

## REVIEW

# The role of laboratory diagnostics in emerging viral infections: the example of the Middle East respiratory syndrome epidemic

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Rapidly emerging infectious disease outbreaks place a great strain on laboratories to develop and implement sensitive and specific diagnostic tests for patient management and infection control in a timely manner. Furthermore, laboratories also play a role in real-time zoonotic, environmental, and epidemiological investigations to identify the ultimate source of the epidemic, facilitating measures to eventually control the outbreak. Each assay modality has unique pros and cons; therefore, incorporation of a battery of tests using traditional culture-based, molecular and serological diagnostics into diagnostic algorithms is often required. As such, laboratories face challenges in assay development, test evaluation, and subsequent quality assurance. In this review, we describe the different testing modalities available for the ongoing Middle East respiratory syndrome (MERS) epidemic including cell culture, nucleic acid amplification, antigen detection, and antibody detection assays. Applications of such tests in both acute clinical and epidemiological investigation settings are highlighted. Using the MERS epidemic as an example, we illustrate the various challenges faced by laboratories in test development and implementation in the setting of a rapidly emerging infectious disease. Future directions in the diagnosis of MERS and other emerging infectious disease investigations are also highlighted.

**Keywords:** coronavirus, middle east, MERS, diagnosis, epidemic

## Introduction

The ongoing threat of emerging viral infections to global pub-

lic health is well evidenced by the recent epidemics caused by Middle East respiratory syndrome coronavirus (MERS-CoV), avian influenza A viruses, Ebola virus, and Zika virus (To *et al.*, 2013, 2014, 2015; Chan *et al.*, 2015b, 2015c, 2016). Prompt and accurate diagnosis is the first step in the successful control of any of these epidemics, and is particularly important for emerging viral infections because they may spread rapidly and may be associated with severe complications (Sridhar *et al.*, 2015). Definitive diagnosis of these emerging viral infections usually requires laboratory confirmation because their clinical features and epidemiological risk factors may be similar to those of other related infections. The characteristics of an ideal laboratory assay for diagnosing these infections include high sensitivity, high specificity, short turn-around time, low cost, low expertise, and facility requirement, suitability for use in different specimen types, availability for point-of-care testing (POCT), and capability to quantify viral load. Unfortunately, despite the recent advances in the field, no single laboratory diagnostic test has all of these characteristics. It is therefore important to understand the clinical applications of the various types of laboratory diagnostic assays and their roles in the control of emerging viral epidemics. In this review, we use the MERS epidemic as an example to illustrate the advantages, disadvantages, practical uses, and impact on epidemic control of the major types of laboratory diagnostic assays available for emerging viral infections.

## Overview of MERS-CoV and MERS

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel lineage C betacoronavirus first isolated from a Saudi Arabian man with severe acute community-acquired pneumonia and acute kidney injury in 2012 (Zaki *et al.*, 2012). As of 5 December 2016, 1864 cases of human MERS cases, including 659 fatalities, have been reported by the World Health Organization (<http://who.int/emergencies/mers-cov/en/>). The epidemic has continued to expand since 2012, with most human cases of MERS being reported in the Middle East as a result of animal-to-human transmissions from infected animal reservoirs (dromedary camels and possibly bats in the region) and person-to-person transmissions in health-care-associated outbreaks (Reusken *et al.*, 2013a; Haagsmans *et al.*, 2014; Wang *et al.*, 2014; Chan *et al.*, 2015b). Moreover, sporadic cases and clusters of human MERS infection have also occurred in other areas with imported cases of MERS, such as the Republic of Korea (Cho *et al.*, 2016). The clinical

