Chapter 4

Competitive ELISA for the Detection of Serum Antibodies Specific for Middle East Respiratory Syndrome Coronavirus (MERS-CoV)

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Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) is the etiological agent of MERS, a severe respiratory disease first reported in the Middle East in 2012. Serological assays are used to diagnose MERS-CoV infection and to screen for serum antibodies in seroepidemiological studies. The conventional enzyme-linked immunosorbent assay (ELISA) is the preferred tool for detecting serum antibodies specific for pathogens; however, the utility of conventional ELISA with respect to detection of MERS-CoV antibodies is limited due to the number of false-positives caused by cross-reactivity of serum antibodies with antigens that are conserved among coronaviruses. The competitive ELISA (cELISA) uses a pathogen-specific monoclonal antibody (MAb) that competes with serum antibodies for binding to an antigen; therefore, it is used widely for serological surveillance of many pathogens. In this chapter, I describe detection of serum antibodies using cELISA based on MAbs specific for MERS-CoV.

Key words  MERS-coronavirus, Serological assay, Competitive ELISA, Monoclonal antibody, Neutralizing antibody

1 Introduction

Middle East respiratory syndrome (MERS) is a severe respiratory illness first reported in the Middle East in 2012; it is caused by a newly recognized coronavirus called MERS-CoV [1]. Humans are infected with MERS-CoV through direct or indirect contact with dromedary camels, indicating that dromedary camels are the amplifying host and a major source of zoonotic infection [2, 3]. Since the discovery of MERS-CoV, serological antibody assays have been developed to assess antibody responses of infected patients and to investigate the seroprevalence of MERS [4–6]. Virus neutralization assays are the gold standard for detecting antibodies specific for MERS-CoV because they are both specific and sensitive [7]. However, conventional virus neutralization assays require handling of infectious MERS-CoV under biosafety level (BSL)-3 conditions.
Furthermore, it takes several days to obtain results because detectable levels of virus replication in virus infected cells are required.

General laboratories lacking heightened biocontainment facilities can use replication-incompetent pseudotyped viruses bearing viral glycoproteins to detect neutralizing antibodies and to investigate the mechanism underlying virus entry into host cells. Indeed, studies show the utility of a high-throughput pseudotyped virus system for detecting neutralizing antibody responses against MERS-CoV and to search for drugs that inhibit entry of MERS-CoV into cells [8–10]. The major advantage of the MERS-CoV pseudotype is that it can be handled without the need for BSL-3 conditions. However, the pseudotype system is not always a versatile approach since it requires equipment for cell culture, along with machines able to measure fluorescence or chemiluminescence to detect pseudotype infection; such a system is not readily adaptable to rural areas or developing countries in which the availability of expensive equipment is limited. Furthermore, the method used to generate the pseudotype virus is tricky; for example, carboxyl-terminal truncation of the MERS-CoV spike (S) protein might be needed to generate a high titer of the vesicular stomatitis virus-based MERS-CoV pseudotype [10].

Enzyme-linked immunosorbent assay (ELISA) is used widely in general clinical laboratories to measure serum antibody responses. Binding of serum antibodies to an antigen attached to a microplate generates a colorimetric reaction that is detected by a microplate reader; most importantly, ELISAs do not require specialized techniques. However, the usefulness of these assays for detecting anti-MERS-CoV antibodies is limited by the fact that antibodies that bind to conserved proteins expressed by coronaviruses are often cross-reactive; therefore, these assays often yield false-positive reactions [7, 11, 12].

The competitive ELISA (cELISA) was developed to detect serum antibody responses against many viruses [13–16]. It is based on a labeled monoclonal antibody (MAb) that is specific for a target antigen; this antibody competes with serum antibodies for binding to the antigen, thereby enabling detection and measurement of pathogen-specific antibodies (Fig. 1). Furthermore, since cELISA does not require a species-specific secondary antibody, it has an advantage over conventional ELISA in that it can detect serum antibodies in any animal species, making it a useful tool for seroepidemiological surveillance of MERS. Neutralizing responses to MERS-CoV in test serum can be measured using cELISA when the labeled MAb recognizes neutralizing epitopes in the MERS-CoV S protein [17]. Comparison with the results generated by a neutralization assay using live MERS-CoV shows that the results of cELISA correlate well with neutralization antibody titers [17]. The following protocol describes detection of serum antibodies using a cELISA based on a MAb specific for MERS-CoV.
2 Materials

1. MAb 45C2 (750 μg/ml) recognizing the receptor binding domain (RBD) of the MERS-CoV S protein, biotinylated using Biotin Labeling Kit-NH2 (see Note 1).

