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ORIGINAL ARTICLE

A sensitive and specific antigen detection assay for Middle East respiratory syndrome coronavirus

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Since its emergence in 2012, more than 900 laboratory-confirmed cases of Middle East respiratory syndrome (MERS) have been reported with a fatality rate of more than 30%. However, no antigen detection assay for commercial use is available for diagnosis. In this study, the full-length nucleocapsid protein (NP) gene of MERS coronavirus (MERS-CoV) was cloned and expressed in *Escherichia coli*. A MERS-CoV NP capture enzyme-linked immunosorbent assay (ELISA) using two MERS-CoV-NP-specific monoclonal antibodies (MAbs) generated was developed. The ELISA was evaluated using 129 nasopharyngeal aspirates (NPAs) positive for various respiratory viruses and simulated positive NPAs by adding serial dilutions of MERS-CoV. Using a cutoff OD of 0.19, all 129 NPAs positive for respiratory viruses showed very low OD, with a specificity of 100%. For the two simulated MERS-CoV-positive NPAs with serial dilutions of live MERS-CoV, all samples with $\geqslant 10$ 50% tissue culture infective dose (TCID$_{50}$)/0.1 mL showed positive results. For the 10 additional NPAs with 20 and 200 TCID$_{50}$/0.1 mL of live MERS-CoV added, all were positive. A highly sensitive and specific MAbs-based antigen capture ELISA has been developed for MERS. This sensitive and specific antigen capture ELISA should be useful for detection of MERS-CoV in human and dromedaries and in field studies.

INTRODUCTION

Since its emergence in 2012, Middle East respiratory syndrome (MERS) has affected more than 20 countries globally with a total of more than 900 laboratory-confirmed cases and a fatality rate of over 30%. Similar to severe acute respiratory syndrome (SARS), MERS is also caused by a coronavirus (CoV), named MERS-CoV;[1–4] but unlike the SARS epidemic which has rapidly died off after the intermediate amplification animal hosts were segregated from humans by closure of wild animal markets in southern China, the MERS epidemic has persisted for more than two years with no signs of abatement. The persistence of the epidemic may be partly due to the difficulty to control the contact between the people and dromedary camels, a major zoonotic source of MERS-CoV.[5–9] Since there is still no effective anti-viral agent for the treatment of MERS-CoV infections, rapid and accurate laboratory diagnosis is essential for commencement of infection control measures.

Although MERS-CoV can be cultured and antibody detection methods are available, laboratory diagnosis of human MERS-CoV infections is mainly achieved by quantitative real-time RT-PCR, using the RNA-dependent RNA polymerase, the region upstream to the envelope gene or the nucleocapsid gene of MERS-CoV as the target.[10–13] However, the cost of quantitative real-time RT-PCR is still high and such expertise may not be available, particularly in the clinical microbiology laboratories in the Middle East where most of the cases were identified. Enzyme-linked immunosorbent assay (ELISA) for antigen detection offers an inexpensive and user-friendly way for laboratory diagnosis of respiratory viral infections, including SARS.[14,15] As for MERS, antigen detection ELISA will be useful not only for laboratory diagnosis of patients with suspected MERS but also for field studies on dromedaries as well as other suspected animal reservoirs. So far, such an antigen detection assay is not available for MERS-CoV. In this article, we report the development and evaluation of a monoclonal antibody (MAb)-based capture ELISA for MERS-CoV nucleocapsid protein (NP) detection.