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presentation of MERS may range from asymptomatic infection detected during contact tracing in outbreak investigations to rapidly fatal disease (Chan *et al.*, 2015b). The disease is especially severe in elderly men with co-morbidities (Assiri *et al.*, 2013a). Severe MERS is characterized by rapidly progressive acute pneumonia with fever and respiratory failure not responsive to broad-spectrum antibacterial treatment, and may be associated with extrapulmonary manifestations, including acute kidney injury, hepatic dysfunction, gastrointestinal symptoms, and seizures (Chan *et al.*, 2012, 2013c; Zaki *et al.*, 2012; Assiri *et al.*, 2013a; Arabi *et al.*, 2015). A number of repurposed drugs, antiviral peptides, and monoclonal antibodies have demonstrated anti-MERS-CoV activity *in vitro* and/or in animal models, but none of them have been proven to be effective in randomized controlled trials yet (Chan *et al.*, 2013b, 2015d; Gao *et al.*, 2013; Lu *et al.*, 2014a; Jiang *et al.*, 2014; Tang *et al.*, 2014; Ying *et al.*, 2014). Various vaccines have been developed and some are undergoing clinical trials and/or testing in camels (Uyeki *et al.*, 2016).

### Specimen collection: what, when, and how?

Like all other infectious diseases, appropriate specimen collection is the most important step in the laboratory diagnosis of MERS and requires knowledge of viral kinetics in various specimen types with relation to time since symptom onset. Like SARS-CoV, MERS-CoV viral loads in respiratory specimens peak in the second week after symptom onset (Chan *et al.*, 2004; Oh *et al.*, 2016). Therefore, a patient testing 'negative' for MERS soon after symptom onset should undergo repeated testing if epidemiological history is suggestive of MERS.

Lower respiratory tract specimens (including tracheal aspirates, bronchoalveolar lavage fluid, well collected sputum

specimens) contain the highest viral RNA loads and should be collected whenever possible (Corman *et al.*, 2016; Oh *et al.*, 2016). However, invasive procedures to obtain lower respiratory specimens may not always be feasible, especially in patients with mild illness. Upper respiratory tract specimens (nasopharyngeal swabs, oropharyngeal swabs, and/or nasopharyngeal aspirates) should be taken for such cases with pooling of swabs in a single container to maximize RNA load as viral loads in the upper respiratory tract are consistently lower than in the lower respiratory tract (Memish *et al.*, 2014b; Corman *et al.*, 2016). Risk assessment regarding requisite transmission-based precautions, patient placement, and personal protective equipment during specimen collection should be conducted due to the possibility of aerosol generation. Specimens should be sent to the laboratory in viral transport medium containing a balanced salt solution, bovine serum albumin, pH buffer, phenol red, and antimicrobials as soon as possible. If specimen processing is likely to be delayed, storage in an ultra-low freezer (-80°C) is recommended.

Extrapulmonary specimens that have been reported to contain detectable MERS-CoV RNA include blood, stool, and urine (Drosten *et al.*, 2013; Poissy *et al.*, 2014; Corman *et al.*, 2016). These specimens may provide further opportunities for MERS diagnosis when lower respiratory tract specimens are unavailable. However, viral loads in these specimen types are generally lower than in the lower respiratory tract, although there are reported exceptions (Abroug *et al.*, 2014). Detection of MERS-CoV in stool specimens may have infection control implications, while detection of MERS-CoV RNA in whole blood or serum in particular may be a prognostic marker of poor outcome (Guery *et al.*, 2013; Kim *et al.*, 2016c).

For serology testing, acute and convalescent serum specimens should ideally be collected 14 to 21 days apart to enable documentation of seroconversion or 4-fold rise in neu-

