Thus, cELISAs using the three MABs could detect anti-MERS-CoV antibodies. The 45C2 MAB was particularly effective in the cELISA as it showed high sensitivity and low background noise (Supplementary

Fig. S5). Consequently, it was selected for further examination to optimize the cELISA.

3.5. Validation of the MERS-CoV cELISA

The 45C2-based cELISA was tested with serum samples from a rat that had been infected with MERS-CoV, two rabbits that were immunized with recombinant MERS-CoV S protein, and rabbits that were immunized with recombinant MERS-CoV N protein, SARS S protein, or UV-inactivated SARS-CoV particles. The serum from the MERS-CoV-infected rat and the rabbit sera specific for recombinant MERS-CoV S protein strongly inhibited the binding of 45C2, whereas the serum samples from rabbits immunized with recombinant MERS-CoV N protein, recombinant SARS-CoV S protein, or UV-inactivated SARS-CoV particles had no effect. The serum from an uninfected rat also had no effect (Fig. 4). Thus, the 45C2-based cELISA may be useful for detecting serum anti-MERS-CoV antibodies in experimental animal models (Fig. 4).

Since more than 90% of serum samples collected from dromedary camels in Ethiopia in 2010–2011 were reactive to the S1 antigen of MERS-CoV (Reusken et al., 2014), we tested whether the 45C2-based cELISA could also accurately detect dromedary camel serum samples that were positive for anti-MERS-CoV antibodies, as determined by neutralization assays. Thus, we performed the cELISA on 66 serum samples that were collected from dromedary camels in 2013 in Ethiopia. The cut-off values in the cELISA were determined by using 42 negative control sera from multiple species including rabbits, rats, mice, and healthy human donors. Of the 66 samples, 63 were positive in the conventional MERS-CoV neutralization assay, while the remaining three were negative (Table 1). The neutralization titers of the positive samples ranged from 20 to 20,480. When the cELISA was performed, 62 of the 63 neutralization-positive sera competed for 45C2 binding to the MERS-CoV antigen. All three neutralization-negative sera showed a negative reaction in the cELISA. The sensitivity and specificity of the cELISA were 98% and 100%, respectively (Table 1).

We then compared the percent inhibition values of the 63 camel sera in the 45C2-based cELISA with their neutralization titers. There was a significant positive correlation between percent inhibition at a 256-fold serum dilution in the cELISA and the neutralization titers (Pearson's correlation coefficient $r = 0.74$, Fig. 5). There were also significant positive correlations when percent inhibition at different serum dilutions was used ($r = 0.76$ at 512-fold serum dilution and

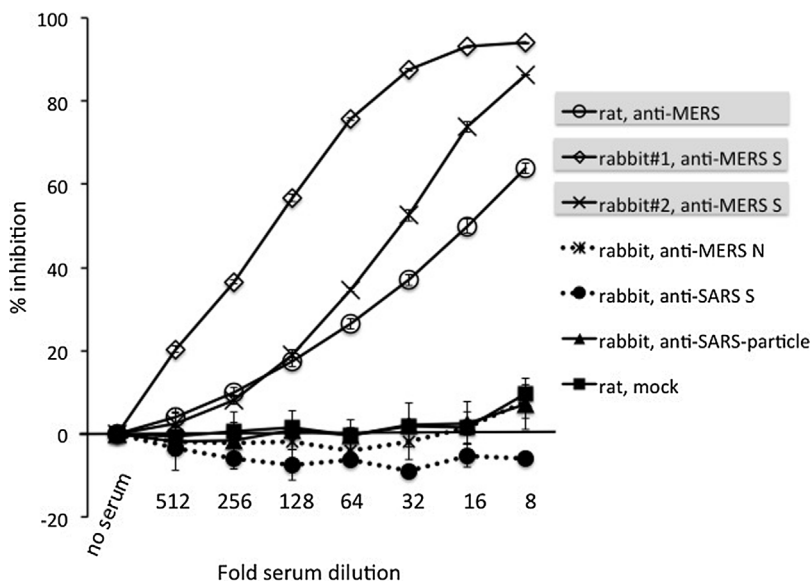


Fig. 4. Ability of the competitive ELISA based on the 45C2 monoclonal antibody (MAb) to detect MERS-CoV S protein-specific antibodies from infected rats and immunized rabbits. The 45C2 MAb was mixed with serially diluted sera from a rat that had been infected with MERS-CoV, two rabbits that had been immunized with the S protein of MERS-CoV, a rabbit that had been immunized with the N protein of MERS-CoV, a rabbit that had been immunized with the S protein of SARS-CoV, a rabbit that had been immunized with UV-inactivated SARS-CoV particles, and a rat that had been mock infected with MERS-CoV. The mixtures were then subjected to cELISA using whole UV-inactivated MERS-CoV antigen. The percent inhibitions relative to the ‘no antibody’ control that were obtained from three independent experiments and are expressed as means and standard deviations are shown. Serum samples that contain neutralization antibodies to live MERS-CoV are shown in the gray boxes.

Table 1
Positivity of 66 dromedary camel sera for antibodies against MERS-CoV in the competitive ELISA and the live MERS-CoV-based neutralization assay.

cELISA	MERS-CoV NT	
	positive	negative
positive	62	0
negative	1	3
Total	63	3

cELISA, competitive ELISA; NT, neutralization test.
Sensitivity = 98% (62/63).
Specificity = 100% (3/3).

