MIDDLE EAST RESPIRATORY SYNDROME (INFECTION OF DROMEDARY CAMELS WITH MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS)

SUMMARY

Description of the disease: *Middle East respiratory syndrome (MERS) is a viral respiratory infection of humans and dromedary camels that is caused by a coronavirus called Middle East respiratory syndrome coronavirus (MERS-CoV). MERS-CoV is an enveloped, positive-sense, single-stranded RNA virus of the genus* Betacoronavirus*, first identified in 2012. Since July 2015, infection with MERS-CoV has been reported in over 21 countries and so MERS-CoV has become a great concern for public health.*

Dromedary camels (Camelus dromedarius) *have been confirmed by several studies to be the natural host and zoonotic source of MERS-CoV infection in humans. Other species may be susceptible to infection with MERS-CoV, however, their epidemiological significance has not been proven. MERS-CoV has been reported to cause little to no disease in camels, and infections have sometimes been associated with mild respiratory signs consisting of nasal discharge, lacrimation and mild fever in young camels. While the impact of MERS-CoV on animal health is very low, human infections have a significant public health impact.*

Detection of the agent: *Nasal swabs are the preferred samples for laboratory detection of MERS-CoV infection in camels. They can be screened for MERS-CoV RNA using a real-time RT-PCR targeting the upstream region of the envelope gene. The presence of viral nucleic acid can be confirmed by either a positive RT-PCR result on both the two specific genomic targets, such as by testing with a real-time RT-PCR targeting the open reading frame 1a gene or a single positive target with sequencing of a second target.*

Serological tests: *Serum samples can be screened for the presence of MERS-CoV-specific antibodies by using a MERS-CoV enzyme-linked immunosorbent assay, a MERS-CoV neutralisation assay or a MERS-CoV pseudo-particle neutralisation assay.*

Requirements for vaccines: *Research on MERS-CoV camel vaccine development is ongoing.*

A. INTRODUCTION

Middle East respiratory syndrome coronavirus (MERS-CoV) is a zoonotic virus from dromedary camels (*Camelus dromedarius*) causing significant mortality and morbidity in humans in the Arabian Peninsula, and was first reported from Saudi Arabia in 2012 (Zaki *et al.,* 2012). Sporadic human cases of MERS have occurred and continue to occur over a wide geographical range with most cases reported from the Arabian Peninsula.

MERS-CoV belongs to lineage C of the genus *Betacoronavirus* in the family *Coronaviridae* under the order *Nidovirales*. MERS-CoV is an enveloped positive-sense single-stranded RNA virus and its single-stranded RNA genome has a size of approximately 30 kb (Chan *et al.,* 2015).

Dromedary camels have been shown to be the natural reservoir from where spill-over to humans can occur (Haagmans *et al.,* 2014). Human-to-human infection is also reported, especially in healthcare settings (Hui *et al.,* 2018). Although Africa has the largest number of dromedary camels, and MERS-CoV is endemic in these camels,

locally acquired zoonotic human MERS is confined to the Arabian Peninsula and has not been reported from Africa to date. There are viral genetic and phenotypic differences in viruses from different parts of Africa that may be relevant to differences in zoonotic potential, highlighting the need for studies of MERS-CoV at the animal–human interface (Chu *et al.*, 2018). The lack of routine surveillance information about MERS-CoV circulation in dromedary camels restricts the understanding of the transmission dynamics and epidemiology in dromedary camel populations (Aguanno *et al.,* 2018).

Published studies have indicated that MERS-CoV or viral RNA from MERS-CoV have been identified in dromedary camels in countries in the Middle East and North Africa; antibodies to MERS-CoV have been identified in samples taken from camels in the Middle East and Africa. Antibodies to MERS-CoV have been detected with a prevalence range of from 0 to 100% in populations of camels in the Middle East and African countries. MERS-CoV is mainly acquired in dromedaries when they are less than 1 year of age, and the proportion of seropositivity increases with age to a seroprevalence of 100% in adult dromedaries (Wernery *et al.*, 2017). In general, only minor clinical signs of disease have been observed in infected dromedary camels and most MERS-CoV infections do not appear to cause any clinical signs (Chu *et al.*, 2014). MERS-CoV infections have also been detected in camels with MERS-CoV antibodies, both in calves with maternal antibodies as well as older camels that had already acquired antibodies from a previous infection. However, virus replication and the virus load are generally lower in infected seropositive animals compared with seronegative camels (Meyer *et al.*, 2016).