**Table 1. Characteristics and applications of different diagnostic methods for MERS**

Diagnostic test	Examples of commonly used assays / cell lines	Advantages	Disadvantages	Other applications
Viral culture	Vero, LLC-MK2, Caco-2, and Huh-7 cell lines	<ul style="list-style-type: none"> <li>Especially useful in the initial phase of emerging epidemics before other diagnostic assays are available</li> <li>Considered as the gold standard of virological diagnosis</li> </ul>	<ul style="list-style-type: none"> <li>Requires BSL-3 facility</li> <li>Requires at least 3-5 days</li> </ul>	<ul style="list-style-type: none"> <li>Pathogenesis studies</li> <li>Antiviral and vaccine evaluation studies</li> </ul>
NAAT				
Monoplex RT-PCR	upE and ORF1a assays	<ul style="list-style-type: none"> <li>Fast (hours to 1-2 days)</li> </ul>	<ul style="list-style-type: none"> <li>Requires expertise and specialized equipment</li> </ul>	<ul style="list-style-type: none"> <li>Contact tracing and animal source finding</li> </ul>
Multiplex RT-PCR	Combined upE and ORF1a assay	<ul style="list-style-type: none"> <li>Does not require BSL-3 facility</li> </ul>	<ul style="list-style-type: none"> <li>Risk of amplicon carryover</li> </ul>	<ul style="list-style-type: none"> <li>Viral shedding patterns</li> <li>Prognostic markers</li> <li>Antiviral and vaccine evaluation studies</li> </ul>
Non-PCR-based assays	RT-LAMP and RT-RPA			
Antigen detection assays	Nucleocapsid protein detection	<ul style="list-style-type: none"> <li>Fast (minutes to hours)</li> <li>May allow point-of-care testing</li> </ul>	<ul style="list-style-type: none"> <li>Not yet thoroughly validated for human clinical specimens</li> <li>Sensitivity is usually lower than NAAT</li> </ul>	<ul style="list-style-type: none"> <li>Histological examination of post-mortem human samples and animal models</li> </ul>
Antibody detection assays	IFA, ELISA, PRNT	<ul style="list-style-type: none"> <li>Most do not require BSL-3 facility</li> <li>Enables retrospective diagnosis</li> </ul>	<ul style="list-style-type: none"> <li>Some assays may show cross-reactivity with other CoVs</li> <li>Requires cumbersome confirmatory assays</li> </ul>	<ul style="list-style-type: none"> <li>Contact tracing in outbreak investigations</li> <li>Seroepidemiological studies in human and animals</li> </ul>

Abbreviations: BSL-3, biosafety level 3; CoVs, coronaviruses; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; NAAT, nucleic acid amplification tests; PRNT, plaque reduction neutralization test; RT-LAMP, reverse transcription-loop-mediated isothermal amplification; RT-PCR, reverse transcription-polymerase chain reaction; RT-RPA, reverse transcription isothermal recombinase polymerase amplification.

tralizing antibody titer. When no serum specimen from the acute phase is available, a convalescent phase serum specimen may also be used to establish retrospective diagnosis with a panel of antibody tests (see Serology section below).

The choice of investigation will depend on the specimen type, timing post-symptom onset and local test availability. The advantages and shortcomings of different tests are detailed below and summarized in Table 1.

### **Viral culture: an important tool for virus discovery, pathogenesis studies, and evaluation of countermeasures**

Isolation of infectious MERS-CoV in cell culture inoculated with the patient's bodily fluids and/or tissues establishes the diagnosis of MERS (Zaki *et al.*, 2012). Although the routine use of viral culture for diagnosing MERS in standard clinical microbiology laboratories is limited by the method's relatively slower turn-around time than molecular diagnostics and requirement of a biosafety level 3 facility, this time-tested diagnostic tool has played important roles in the discovery and studies on the pathogenesis and antivirals of MERS-CoV. Unlike the other human-pathogenic CoVs which are notoriously difficult to culture in cell lines, MERS-CoV replicates rapidly with induction of prominent cytopathic effects in a broad range of cell lines (Muller *et al.*, 2012; Chan *et al.*, 2013a). MERS-CoV produces focal cytopathic effects with rounded refractile cells in susceptible cell lines within 5 days after inoculation during primary isolation (Chan *et al.*, 2013a). The spread of these changes throughout the cell monolayers leads to rounding and detachment of cells within 1 to 2 days. Syncytium formation caused by fusion activity of the MERS-CoV spike (S) protein may be seen in Calu-3, Caco-2, Huh-7, and LLC-MK2 cell lines (Zaki *et al.*, 2012; Chan *et al.*, 2013a; de Wilde *et al.*, 2013). These rapid and prominent cytopathic effects allowed Zaki and colleagues to successfully isolate the first MERS-CoV strain from cell lines which were commonly used in clinical virology laboratories (Vero and LLC-MK2) shortly after inoculation of the index patient's sputum sample into these cell lines (Zaki *et al.*, 2012).