2. Positive control antibody (serum from a rabbit immunized with the MERS-CoV S protein expressed in a recombinant baculovirus expression system, inactivated at 56 °C for 30 min) [10].

3. Test serum (inactivated at 56 °C for 30 min).

4. MERS-CoV antigen cell slurry (see Note 2) or purified recombinant RBD antigen (rRBD, see Note 3).

5. Wash buffer: Phosphate-buffered saline (PBS, pH 7.4) containing 0.05% tween 20 (PBS-T).

6. Blocking buffer: PBS-T containing 2% bovine serum albumin.

7. High Sensitivity Streptavidin-HRP.

8. Substrate: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) tablets dissolved in ABTS™ buffer. One ABTS tablet (50 mg) is dissolved in 50 ml ABTS™ buffer working solution.

9. 96-well ELISA plate.
10. 96-well U-bottom microplate.
11. 12-channel pipette.
12. Dispenser trays.
13. ELISA plate washer.
14. ELISA plate reader capable of reading at wavelengths of 405 and 495 nm.

3 Methods

3.1 cELISA

1. Coat the ELISA plate with 100 μl MERS-CoV antigen cell slurry (diluted 1:800 in PBS) or 100 μl of purified recombinant RBD antigen (47 ng/ml in PBS) (Fig. 2). Seal the plate and incubate overnight at 4 °C.

2. Wash the ELISA plate twice with 300 μl PBS-T.

3. Add 200 μl blocking buffer to each well and seal the plate. Incubate at 37 °C for 2 h.

4. Prepare the series of twofold dilutions in the U-bottom microplate (Fig. 3) (see Note 4). Add 82.5 μl blocking buffer to line A and 55 μl blocking buffer to lines B–H.

5. Mix 27.5 μl test serum with 82.5 μl blocking buffer to make a fourfold dilution (line A).

6. Take 55 μl of the fourfold diluted serum sample (line A) and mix with 55 μl blocking buffer to make an eightfold dilution (line B).

7. Take 55 μl of the eightfold diluted serum sample (line B) and mix with 55 μl blocking buffer to make a 16-fold dilution (line C).

Fig. 2 Coating of the 96-well ELISA plate with MERS-CoV antigen
Fig. 3 Dilution of test serum in the U-bottom microplate. (a) Blocking buffer is added to the U-bottom microplate. (b) A fourfold dilution of each serum sample is added to line A. (c) Serum is diluted serially from line A to G. The nonserum control is placed in line H.
8. Take 55 μl of the 16-fold diluted serum sample (line C) and mix with 55 μl blocking buffer to make a 32-fold dilution (line D).

9. Take 55 μl of the 32-fold diluted serum sample (line D) and mix with 55 μl blocking buffer to make a 64-fold dilution (line E).

10. Take 55 μl of the 64-fold diluted serum sample (line E) and mix with 55 μl blocking buffer to make a 128-fold dilution (line F).

11. Take 55 μl of the 128-fold diluted serum sample (line F) and mix with 55 μl blocking buffer to make a 256-fold dilution (line G). Discard 55 μl of the mixture.

12. Place the nonserum control in line H.

13. In the dispenser tray, prepare a 2340-fold dilution of biotinylated MAb 45C2 (e.g., mix 6 μl of biotinylated 45C2 with 14 ml of blocking buffer).

14. Using the 12-channel pipette, take 55 μl of diluted biotinylated 45C2 (prepared in 13 above) and mix with 55 μl of serially diluted serum sample (final serum dilutions range from 8 to 512) and nonserum control in a U-bottom microplate (prepared in 12 above). Pipette tips should be replaced after mixing each sample (Fig. 4).

15. After blocking, wash the ELISA plate twice with 300 μl PBS-T.

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**Fig. 4** Biotinylated monoclonal antibody 45C2 is added to the U-bottom microplate plate containing diluted serum samples
16. Transfer 100 μl of the serum–antibody mixture from the U-bottom microplate (prepared in 14 above) to the ELISA plate (prepared in 15 above) (Fig. 5). The mixture can be transferred from line A to line G without changing tips, but for line H the mixture should be transferred using new tips. Seal the plate and incubate at 37 °C for 1 h.

