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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 TMPRSS2 and Vero-TMPRSS2 (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 8000*g* 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/cm^2) 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 8000*g* 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