A recent comparison between Vero and Caco-2 cell lines for the isolation of MERS-CoV showed that the isolation rate of MERS-CoV was significantly higher in Caco-2 than in Vero cells (45.5% vs 19.1%,  $P = 0.013$ ) (Muth *et al.*, 2015). The isolation rate of MERS-CoV in cell culture was higher in respiratory samples with higher viral RNA loads (66.7% vs 5.9% in samples with  $\geq 10^7$  copies/ml and  $< 10^7$  copies/ml, respectively), lower respiratory tract samples (0.0% in nasopharyngeal aspirate, 33.3% in sputa, and 48.6% in endotracheal aspirates), and samples which were collected earlier after diagnosis (58.6% vs 22.2% in samples collected within and after 5 days of diagnosis, respectively). These factors should be considered in laboratories which are attempting to isolate MERS-CoV from clinical specimens.

The broad tissue tropism of MERS-CoV in cell lines of different human organ tissue origins corroborated with the protean clinical manifestations of MERS in human. The high viral load of MERS-CoV in human lung, kidney, colonic, and hepatic cell lines correlate with the predominantly lower

respiratory tract involvement and extrapulmonary manifestations of acute kidney injury, diarrhea, and hepatic dysfunction, respectively (Chan *et al.*, 2015b). Most of these *in vitro* observations were subsequently validated in *ex vivo* organ tissue culture and/or animal models (Chan *et al.*, 2013f; Zhou *et al.*, 2014; Chu *et al.*, 2016; Yeung *et al.*, 2016). The replication of MERS-CoV in monocytes, dendritic cells, and T lymphocytes with aberrant induction of inflammatory cytokines/chemokines and activation of extrinsic and intrinsic apoptosis pathways partly explained the pathogenesis of virus dissemination, cytokine/chemokine storm, and lymphopenia in severe MERS (Lau *et al.*, 2013a; Chu *et al.*, 2014, 2016; Zhou *et al.*, 2014, 2015; Scheuplein *et al.*, 2015; Tynell *et al.*, 2016). Moreover, MERS-CoV could be isolated from numerous non-human cell lines, including those of non-human primate, camel, and bat origins (Muller *et al.*, 2012; Chan *et al.*, 2013a; Eckerle *et al.*, 2014). In contrast, cell lines of mouse and rat origins were not susceptible (Chan *et al.*, 2013a). These *in vitro* biological characteristics of MERS-CoV provided insights on the possible clinical manifestations, animal reservoirs, and animal species which were susceptible to MERS-CoV infection for animal model development at an early stage of the epidemic (de Wit *et al.*, 2013a; Munster *et al.*, 2013; Coleman *et al.*, 2014; Falzarano *et al.*, 2014; Yao *et al.*, 2014; Zhao *et al.*, 2014; Agrawal *et al.*, 2015; Chan *et al.*, 2015d; Haagmans *et al.*, 2015). Viral culture of MERS-CoV also facilitated the identification and evaluation of anti-MERS-CoV drugs. Screening of potential candidate anti-MERS-CoV agents in chemical libraries consisting of a large number of clinically approved drugs and validation of their *in vitro* anti-MERS-CoV activity in cytopathic effect inhibition, viral load reduction, and plaque reduction assays using cell culture systems successfully identified repurposed drugs, such as type I interferons and lopinavir-ritonavir, for further testing in animal models (Chan *et al.*, 2013b; Dyal *et al.*, 2014; de Wilde *et al.*, 2014). Similarly, the anti-MERS-CoV effects of newly designed antiviral peptides and monoclonal antibodies were also validated in cell culture (Gao *et al.*, 2013; Jiang *et al.*, 2014; Lu *et al.*, 2014a; Tang *et al.*, 2014; Ying *et al.*, 2014). The availability of viral culture for MERS-CoV in reference research laboratories is crucial for further deepening our understanding on and finding countermeasures for MERS.