Clinical signs of disease in camels that have been described after experimental and field infections are nasal discharge, fever and loss of appetite (Adney *et al.,* 2014; Hemida *et al.,* 2014; Khalafalla *et al.,* 2015). A systemic review of the global status of MERS-CoV in dromedary camels is provided by Sikkema *et al.* (2019).

Under experimental conditions, the disease observed in young adult dromedary camels was clinically benign with the absence of overt illness and with a large quantity of MERS-CoV and viral RNA detected in nasal swab specimens from camels. Histopathological examination revealed that the infectious virus was detected in the upper respiratory tract including nasal turbinates, olfactory epithelium, pharynx, and larynx. Specifically, the respiratory epithelium in the nasal turbinates is the predominant site of MERS-CoV replication in camels. In the lower respiratory tract, infectious virus was detected in the trachea. No viral antigen or lesions were detected in the alveoli. The large quantities of MERS-CoV shed in nasal secretions suggest that camel-to-camel and camel-to-human transmission may occur readily through direct contact and large droplet, or possibly fomite transmission (Adney *et al.,* 2014).

MERS-CoV antibodies have been detected in llamas and alpacas (David *et al.,* 2018; Reusken *et al.*, 2016). Naturally, other species of animals including sheep, goats, cattle, water buffalo and wild birds have tested negative for the presence of antibodies to MERS-CoV (Hemida *et al.,* 2013; Reusken *et al.*, 2016). However, recently, a single report from Africa that followed surveillance of other domestic mammalian species, such as sheep, goat, cow and donkeys, that were in contact with infected camels found the animals to be seropositive for MERS-CoV; domestic livestock in contact with MERS-CoV-infected camels may therefore be at risk of infection (Kandeil *et al.*, 2019). It has also been shown that Bactrian camels and Bactrian X dromedary hybrids can get naturally infected with MERS-CoV, when brought to countries where dromedaries are reared (Lau *et al.,* 2020).

B. DIAGNOSTIC TECHNIQUES

Diagnosis should always use a combination of techniques based on history, the purpose of the testing and the stage of the suspected infection. For a definitive interpretation, combined epidemiological, clinical and laboratory information should be evaluated carefully.

Nasal swabs are preferred specimens for laboratory detection of MERS-CoV infection in camels. Viral RNA can be extracted using any of the commercial kits and then screened for MERS-CoV RNA using a conventional or a real-time reverse-transcription polymerase chain reaction (RT-PCR) targeting the upstream regions of the envelope (UpE) gene. Positive samples should be confirmed by testing with a real-time RT-PCR targeting the open reading frame 1a (ORF1a) gene or 1b (ORF1b) gene. The presence of viral nucleic acid can be confirmed by either a positive RT-PCR result on both the two specific genomic targets, such as by testing with a real-time RT-PCR targeting the open reading frame 1a (ORF1a) gene or a single positive target with sequencing of a second target. Serum samples can be screened for the presence of MERS-CoV-specific antibodies by using a MERS-CoV enzyme-linked immunosorbent assay (ELISA), a MERS-CoV neutralisation assay or a MERS-CoV pseudo-particle neutralisation test.

The collection of specimens and their transport to the laboratory should comply with the standards of Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*.

All the test methods described below should be validated in each laboratory using them (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Table 1. Test methods available for diagnosis of MERS and their purposes

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Real-time RT-PCR	–	+++	+	+++	+++	–
Antigen detection	–	+	+	++	++	–
Virus isolation and identification	–	+	–	+++	–	–
Detection of immune response						
Indirect IgG ELISAs	++	–	++	–	++	+
Pseudo-particle neutralisation assay	+	–	+	–	+	+++
PRNT	+	–	+	–	+	+++
VNT	+	–	+	–	+	+++

Key: +++ = recommended for this purpose; ++ = recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; IgG ELISA = immunoglobulin G enzyme-linked immunosorbent assay; PRNT = plaque reduction neutralisation test; VN = virus neutralisation.