MATERIALS AND METHODS

Cloning and purification of (His)$_6$-tagged recombinant NP (rNP) of MERS-CoV

A clinical isolate of MERS-CoV was kindly provided by RA Fouchier and colleagues.[2] All work with infectious MERS-CoV was performed inside a biosafety level-2 cabinet with standard operating procedures in approved biosafety level-3 facilities.[16–18] Viral culture and RNA extraction were performed as we described previously.[9,16] To produce a plasmid for protein purification, primers (5′-GAA TTC ATG GCA TCC CCT GCT GCA CCT-3′ and 5′-GTC GAC ATC AGT GTT AAC ATC AAT CAT-3′) were used to amplify the gene encoding the NP of MERS-CoV by RT-PCR. The sequence coding for amino acid residues 1–413 of the NP was amplified and cloned into the *Eco*RI and *Sal*I sites of expression vector pET-28a(+) (Novagen, Merck Millipore, Billerica, MA, USA) in

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frame and upstream of the series of six histidine residues. The rNP protein was expressed in *Escherichia coli* ER2566 cells (Invitrogen, Life Technologies, Grand Island, NY, USA) and purified using Ni-NTA affinity chromatograph column (Novagen, Merck Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Production of anti-MERS-CoV-rNP MAbs

Anti-MERS-CoV-rNP MAbs were produced using a protocol as described previously.[19] Briefly, six-week-old female BALB/c mice were immunized with 100 μg purified MERS-CoV-rNP emulsified in an equal volume of Freund's complete adjuvant (Sigma-Aldrich, Saint Louis, MO, USA) subcutaneously. Two booster immunizations using the same antigen emulsified in incomplete Freund's adjuvant were carried out on days 14 and 28. Blood samples were obtained from the tail veins of the immunized mice and tested for titers against purified MERS-CoV-rNP by ELISA on MERS-CoV-rNP-coated microplates. After the final booster dose, the spleens of the mice were removed, and splenocytes were fused with the mouse myeloma cell line Sp2/0-Ag-14. Culture supernatant from individual hybridoma clones was then screened against purified MERS-CoV-rNP by ELISA. After subcloning three times by limiting dilution, stable hybridoma cells were injected into the abdominal cavity of a mouse to produce ascitic fluid. The MAbs were purified first by precipitating with 50% ammonium sulfate, then with diethylaminoethyl column by high performance liquid chromatography. The concentrations of purified IgG were determined by their absorbance at OD_{280}. All animal procedures were approved officially by the Xiamen University Institutional Committee for the Use and Care of Laboratory Animal.

Western blot analysis

Western blot analysis was performed according to our published protocol.[20] Briefly, 1.5 μg purified $(His)_6$-tagged rNPs of MERS-CoV, human CoV (HCoV) 229E and HCoV-OC43 respectively were subjected to 12% SDS-PAGE and were transferred to 0.22 μm nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were blocked with 5% skim milk in Tris-buffered saline and then incubated with mouse anti-MERS-CoV-rNP immune sera at 37 °C for 60 min. Antigen–antibody interaction was detected with 1:4000 diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Novex, Life Technologies, Grand Island, NY, USA) and visualized by 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrates system for membranes (Sigma Aldrich, Saint Louis, MO, USA).

Capture ELISA for detection of MERS-CoV NP

The capture ELISA for MERS-CoV-NP detection was developed as described previously with minor modifications.[21] Microplates (Sigma Aldrich, Saint Louis, MO, USA) were pre-coated with the first anti-MERS-CoV-rNP MAb 1F6 and incubated at 37 °C overnight with a blocking reagent (phosphate-buffered saline with 2% sucrose, 0.2% casein-Na, and 2% gelatin). Inactivated MERS-CoV, HCoV-229E, HCoV-OC43, two influenza virus A strains, one influenza virus B strain and one respiratory syncytial virus (RSV) strain, and purified rNPs of MERS-CoV, HCoV-229E, and HCoV-OC43 were diluted in phosphate-buffered saline with 2% skim milk. The inactivated viral lysates were diluted to the same virus titer of 10^6 $TCID_{50}$ and further serially diluted. Specifically, 50 μL of viral lysis buffer was added to the coated well and a 50 μL of sample (fixed concentrations of purified NP or viral cell culture lysates) was then added to the wells in duplicates. The plate was shaken for 2 min and incubated at 37 °C for 30 min. After the wells were washed

five times, 100 μL of the second anti-MERS-CoV-rNP MAb 7C4 conjugated with HRP was added and the plate was incubated at 37 °C for 30 min. After five washes, detection was carried out by adding of 100 μL TMB per well with incubation for 10 min followed by the addition of 50 μL 0.2 M H_2SO_4. The OD 450/630 nm was measured with a microplate reader. The cutoff value was set as $0.15 +$ mean value of 20 human nasopharyngeal aspirates (NPAs) negative for influenza A, B, and C viruses, adenovirus, RSV, parainfluenza viruses 1–4, human rhinoviruses, human metapneumovirus, HCoV-HKU1, HCoV-OC43, HCoV-229E, HCoV-NL63, and MERS-CoV.