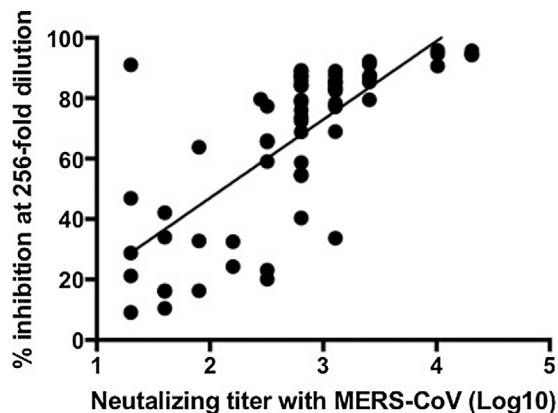


Fig. 5. Correlation between percent inhibition in the 45C2-based competitive ELISA and the neutralization titers of 63 camel sera, as determined by using live MERS-CoV. Sixty-three sera from dromedary camels from Ethiopia were tested at various dilutions in the 45C2-based competitive ELISA and the neutralization test. The percent inhibition of each sample at the 256-fold dilution in the competitive ELISA is plotted on the y-axis. The neutralization titers (Log_{10}) are plotted on the x-axis. The Pearson’s correlation coefficient (r) was 0.74.

$r = 0.71$ at 128-fold serum dilution) (Fig. S6).

4. Discussion

The most commonly used ELISAs are IgG/IgM ELISAs that rely on a species-specific secondary antibody for detection. Moreover, to reduce non-specific reactions, the serum samples should be treated in a specific

manner in some circumstances (Qing et al., 2003). These disadvantages together with the fact that MERS-CoV is a zoonotic virus led us to develop a cELISA, as this approach would enable us to study the prevalence of positivity for MERS-CoV antibodies in multiple animal species as well as assess the risk of zoonotic infections with MERS-CoV in humans.

In the present study, we generated novel MAbs that specifically recognized the S protein of MERS-CoV and developed a cELISA on the basis of this MAb. Relative to neutralization tests with live MERS-CoV (the gold standard in the field for detecting anti-MERS-CoV antibodies), the cELISA was able to detect dromedary camel sera that were positive ($n = 63$) and negative ($n = 3$) for MERS-CoV-specific antibodies with a sensitivity and specificity of 98% and 100%, respectively. Moreover, the cELISA was able to detect anti-MERS-CoV antibodies in sera from MERS-CoV-infected rats and rabbits that had been immunized with MERS-CoV S protein. In addition, negative control samples, including sera from rabbits and mice that had been immunized with SARS-CoV particles, the N protein of MERS-CoV, or the S protein of SARS-CoV, did not inhibit the binding of the MAb to the MERS-CoV antigen at even the lowest serum dilution (8-fold). Because the anti-sera against other coronaviruses were not available in this study, we did not test specificity of cELISA using them. However, to ensure the accuracy and versatility of the cELISA, the labeled MAb was characterized in terms of its antigen specificity (Fig. 1 and Supplementary Fig. S1). This confirms that the specificity of the cELISA is high. Thus, the cELISA detects MERS-CoV antibody in a non-species-dependent and highly-specific manner. Furthermore, we found that the three MAbs that we selected for epitope analyses (including 45C2) were highly specific for the RBD of the S protein of MERS-CoV. When we compared the RBD amino acid sequences of > 170 strains of MERS-CoV in the NCBI database, we found that the amino acid residues that were critical for the recognition of the three MAbs are conserved in all strains (data not shown). This indicates that all MERS-CoV strains that have been reported to date are likely to be recognized by the three MAbs. Thus, all three MAbs may be useful for detecting MERS-CoV-specific antibodies in cELISA. To the best of our knowledge, this is the first report of a cELISA that is based on a MERS-CoV S protein-specific MAb.

We determined the putative epitope of the 45C2 MAb that was used in the cELISA by analyzing the amino acid substitutions of the mutant MERS-CoV viruses that had escaped neutralization by 45C2. The two mutations of the 45C2 escape viruses were both located on the RBD, specifically within the site that binds to the human host receptor DPP4, which mediates the infection with the virus (Wang et al., 2013; Ying

et al., 2015). These observations suggest that the 45C2 MAb reacts with the DPP4-binding site in the RBD and that this is responsible for its ability to neutralize MERS-CoV infection of Vero cells. This is supported by the fact that, in cELISAs, serum antibodies inhibit the labeled antibody either by blocking the labeled antibody epitope directly or by recognizing a neighboring epitope and therefore providing spatial competition (Hirota et al., 2012). Given that a neutralizing epitope appears to be the target for the competition between the 45C2 MAb and the test serum antibodies, it is likely that the inhibitory serum antibodies that are detected by our cELISA will be neutralizing antibodies.

Our cELISA differed from the neutralization test in only one of the 66 dromedary camel serum samples: a sample that was positive on the neutralization test was negative on the cELISA. There was also a significant positive correlation between the percent inhibition values in the cELISA and the neutralization titers, although the correlation coefficient r did vary slightly between the different serum dilutions.

This study has some limitations. In particular, we have not yet tested serum samples from convalescent patients with MERS to validate the efficacy of our cELISA for the serological diagnosis of MERS in humans. However, our study did show that cELISA can be used to detect MERS-CoV antibodies in infected or immunized rats or rabbits in this study, or dromedary camels. Our cELISA also has the advantage that it does not have to be performed in a high containment laboratory, unlike the neutralization test. Thus, this cELISA may be useful for serologically diagnosing MERS-CoV infections in various animal species and as such may play an important role in sero-epidemiological investigations on MERS-CoV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2017.10.008>.

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