1. Detection of the agent

1.1. Specimen collection and storage

Nasal swabs are the samples of choice for virus isolation. Sterile plain cotton swabs are taken from both nostrils by inserting the swabs deep into the nasal cavity and turning them 10 to 20 times in the nose. These swabs are immediately submerged into viral transport medium supplemented with antibiotics (e.g. tissue culture medium 199 with 5% bovine serum albumin (BSA), benzylpenicillin (2×10^6 IU/litre), streptomycin (200 mg/litre), polymyxin B (2×10^6 IU/litre), gentamicin (250 mg/litre), nystatin (0.5×10^6 IU/ litre), ofloxacin hydrochloride (60 mg/litre) and sulfamethoxazole (0.2 g/litre). The samples can be stored in a cool box with ice packs if a –80°C freezer is reachable in 48 hours or they can be frozen in a liquid nitrogen tank immediately after the sampling. Swabs should be processed immediately upon arrival at the laboratory or kept in a –80°C freezer until use.

1.2. Isolation in cell culture

i)   Test procedure (Chu *et al.,* 2014; Woo *et al.,* 2016)

a)   The day before inoculation of the samples, prepare wells of African green monkey kidney (Vero) cells (24-well format) that can reach 80% confluence the next day. Apart from Vero cells, other common cell lines, such as rhesus monkey epithelial kidney cells (LLC-MK2) and human liver cancer cells (Huh 7), can also be used for virus isolation.

b)   Thawed samples are vortexed briefly, followed by a brief centrifugation at 1000 *g* for 5 minutes.

c)   Samples can be filtered through a 0.45 μm filter (optional).

d)   Retrieve the prepared Vero cell culture and wash the cells gently with sterile phosphate-buffered saline (PBS) for three times.

e)   Add 300 μl of a sample to a designed well of Vero cells.

f)   Incubate the mixture at 37°C in a CO_2 incubator for 1 hour.

g)   Retrieve the plate and add 700 μl of virus culture medium (DMEM [Dulbecco's modified Eagle's medium] with 1% Pen/Strep, 1% sodium pyruvate and 2% fetal calf serum) into each well. Treated cells are then cultured in a CO_2 incubator for 24 hours.

h)   Inoculum is removed after the incubation and 1 ml of fresh virus culture medium is added into each well. Treated cells are then cultured for another 3–5 days.

i)   Check for cytopathic effect (CPE) under microscope every day. Infected cells show CPE effects with rounded, aggregated and granulated giant cells detaching from the monolayer at about day 3 post-infection.

j)   Harvest the supernatant from cultures where 60% of cells have CPE.

k)   Aliquot the supernatant and keep them at a –80°C freezer until use.

l)   Confirm the identity of virus isolate using RT-PCR specific for MERS-CoV.

1.3. Real-time reverse-transcription polymerase chain reaction (RT-PCR) assay

Currently described tests are an assay targeting the upstream regions of the E protein gene (UpE) and an assay targeting the open reading frame 1b (ORF 1b) and/or an assay targeting ORF 1a. The assay for the UpE target is recommended for screening, whereas the ORF 1b or ORF 1a assay is recommended for confirmation. There are many commercially available kits for the UpE target MERS-CoV real-time RT-PCR assays, but these kits lack proper validation for use with camel samples (Mohamed *et. al.,* 2017). Therefore, any kit to be used must be validated according to WOAH standards (Chapter 1.1.6) in each individual laboratory before it can be used for routine diagnosis.

1.3.1.   Real-time RT-PCR targeting the upstream of the E protein gene (UpE)

The protocol recommended is based on the method described by Corman *et al.* (2012a).

i)   Test procedure

a)   Nasal swabs are preferred samples for laboratory detection of MERS-CoV infection in camels. Different methods for RNA isolation have been described and a large variety of commercial kits are available; the RNA extraction step should be appropriate to the sample to be tested and must also be validated in the laboratory.

b)   Reverse transcription is performed according to the manufacturer's instructions. Many one- and two-step RT-PCR kits formulated for application with probes are commercially available and should all provide satisfactory results.

c)   Prepare a master mix for the number of samples under test plus one extra sample. For example, 12.5 μl of 2 × reaction buffer provided with the one-step RT-PCR system with Taq polymerase containing 0.4 mM of each dNTP and 3.2 mM magnesium sulphate, 1 μl of reverse transcriptase/Taq mixture, 0.4 μl of a 50 mM magnesium sulphate solution, 1 μg of non-acetylated BSA, 400 nM concentrations of primer upE-Fwd (GCA-ACG-CGC-GAT-TCA-GTT)

and primer upE-Rev (GCC-TCT-ACA-CGG-GAC-CCA-TA), as well as 200 nM of probe upE-Prb (6-carboxyfluorescein [FAM])-CTC-TTC-ACA-TAA-TCG-CCC-CGA-GCT-CG-6-carboxy-N,N,N,N'- tetramethylrhodamine [TAMRA]).