Evaluation of MERS-CoV-NP capture ELISA

NPAs from 129 patients with viral respiratory tract infections (influenza A virus ($n = 10$), influenza B virus ($n = 10$), influenza C virus ($n = 2$), adenovirus ($n = 10$), RSV ($n = 10$), parainfluenza virus 1 ($n = 5$), parainfluenza virus 2 ($n = 2$), parainfluenza virus 3 ($n = 10$), parainfluenza virus 4 ($n = 10$), human rhinoviruses ($n = 30$), human metapneumovirus ($n = 10$), HCoV-HKU1 ($n = 5$), HCoV-OC43 ($n = 5$), HCoV-229E ($n = 5$), and HCoV-NL63 ($n = 5$)) and simulated MERS-CoV positive NPAs were used for the evaluation of the ELISA. NPAs positive for influenza C virus, parainfluenza virus 4, human rhinoviruses, human metapneumovirus, and HCoVs were determined by RT-PCR and those for the other respiratory viruses were determined by indirect immunofluorescence assay as described in our previous publications.[22–26] MERS-CoV culture and titration were performed as we described previously.[16] Simulated MERS-CoV positive NPAs were prepared by adding serial dilutions (1–500 000 $TCID_{50}$/0.1 mL) of MERS-CoV to two different NPAs and 20 $TCID_{50}$/0.1 mL and 200 $TCID_{50}$/0.1 mL of MERS-CoV to 10 additional NPAs negative for the above mentioned respiratory viruses. The ELISA was performed as described above, using 50 μL of each NPA sample.

RESULTS

Production and characterization of MERS-CoV-rNP

The full-length NP gene of MERS-CoV was cloned and expressed in *E. coli* strain ER2566 with a stretch of six histidine residues at both the N and C termini. The MERS-CoV-rNP, with a molecular weight of 46 kDa, was purified by Ni-NTA affinity chromatography and was used to immunize BALB/c mice. Western blot showed that mouse anti-MERS-CoV-rNP immune sera had good reactivity with MERS-CoV and MERS-CoV-rNP, indicating that the expressed MERS-CoV-rNP has similar antigenicity to native NP of MERS-CoV (Figure 1). As
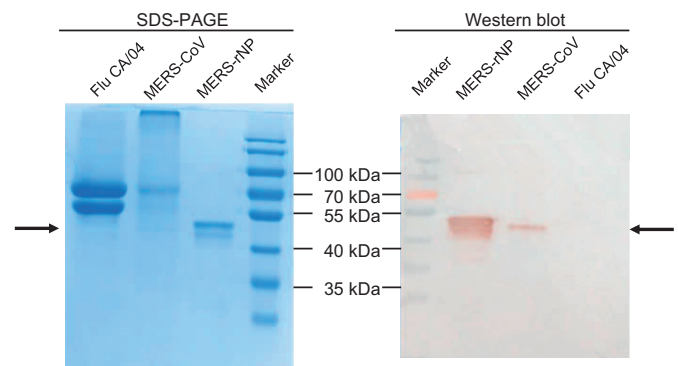

Figure 1 Western blot analysis of mouse anti-MERS-CoV-rNP immune sera. Proteins loaded include: MERS-CoV-rNP, lysate of MERS-CoV (MERS-CoV), lysate of influenza A virus strain CA/04 (Flu CA/04), molecular weight marker. The arrows indicate the size of the MERS-CoV-rNP.

a control, the mouse anti-MERS-CoV-rNP immune sera did not react with influenza A virus strain CA/04 (Figure 1). The high ELISA titer of mouse anti-MERS-CoV-rNP immune sera confirmed that the MERS-CoV-rNP has good immunogenicity in BALB/c mice, indicating that the MERS-CoV-rNP was useful for generation of anti-MERS-CoV-rNP MAbs (Figure 2).