### **Nucleic acid amplification tests: the platinum standard for MERS diagnosis**

Given the limitations of viral culture, more rapid and readily available laboratory assays were required for diagnosing MERS. Specific primers and a standardized laboratory protocol were quickly developed after the successful isolation of the first MERS-CoV strain and sequencing of its complete genome early in the epidemic in September 2012 (Palm *et al.*, 2012). The positive sense, single-stranded RNA genome of MERS-CoV has a size of approximately 30 kb and is arranged in the order of 5'-replicase [open reading frame (ORF) 1a/b]-structural proteins [S-envelope (E)-membrane (M)-nucleocapsid (N)]-poly(A)-3' (Woo *et al.*, 2012; Chan *et al.*, 2013d; Lau *et al.*, 2013b). A number of monoplex reverse transcrip-

tion-polymerase chain reaction (RT-PCR) assays using primers that target conserved gene regions of the MERS-CoV genome were developed and evaluated for screening and/or confirmatory tests.

These gene targets include the leader sequence at the 5'-untranslated region, and ORF1a, ORF1b, RNA-dependent RNA polymerase (RdRp), ORF4a, upE (upstream of envelope (E) gene), M, and N gene regions (Corman *et al.*, 2012a, 2012b; Lu *et al.*, 2014b; Chan *et al.*, 2015a; Douglas *et al.*, 2015). The most commonly adopted diagnostic protocol utilizes the upE assay as a screening test, followed by either the ORF1a or ORF1b assays for confirmation. In general, these assays were highly sensitive and specific, with their technical limits of detection ranging from 1.6 to 263.0 RNA copies/reaction (Chan *et al.*, 2015b; Kim *et al.*, 2016b). The limits of detection appear to be the lowest in the assays targeting the abundantly expressed leader sequence at the 5'-untranslated region and the N gene, although clinical comparison among the various assays have not been reported. Most of these assays were evaluated using clinical specimens, including respiratory (nasopharyngeal aspirate, sputum, endotracheal aspirate, bronchoalveolar lavage fluid, and/or nose and mouth exudates) and/or extrapulmonary specimens (serum, urine, and/or stool). A number of regional and international external quality assessments showed that the majority (>80%) of participating laboratories were capable of detecting MERS-CoV RNA by RT-PCR assays with high accuracy, but false-negative results might occur in a minority of samples with low viral loads (Pas *et al.*, 2015; Seong *et al.*, 2016; Zhang *et al.*, 2016).

Recent advances in molecular diagnostics for MERS include the development of commercial monoplex and multiplex RT-PCR kits, and other novel non-PCR-based diagnostics. Most of the commercial assays utilize primers that target the upE and/or ORF1a gene regions (Corman *et al.*, 2014; Kim *et al.*, 2016b) (<http://www.fast-trackdiagnostics.com/products/ftd-mers-cov/>; <http://eng.bioneer.com/diagnostic/HumanMDxkits/Accupower-MERS-CoV-Multiplex-overview.aspx>; [http://seegene.com/neo/en/products/respiratory/anyplex\\_mers\\_cov.php](http://seegene.com/neo/en/products/respiratory/anyplex_mers_cov.php)). Internal controls of these assays include primers against human glyceraldehyde 3-phosphate dehydrogenase gene, which is a housekeeping gene found in clinical specimens, spiked RNA, tobacco mosaic virus DNA, or phocine herpesvirus DNA spiked in the PCR mixtures. Analytical and clinical evaluation of these assays showed that they generally had high sensitivity and specificity, but false-negative or invalid results may occur in specimens containing high levels of PCR inhibitors, such as sputum specimens (Kim *et al.*, 2016b). Sputum homogenization prior to nucleic acid extraction with proteinase K and DNase treatment may be more effective than either phosphate-buffered saline treatment or N-acetyl-L-cysteine and sodium citrate treatment for improving the sensitivity of these assays (Sung *et al.*, 2016). The performance of multiplex assays may be improved by the use of self-avoiding molecular recognition system-artificially expanded genetic information systems (SAMRS-AEGIS) primers to reduce non-specific reactions generated among the multiple primers in the same assay (Glushakova *et al.*, 2015; Yaren *et al.*, 2016). The main advantage of these commercial diagnostic kits is their ease for use

in laboratories without technical expertise for designing and performing in-house-developed RT-PCR assays. The major disadvantage is their relatively high costs which may limit their use in resource-limited regions.