d) Add 20 μl PCR reaction mix to each PCR tube or well of a real-time PCR plate followed by 5 μl of the prepared RNA to give a final reaction volume of 25 μl. Spin for 1 minute in a suitable centrifuge.

e) Place the tubes or the plate in a real-time thermal cycler for PCR amplification and run the following programme: 55°C for 20 minutes, followed by 95°C for 3 minutes and then 45 cycles of 95°C for 15 seconds, 58°C for 30 seconds.

f) BSA can be omitted if using a PCR instrument with plastic tubes as this component only serves to enable glass capillary-based PCR cycling.

g) The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

h) Real-time RT-PCR-based diagnostics should be interpreted with caution because a positive RT-PCR result does not necessarily indicate the presence of infectious virus (MacLachlan *et al.,* 1994). Furthermore, results of this procedure are difficult to correlate quantitatively to virus titre due to the imbalance between viral genomic and sub-genomic transcripts (Corman *et al.,* 2012a).

i) For the results to be valid, the positive control should give the amplification curve and no curve should be observed in the negative control.

j) The threshold cycle (Ct value) used in the interpretation of the results should be defined by individual laboratories using the appropriate reference material.

1.3.2. Real-time RT-PCR targeting the open reading frame 1b (ORF 1b)

The assay for ORF 1b has the same conditions as for the UpE real-time RT-PCR, except primer and probe sequences are: ORF1b-Fwd (TTC-GAT-GTT-GAG-GGT-GCT-CAT), primer ORF1b-Rev (TCA-CAC-CAG-TTG-AAA-ATC-CTA-ATT-G), and probe ORF1b-Prb (6-carboxyfluorescein [FAM])-CCC-GTA-ATG-CAT-GTG-GCA-CCA-ATG-T-6-carboxy N,N,N,N'-tetramethylrhodamine [TAMRA]) (Corman *et al.,* 2012a).

1.3.3. Real-time RT-PCR targeting the open reading frame 1a (ORF 1a)

The procedure to perform this test is described by Corman *et al.* (2012b). The conditions are also the same as for the UpE real-time RT-PCR, except primer and probe sequences are: Orf1a-Fwd (CCA-CTA-CTC-CCA-TTT-CGT-CAG) and Orf1a-Rev (CAG-TAT-GTG-TAG-TGC-GCA-TAT-AAG-CA), as well as 200 nM of probe Orf1a-Prb (6-carboxyfluorescein [FAM]-TTG-CAA-ATT-GGC-TTG-CCC-CCA-CT-6-carboxy-N,N,N,N'-tetramethylrhodamine [TAMRA]) (Corman *et al.,* 2012b).

1.4. Antigen detection

1.4.1. MERS-CoV immunochromatographic test (ICT)

This procedure is used to detect MERS-CoV directly from samples of suspected camels and can be used in the field or laboratories for rapid diagnosis. The assay is based on the immune-chromatographic detection of nucleocapsid protein to MERS-CoV using a rapid strip test (Song *et al.,* 2015). Commercial kits that detect recombinant nucleocapsid antigen of MERS CoV are available. The kits should be validated for the specific species of interest and for the specific purpose(s) for which they are to be used. Please see the WOAH Register for kits certified by the WOAH[1].

The procedure is fit for the qualitative detection of MERS-CoV antigens from nasal, nasopharyngeal swabs or nasal aspirates in dromedary camels for the following purposes:

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1   http://www.woah.org/en/scientific-expertise/registration-of-diagnostic-kits/background-information/

i) Detection of MERS-CoV infected herds (herd test) with acutely infected animals with high virus loads;

ii) A supplemental test to estimate prevalence of infection to facilitate risk analysis, e.g. surveys, herd health schemes and disease control programmes.