Generation and characterization of anti-MERS-CoV-rNP MAbs

A total of 24 MAbs were generated against MERS-CoV-rNP by ELISA. Twelve of them were confirmed to be MERS-CoV-rNP-specific MAbs by ELISA using MERS-CoV-rNP as well as HCoV-229E-rNP and HCoV-OC43-rNP (Figure 3). The cloning, expression, and purification procedures for HCoV-229E-rNP and HCoV-OC43-rNP were the same as described for those of MERS-CoV-rNP (data not shown). The other 12 MAbs showed different degrees of cross-reactivity among the three rNP proteins, suggesting that the NPs of different CoVs might have some common epitopes.

Development of capture ELISA for detection of MERS-CoV NP

To establish a capture ELISA for MERS-CoV NP detection, two anti-MERS-CoV-rNP-specific MAbs (1F6 and 7C4) were selected. MAb 1F6 was coated onto the microplate and MAb 7C4 was conjugated with HRP. This ELISA showed a high sensitivity, with detection limit to MERS-CoV-rNP of less than 1 ng/mL; and a high specificity, with no detection for the HCoV-OC43-rNP and HCoV-229E-rNP (Figure 4A). The high sensitivity was further confirmed with a detection limit of 10^3 TCID$_{50}$ of inactivated viral lysate of MERS-CoV culture and the high specificity was further confirmed with no detection for inactivated viral lysates of two HCoVs (HCoV-OC43 and HCoV-229E), two influenza A virus strains (CA/4/2009(H1N1) and Brisbane/10/2007(H3N2)), one influenza B virus strain (Florida/04/2006), and one RSV strain (Figure 4B).

Evaluation of MERS-CoV-NP capture ELISA

The mean value of 20 human NPAs negative for influenza A, B, and C viruses, adenovirus, RSV, parainfluenza viruses 1–4, human rhinoviruses, human metapneumovirus, HCoV-HKU1, HCoV-OC43, HCoV-229E, HCoV-NL63, and MERS-CoV was 0.04. Using a cutoff of 0.19 (0.15 + 0.04), all 129 NPAs positive for various viral respir-
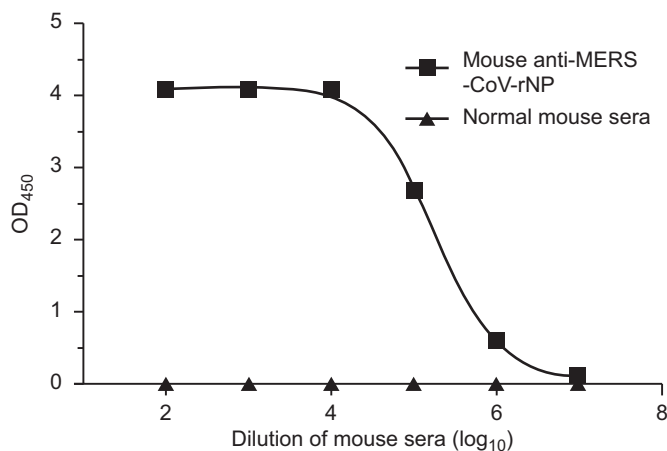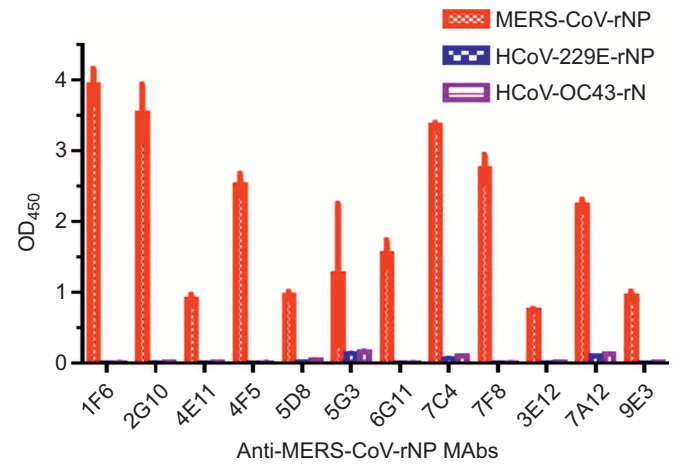
Figure 3 Reactivity of anti-MERS-CoV-rNP MAbs in ELISA. The MERS-CoV-rNP was coated at 100 ng/well. 1:5000 dilution of HRP-conjugated goat anti-mouse antibody was used as the secondary antibody. Normal mouse sera were used as negative control.