17. Dilute the High Sensitivity Streptavidin-HRP with blocking buffer (final dilution, 1:8000).

18. Wash the ELISA plate three times with 300 μl PBS-T.

19. Add 100 μl of the diluted High Sensitivity Streptavidin-HRP to each well. Seal the plate and incubate at 37 °C for 1 h.

20. Wash the ELISA plate three times with 300 μl PBS-T.

21. Add 100 μl ABTS substrate. Incubate the plate at room temperature for 15–30 min. The O.D. values in the control non-serum wells should not be higher than the limit of the microplate reader (e.g., O.D. 405 nm >3.5) (Fig. 6).

22. Measure the O.D. at 405 nm at the reference wavelength of 495 nm.

3.2 Evaluate cELISA Results

1. Calculate the inhibition rate (%) of each serum sample as follows:

\[
\text{% inhibition} = \left(1 - \frac{\text{O.D. value with serum}}{\text{O.D. value without serum}}\right) \times 100
\]

2. The cutoff values of % inhibition for each serum dilution can be determined using at least ten negative serum samples.
3. The antibody titer is determined by performing cELISA with twofold serial dilutions (from 8- to 512-fold) of serum samples and is expressed as the reciprocal of the highest dilution at which % inhibition is above the cut-off value for each serum dilution.

4. Comparison with the results of a neutralization assay using live MERS-CoV allows for confirmation that the results of the cELISA correlate well with neutralizing antibody titers [17].

**Fig. 6** Schematic representation of the colorimetric reaction after addition of ABTS solution to the ELISA plate. After measurement of O.D. at 405 nm (reference wavelength, 495 nm), the percent inhibition at each serum dilution can be determined.

\[
\text{% inhibition} = \left( 1 - \frac{\text{O.D. value in 1A}}{\text{O.D. value in 1H}} \right) \times 100
\]

1. To generate MAb 45C2 (specific for the MERS-CoV antigen), BALB/c mice are immunized with purified, UV-inactivated MERS-CoV particles [17]. Hybridoma cells are produced by fusing mouse myeloma cells with splenic cells isolated from the
immunized mice. ELISA was used to screen culture supernatants of hybridoma cells for antibodies specific for the MERS-CoV antigen. Neutralizing activity of the MAb is confirmed in a plaque-reduction assay using live MERS-CoV. Epitope mapping experiments have indicated that MAb 45C2 recognized the RBD of MERS-CoV. The detailed characterization of MAb 45C2 has been published by Fukushi et al. [17]. Ask the author to share the MAb; this can be done formally using a material transfer agreement (MTA). A MAb specific for the MERS-CoV RBD is also available commercially (Absolute antibody, Wilton, UK); however, detailed characterization (specificity, epitopes, etc.) of this MAb might be required.

2. To prepare the MERS-CoV antigen cell slurry, Vero cells are grown in a T75 flask and inoculated with MERS-CoV at a multiplicity of infection (m.o.i.) of 1.0. After 26 h, the cells are lysed in 1 ml of PBS containing 1% NP40 to extract viral antigens from infected cells. After centrifugation at 8000 × g for 10 min, the supernatant is collected and used as the source of MERS-CoV antigen in a cELISA. The MERS-CoV was inactivated by UV irradiation (312 nm, 2.5 mW/cm²) for 10 min in a trans-illuminator before use. Viral inactivation is confirmed to be complete by inoculating an aliquot of antigen onto Vero cells, followed by cultivation of cells for at least 3 weeks. A preliminary experiment determines the optimum dilution of the antigen used for the cELISA as 1:800 (in PBS).

3. Recombinant RBD (rRBD) can be used as an alternative antigen for the cELISA. To prepare rRBD from MERS-CoV, the mammalian expression plasmid pCAGGS-RBD, which encodes histidine-tagged MERS-CoV RBD (amino acid 358-588), is transfected to 293T cells. At 2 days post-transfection, the rRBD is purified from the supernatant using a His-Bind Purification Kit. The amount of purified rRBD protein is determined using a BCA protein assay kit. Ask the author to share the rRBD; this can be done formally using an MTA. A commercial MERS-CoV S antigen (e.g., MERS-CoV S1 subunit protein; CD Creative Diagnostics, Shirley, NY, or Sino Biological, Beijing, China) might be available for use in the cELISA.

4. To screen serum antibodies from a large number of samples, it is better to test first at a single serum dilution (fourfold dilution) and then serially dilute only samples that are antibody-positive to measure antibody titers.

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