For sample preparation, use individual and new sterile swab for each camel. Collect the nasal swab specimens using sterile swab. Insert the swab through the nostril which presents more secretion. Rotate the swab a few times on the respiratory epithelium of the nose. The swab specimen should be placed immediately into sterile tubes containing 2–3 ml of viral transport media or the assay diluent tube.

i) Test procedure

a) Insert each swab sample without transport medium into the assay diluent tube, swirl the swab head against the inside of the assay diluent tube, squeeze remaining buffer from swab and take out the swab. Treat swabs collected in a transport medium in the same manner.

b) Dispense 100 µl from the assay diluent tube into a test tube.

c) Directly pipette 100 µl of the swab sample into the same test tube and mix well by vortex or by any other means.

d) Remove the test strip from the foil pouch and place immediately into the test tube.

e) Read the test result after 10~15 minutes, samples should not be interpreted after 20 minutes.

f) Each strip used in this assay contains a control line that indicates that the assay is working.

g) With every batch of sample always run a confirmed RT-PCR negative sample and a confirmed RT-PCR positive sample as negative and positive controls, respectively.

The presence of the purple line on both the control (C) and test (T) position is the threshold determination. The test sample is positive when two lines (C line and T line) both appear and negative when only the C line appears. Lines consist of the immune reaction of the gold conjugate and target analytes. Gold conjugate consists of colloidal gold and MERS-CoV antibody.

ii) Result interpretation

a) Negative result: Only one control ("C") band appears.

b) Positive result: Test ("T") band and control("C") band appear.

c) Invalid: Control ("C") fails to appear. If the control band is not visible within the result window after performing the test, the result is considered invalid. It is recommended that the sample be re-tested using a new test kit.

iii) Quality control

Each strip used in this assay contains a control line that indicates that the assay is working. With every batch of sample always run a confirmed RT-PCR negative sample and a confirmed RT-PCR positive sample as negative and positive controls, respectively. ICT can be negative when not enough virus is in the sample

2. Serological tests

Several assays are available for detection of MERS-CoV antibodies in dromedary camels. Currently the most widely used technique is the enzyme-linked immunosorbent assay (ELISA) for the detection of IgG. Virus neutralisation tests have also been used to detect antibodies against MERS-CoV in the serum of dromedary camels (Hemida *et al.,* 2013; Meyer *et al.,* 2014; Reusken *et al.,* 2013a; 2013b). Samples collected from animals for antibody testing may contain live virus and appropriate inactivation steps should be put in place.

2.1. Enzyme-linked immunosorbent assay

Several ELISAs have been developed and one test is commercially available. The ELISA is a reliable and sensitive test to detect antibodies against MERS-CoV. The use of the antigenically divergent S1 subunit

of the MERS-CoV spike protein allows for the detection of antibodies specific for MERS-CoV. IgM ELISAs that allow diagnosis of recent infections have not been described.

2.1.1. Indirect IgG ELISA

i) Test procedure

a) Coat each well of the 96-well ELISA plate with 100 µl of recombinant S1 protein at 1 µg/ml in PBS, seal the plate and leave overnight at 4°C.

b) Wash the plates three times with approximately 300 µl PBS per well.

c) Block the plates with 200 µl blocking buffer containing 1% BSA/0.5% Tween20 in PBS for 1 hour at 37°C.

d) Dilute both control (positive and negative) and test sera 1/100 in blocking buffer.

e) Add 100 µl of the diluted sera in designated wells in duplicate.

f) Incubate the plates for 1 hour at 37°C. Avoid drying by putting plates in a humid chamber.

g) Following the incubation step, wash the ELISA plates with wash buffer (0.05% Tween20 in PBS) three times with 300 µl of wash buffer per well.

h) Next, wells are incubated with 100 µl of a goat anti-llama biotin conjugate (diluted 1:1000 in blocking buffer).

j) Incubate for 1 hour at 37°C.

j) The plates are washed four times with PBS.

k) Add 100 µl of ready to use TMB (tetramethyl benzidine) chromogenic substrate to each well and allow the plates to stand at room temperature for a few minutes, while avoiding exposure to direct light.

l) Stop the reaction with 100 µl stop solution, and read the plates using ELISA plate reader at 450 nm.

2.2. MERS-CoV neutralisation assays

Neutralisation tests are the most specific diagnostic serological tests, but these tests can only be performed with live virus and are not recommended for use outside laboratories without appropriate biosecurity facilities. Alternative neutralisation assays not requiring handling of highly virulent MERS-CoV using pseudotyped viruses are available.