atory tract infections were negative, with mean OD $\pm$ SD of 0.067 $\pm$ 0.022 (Figure 5). Therefore, the specificity of the capture ELISA for NPA is 100%. For the two simulated MERS-CoV positive NPAs with serial dilutions (1–500 000 TCID$_{50}$/0.1 mL) of live MERS-CoV added, all samples with $\geqslant$10 TCID$_{50}$/0.1 mL showed positive results (Figure 5). As for the 10 additional NPAs with 20 TCID$_{50}$/0.1 mL and 200 TCID$_{50}$/0.1 mL of live MERS-CoV added, all showed positive results (Figure 5).

DISCUSSION

We report the development of a MAbs-based antigen capture ELISA for MERS-CoV-NP. Our previous experience on SARS-CoV has shown that SARS-CoV-NP detection in SARS patients is highly feasible using an antigen capture ELISA-based method.[14,15] Since NP is also an important, highly immunogenic and abundantly expressed structural protein in MERS-CoV, its detection using similar principles should also be feasible. The NP protein is a more preferable target than the S protein because the NP protein is present in much higher amount than the S protein. In this study, using hyperimmune sera obtained from mice immunized with three doses of MERS-CoV-rNP, we confirmed that MERS-CoV-NP is abundantly present in inactivated MERS-CoV cell cultures (Figure 2). The antigen capture ELISA was designed using two different MAbs (1F6 and 7C4) with high and specific antibody titers against MERS-CoV-rNP (Figure 3). As half of the 24 MAbs generated showed different degrees of cross-reactivity against HCoV-229E and/or HCoV-OC43, suggesting the possibility of common epitopes across the different CoVs, this MAbs-based ELISA should be much more specific than a polyclonal antibodies-based ELISA using hyperimmune sera generated from two different kinds of animals immunized with MERS-CoV-rNP. In addition to the high specificity, we also observed that MERS-CoV-rNP and MERS-CoV-NP can be detected by the present assay with a protein and inactivated viral concentrations of as low as 1 ng/mL and 10^3 TCID$_{50}$/mL respectively (Figure 4).

Evaluation of the diagnostic test using control and simulated clinical specimens showed that the present ELISA is highly sensitive and specific for MERS-CoV. Using more than 120 NPAs positive with various respiratory viruses, we showed that the antigen capture ELISA is highly specific. Most importantly, NPAs positive for the four