Other non-PCR-based assays for MERS include reverse transcription-loop-mediated isothermal amplification (RT-LAMP) and reverse transcription isothermal recombinase polymerase amplification (RT-RPA) (Abd El Wahed *et al.*, 2013; Shirato *et al.*, 2014; Bhadra *et al.*, 2015). These isothermal assays generally have short incubation times and are highly sensitive and specific. They are simple to perform and do not require thermocyclers, and are therefore especially suitable for POCT in resource-limited areas where the expertise and equipment for RT-PCR are not readily available.

In addition to establishing diagnosis, nucleic acid amplification tests have been applied to fulfill a number of other important purposes in the MERS epidemic. Firstly, it was used to investigate the animal reservoir of MERS-CoV and established the link between dromedary camels and human cases of MERS (Haagmans *et al.*, 2014; Lau *et al.*, 2016). The higher rate of detection of MERS-CoV RNA in the nasal and/or rectal swabs of juvenile camels than in those of adult camels further helped to identify juvenile camels as an important source of camel-to-human transmission of MERS (Alagaili *et al.*, 2014; Wernery *et al.*, 2015). Secondly, RT-PCR is commonly employed in contact tracing during healthcare-associated outbreaks of MERS (Assiri *et al.*, 2013b; Memish *et al.*, 2013; Drosten *et al.*, 2014; Oboho *et al.*, 2015). During these outbreak investigations, it was recognized that asymptomatic infection might occur in young and previously healthy persons and that they might serve as the source of further person-to-person transmission of MERS (Memish *et al.*, 2013, 2014a; Omrani *et al.*, 2013). Thirdly, serial testing of different clinical samples of MERS patients by RT-PCR identified the shedding patterns of the virus in respiratory and non-respiratory samples. Notably, viral RNA was detected in 14.6% of stool and 2.4% of urine samples, suggesting that these clinical samples may also be important in the spread of MERS. Fourthly, viral RNA load was found to be a predictive factor for severe disease. High MERS-CoV load in lower respiratory tract specimens was predictive of progression to pneumonia. Blood MERS-CoV RNA positivity at initial diagnosis was associated with worse clinical outcome in terms of a higher rate of requiring mechanical ventilation or extracorporeal membrane oxygenation, as well as death ( $P < 0.05$ ). Fifthly, RT-PCR was commonly employed in *in vitro* and *in vivo* antiviral and vaccine evaluation studies (Zumla *et al.*, 2016). Finally, RT-PCR and sequencing were important for surveying molecular epidemiological changes that may be associated with virus adaption for efficient person-to-person transmission.

### Antigen detection assays: potential for POCT

Molecular diagnostic assays have excellent sensitivity for the diagnosis of MERS-CoV infection. However, these assays require dedicated facilities, expensive equipment and highly trained personnel, which places a great strain on laboratory



infrastructure in endemic areas of MERS. Therefore, there has been considerable interest in developing MERS-CoV antigen detection assays, which are better than PCR diagnosis in terms of convenience.

Specific monoclonal antibodies targeting MERS-CoV proteins are used to demonstrate evidence of MERS-CoV in infected tissues (de Wit *et al.*, 2013b). Such antibodies have also been used to detect the MERS-CoV N protein in respiratory specimens as this antigen is abundantly expressed during the acute phase of illness. Four assays for detection of the MERS-CoV N protein have been described to date (Chen *et al.*, 2015, 2016; Song *et al.*, 2015; Yamaoka *et al.*, 2016). The peptides used to immunize mice for raising monoclonal antibodies were either in the form of recombinant protein synthetically produced by cloning the corresponding DNA fragment into *E. coli* and subsequently purifying the protein, or prepared using a wheat germ extract-based cell free expression system. Researchers either used a single long peptide or a pool of smaller synthetic peptides spanning the length of the MERS-CoV N protein (Chen *et al.*, 2015, 2016; Song *et al.*, 2015; Yamaoka *et al.*, 2016).