2.2.1. Pseudo-particle neutralisation assay

i) Production of human coronavirus (HcoV)-MERS spike pseudo-particles

The pseudo-particle used in this test expresses full-length spike protein of MERS-CoV. It is intended to be used for screening serum samples that can neutralise MERS-CoV virus. Unlike the standard virus neutralisation test for MERS-CoV, no infectious MERS-CoV is involved in this assay and the entire work can be performed safely in standard biosafety level 2 settings. Plasmids (pNL Luc-E-R- and pcDNA-S) required for making pseudo-particles are available upon request (Perera *et al.,* 2013).

ii) Preparation of pseudo-particles

a) Trypsinise a flask of human 293T cells (70–80% confluence, in 75 cm^2 flask).

b) Resuspend the trypsinised cells with 10 ml of DMEM complete medium (DMEM + pyruvate with 10% FBS (fetal bovine serum), 10 mM HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid), 5% Pen/Strep).

c) Harvest the suspended cells, followed by a centrifugation (450 g for 5 minutes).

d) Discard the supernatant and resuspend the cells with fresh DMEM complete medium.

e) Count cells using a haemocytometer.

f) Adjust the cell density to 5×10^5 per ml with DMEM complete medium.

g) Transfer 5×10^6 cells onto a 10 cm^2 dish and incubate the freshly prepared cell culture in a CO_2 incubator for overnight. Make sure cells are evenly distributed on a dish before incubation.

h) On the day of transfection, prepare a solution mixture by adding 15 µg of pNL Luc-E$^-$R$^-$ and 15 µg of pcDNA-S to 350 µl of sterile distilled water. Add 56 µl of 2 M CaCl$_2$ to the mixture and top up the mixture to 450 µl using sterile distilled water. Slowly add dropwise 450 µl of 2× HEPES buffered saline to the diluted DNA mixture. Briefly vortex the solution mixture and incubate it at room temperature for 2 minutes.

i) Evenly dispense the diluted DNA solution dropwise to the 10 cm^2 dish and mix the solution by moving the dish back and forth and sideway several times.

j) Incubate the transfected cells in a CO_2 incubator at 37°C for 16–18 hours.

k) Replace the medium with DMEM complete medium without disturbing the monolayer. Incubate the treated cells for another 2 days.

l) Harvest the supernatant at day 3 post-transfection. Centrifuge the supernatant at 450 *g* for 5 minutes. Filter the centrifuged supernatant with a sterile 0.45 µm filter.

m) Make aliquots of the filtrate and store them at –80°C until use.

iii) Pseudo-particle titration

a) Seed 1×10^4 Vero cells in a well (96-well plate format) similar to the protocol as described above. Culture the cells in a CO_2 incubator at 37°C overnight.

b) Prepare two-fold diluted pseudo-particle solution (range of dilution factors: 1 to 2048) by mixing the pseudo-particle with virus culture medium (DMEM + pyruvate with 2% FBS, 10 mM HEPES, 5% Pen/Strep) immediately before the infection. 200 µl for each studied concentration is sufficient for performing the test in triplicate.

c) Retrieve the cultured cells, discard the culture medium and add 50 µl of virus culture medium to each well.

d) Transfer 50 µl of prepared pseudo-particle solution to the corresponding wells in the plate. Incubate the treated cells in a CO_2 incubator at 37°C overnight.

e) Add 100 µl of virus culture medium in to each well at 24 hours post-infection and incubate the cells for another 48 hours.

f) At 72 hours post-infection, check the luciferase activity of infected cells using a commercially available luciferase assay kit. The final working concentration used for pseudo-particle neutralisation assays is the most diluted concentration that has a maximum luciferase activity in this titration assay.

iv) Pseudo-particle neutralisation assay

a) Prepare Vero cells in a 96-well plate format on the day before infection as described above.

b) Add 16 µl of heat-treated serum (56°C, 30 minutes) into 144 µl of virus culture medium (DMEM + pyruvate, 2% FBS, 10 mM HEPES, 5% Pen/Strep), then mix the diluted serum with 160 µl of diluted pseudo-particle at the predetermined concentration (see above). Keep the mixture on ice for 60 minutes. Include appropriate positive and negative controls for each run.

c) Retrieve the cultured Vero cells, discard the culture medium and transfer 100 µl of incubated pseudo-particle to the corresponding wells in triplicate. Incubate the treated cell in a CO_2 incubator at 37°C for 24 hours.

d) Add 100 µl of virus culture medium to each well at 24 hours post-infection and incubate the culture at 37°C for another 48 hours.

e) At 72 hours post-infection, check the luciferase activity of infected cells using a commercially available luciferase assay kit. Use data from negative controls for background subtraction. Data from positive controls representative 100% luciferase activity. Assay cut-off value is 90% of inhibition of the average luciferase activity from positive controls.

f) This assay screens for the presence of MERS-CoV-specific neutralising antibodies using 20× diluted serum samples. Neutralising antibody titres of positive samples can be

determined in a similar fashion by using two-fold serially diluted sera in the test (starting dilution: 20×).