Figure 2 The ELISA titer of mouse anti-MERS-CoV-rNP immune sera. The MERS-CoV-rNP was coated at 100 ng/well. 1:5000 dilution of HRP conjugated Goat anti-mouse antibody was used as second antibody. Normal mouse sera were used as negative control.
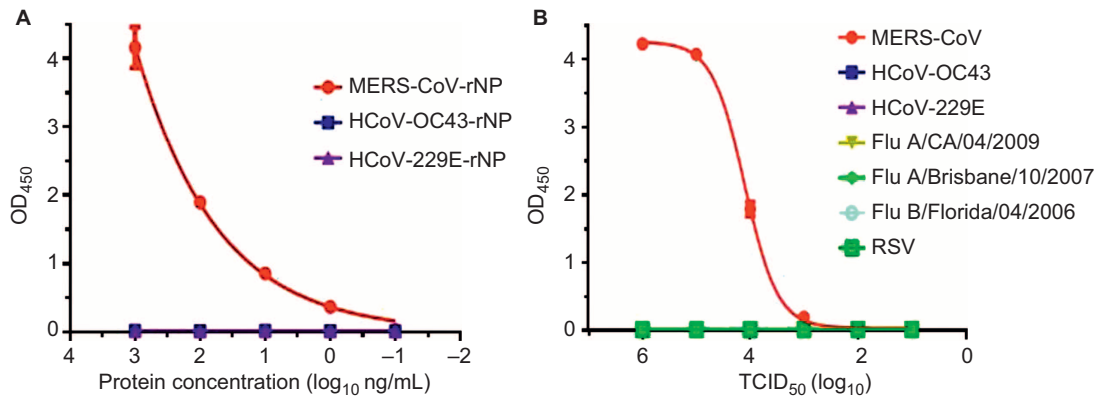
Figure 4 Detection limit of anti-MERS-CoV-rNP-specific MAbs-based capture ELISA for MERS-CoV-NP. (**A**) Detection of MERS-CoV-rNP, HCoV-OC43-rNP, and HCoV-229E-rNP. (**B**) Detection of inactivated cell lysates of MERS-CoV, HCoV-OC43, HCoV-229E, influenza A virus CA/4/2009(H1N1), influenza A virus Brisbane/10/2007(H3N2), influenza B virus Florida/04/2006, and RSV cultures.

human CoVs all showed very low OD values by the present MERS-CoV antigen detection assay, as the MERS-CoV-NP only shares 18.1%, 19.1%, 30.5%, and 30.5% amino acid identities with those of HCoV-229E (alphaCoV), HCoV-NL63 (alphaCoV), HCoV-OC43 (betaCoV), and HCoV-HKU1 (betaCoV) respectively. As there have been no imported MERS cases in Hong Kong so far and we do not have access to any MERS patient samples, we used simulated NPAs spiked with live MERS-CoV for evaluation. Since the assay is able to detect simulated NPA samples with viral load as low as 10 $TCID_{50}$/0.1 mL of live MERS-CoV, this diagnostic tool should be suitable for early diagnosis of MERS, since the viral loads in NPAs of patients during acute infection are usually around 10^6 copies/mL.[27,28]

The present MERS-CoV-NP capture ELISA is potentially useful for detecting MERS-CoV in dromedary camel samples and screening other animal specimens for MERS-CoV. Although the ancestral origin of