2.2.2. Plaque reduction neutralisation test

The plaque reduction neutralisation test (PRNT) may be used to determine the presence of antibodies in naturally infected animals and in vaccinated animals. The test is highly specific and can be used to test serum of any species. The PRNT80 (i.e. 80% reduction) or PRNT90 conducted in a cell culture system is generally accepted as the standard assay system for the quantitative determination for neutralisation antibody activity in serum samples. The following technique uses a 96 well format.

i) Test procedure

a) Samples are first inactivated at 56°C for 30 minutes.

b) Prepare 50 µl of two-fold serial dilutions of heat-inactivated serum in RPMI1640 medium supplemented with clemizole penicillin (penicillin G), streptomycin, and 1% FBS (1% culture medium) using 96-well round-bottom plates. Starting dilution should be 1/10 and include known positive and negative control sera.

c) Dilute MERS-CoV in 1% culture medium to a dilution of 10000 tissue culture infective dose per ml ($TCID_{50}$/ml) of MERS-CoV. Add 50 µl of this suspension to the wells.

d) Incubate for 60 minutes at 37°C in a humidified atmosphere with 5% CO_2.

e) Inoculate onto Huh-7 cells that were grown in flat-bottom 96-well plates.

f) Wash with phosphate buffered saline and add 100 µl of 1% culture medium. Incubate for 8 hours at 37°C in a humidified atmosphere with 5% CO_2.

g) The cells are then fixed with 3.7% formalin for 15 minutes at 20°C. After removal of the formalin plates are soaked in 70% ethanol and kept overnight at 4°C.

h) Cells are stained using an anti-MERS-CoV N protein mouse monoclonal antibody or other specific antisera against MERS-CoV. A secondary peroxidase-labelled goat anti-mouse IgG1 or other appropriate antibody is subsequently applied. The signal can be developed using a precipitate forming TMB substrate. The number of infected cells per well are counted using an inverted microscope or image analyser.

i) The neutralisation titre of each serum sample is determined as the reciprocal of the highest dilution resulting in an at least 80 or 90% reduction in the number of infected cells. A titre of ≥ 20 is considered to be positive.

2.2.3. Virus neutralisation test

i) Test procedure

a) Samples are first inactivated at 56°C for 30 minutes.

b) Prepare 50 µl of two-fold serial dilutions of heat-inactivated serum in Iscove's modified Dulbecco's medium (IMDM) supplemented with clemizole penicillin (penicillin G), streptomycin, and 1% FBS (1% culture medium) using 96-well round-bottom plates. Starting dilution should be 1/10 and include known positive and negative control sera.

c) Dilute MERS-CoV in 1% culture medium to a dilution of 2000 tissue culture infective dose per ml ($TCID_{50}$/ml) of MERS-CoV. Add 50 µl of this suspension to the wells.

d) Incubate for 60 minutes at 37°C in a humidified atmosphere with 5% CO_2.

e) Inoculate the virus suspension onto Vero cells that were grown in flat bottom 96 well plates in 10% culture medium.

f) Incubate for 60 minutes at 37°C in a humidified atmosphere with 5% CO_2.

g) Wash with phosphate buffered saline and add 200 µl 1% culture medium. Incubate for 5 days at 37°C.

h) Determine the endpoint titres.

C. REQUIREMENTS FOR VACCINES

So far, among the available vaccine candidates, only three have been tested in dromedary camels: pVaxA, a DNA-based vaccine, adjuvanted MERS-rCoV, spike protein subunit vaccine and MVA vaccine, a viral-vector-based vaccine (Adney *et al.,* 2019; Haagmans *et al.,* 2016; Muthumani *et al.* 2015).

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NB: There is a WOAH Reference Laboratory for MERS (please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for MERS

NB: FIRST ADOPTED IN 2021.