MERS-CoV is believed to be from bats,[29–31] dromedaries are likely to be the immediate animal reservoir for transmission of MERS-CoV to human. High titers of antibodies to MERS-CoV have been detected in more than 95% of adult dromedaries in the Middle East,[5,9,32,33] and MERS-CoV have also been found from the nasal samples of a small proportion of dromedaries.[8,34–36] As MERS-CoV was also detected in the nasal samples in dromedaries with viral loads of around 10^4 to 10^6 copies/mL,[37] the present ELISA should be readily useful for detecting MERS-CoV in dromedaries. To prevent MERS infections as well as in cases of MERS outbreaks, it may be necessary to screen and isolate dromedaries that are actively shedding MERS-CoV. Since the present ELISA captures MERS-CoV-NP antigen, it should be able to detect MERS-CoV regardless of the source of the specimen, and should be extremely useful for assessing viral shedding patterns in dromedaries as well as searching for other animal sources of MERS-CoV.
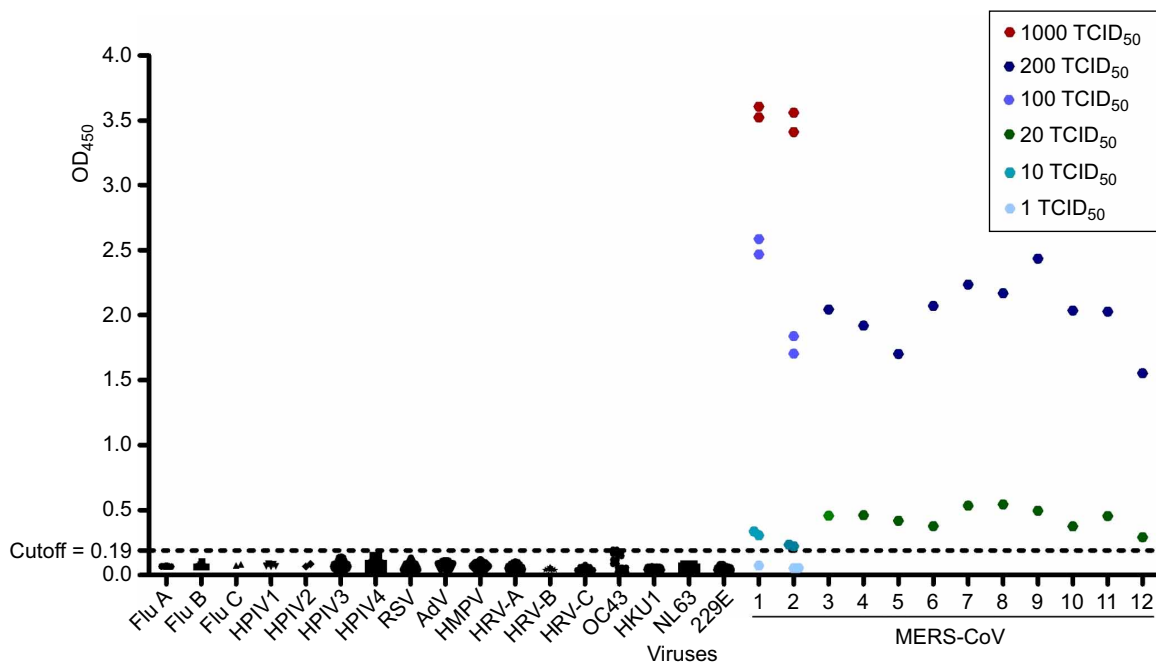

Figure 5 Evaluation of the MERS-CoV-NP capture ELISA using 129 NPAs positive for various viral respiratory tract infections, two simulated MERS-CoV-positive NPAs spiked with serially diluted MERS-CoV cell lysates and 10 simulated MERS-CoV-positive NPAs with 20 $TCID_{50}$/0.1 mL and 200 $TCID_{50}$/0.1 mL of MERS-CoV. The dotted line represents the corresponding cutoff OD_{450} value at 0.19.

This antigen detection ELISA is suitable for clinical use and field studies in the Middle East where most cases of MERS have occurred. So far, most patients with MERS either resided in or had recent travel histories to the Middle East. Therefore, a MERS diagnostic test developed should be able to be used in clinical microbiology laboratories in the Middle East. As for the animal reservoirs, the infected dromedaries also live in the Middle East as well as North Africa. Compared to quantitative real-time RT-PCR, the consumable cost of the present antigen capture ELISA is lower. It also does not require expertise on molecular technology and the expensive set-up for molecular studies, which may not be present in most clinical microbiology laboratories in the Middle East. For example, about 15 million people, including more than two million pilgrims, visit Mecca in Saudi Arabia every year. In particular, a high concentration of visitors is expected during the Hajj period. A user-friendly laboratory test would be necessary for handling such a large number of exposed people as well as potential outbreaks because of mass gathering. Therefore, the present ELISA is likely to be more suitable than quantitative real-time RT-PCR for the use in laboratories in the Middle East and North Africa, where resources and expertise could be limited. As for animals, this test would be extremely useful for tracking the transmission patterns of MERS-CoV among the dromedaries in the same farm as well as across different countries. Further refinement of the test and the development of a rapid antigen detection kit for on-the-spot diagnosis are warranted.

ACKNOWLEDGEMENTS

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