Monoclonal antibodies that produced favorable signal-to-noise ratios in a recombinant MERS-CoV N protein immunoassay were selected for incorporation into either an enzyme-linked immunosorbent assay (ELISA) (Chen *et al.*, 2015; Yamaoka *et al.*, 2016) or a POCT format (Song *et al.*, 2015; Chen *et al.*, 2016).

The analytical sensitivity of antigen detection assays was measured using lower limit-of-detection (LOD) experiments. We have previously described an ELISA assay that had an LOD of 10 TCID<sub>50</sub>/0.1 ml using simulated NPA specimens seeded with serially diluted MERS-CoV cultures (Chen *et al.*, 2015). A lateral flow immunoassay (LFIA), also described by us, had an LOD of at least 10<sup>3.7</sup> TCID<sub>50</sub>/ml MERS-CoV (Chen *et al.*, 2016). An immunochromatographic test (ICT) developed by Song *et al.* had a LOD of 1.5 ng/ml recombinant MERS-CoV N protein while the ELISA assay developed by Yamaoka *et al.* could detect 0.625 ng/ml of N protein (Song *et al.*, 2015; Yamaoka *et al.*, 2016). Due to careful selection of monoclonal antibodies, the published assays were quite specific for MERS-CoV and did not cross-react with other animal or human coronaviruses.

The main advantage of antigen detection assays is their ease of use and rapid results, especially when adapted to a POCT format. POCTs can be performed entirely within a biosafety cabinet, have built-in quality control and require minimal training of laboratory personnel. They offer a rapid and specific 'rule-in' option for clinicians while pending PCR tests, which may require send out to reference laboratories and long turnaround times in regions where laboratory infrastructure is not well developed.

However, there are several obstacles to the application of antigen detection for the diagnosis of MERS in humans. Firstly, such assays have not been validated in clinical specimens from suspected MERS cases in endemic areas. There is no head-to-head comparison with PCR, which is the most commonly employed 'platinum standard' diagnostic method in MERS patients. From experience with other respiratory viruses, the sensitivity is expected to be lower than PCR tests, which may provide false reassurance and lapses in infection

control if false negative results are interpreted without a confirmatory PCR assay (Chan *et al.*, 2002). Furthermore, antigen detection POCTs and ELISAs designed to date have only been evaluated using nasopharyngeal specimens; it is unknown whether such assays can be used on lower respiratory tract specimens, which are often observed to contain higher viral loads than NPA (Drosten *et al.*, 2013; Guery *et al.*, 2013) and would theoretically be even more suitable for antigen detection assays. Lastly, antigen detection assays do not feature in WHO or CDC algorithms for the diagnosis of MERS, limiting interest in developing commercial kits up to this stage (<http://www.cdc.gov/coronavirus/mers/lab/lab-testing.html>; [http://apps.who.int/iris/bitstream/10665/176982/1/WHO\\_MERS\\_LAB\\_15.1\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/176982/1/WHO_MERS_LAB_15.1_eng.pdf)).

Antigen detection assays may have a potential role in the epidemiological surveillance of camels. The ICT developed by Song *et al.* was validated using camel nasal swabs, demonstrating a high sensitivity (93.9%) and specificity (99.6%) compared to upE and ORF1A real time RT-PCR (Song *et al.*, 2015). Our LFIA also showed a moderately high sensitivity of 81% with excellent specificity compared to real time RT-PCR on dromedary camel respiratory specimens (Chen *et al.*, 2016). These studies indicate that such assays can be used for conveniently detecting infected camels in rural endemic areas.

### Antibody detection assays: retrospective diagnosis and contact tracing

Data regarding the kinetics of the antibody response in MERS patients is steadily accumulating. There appears to be considerable person-to-person variation in the robustness and timing of the antibody response, but as with other viral infections, an initial IgM response is followed by rising IgG titers, which is usually detectable 2 to 3 weeks after symptom onset (Drosten *et al.*, 2013; Chan *et al.*, 2015b; Park *et al.*, 2015b). Longitudinal serology of one MERS-infected patient in China showed that anti-S ELISA antibodies rose before anti-N antibodies (Wang *et al.*, 2016a). The differential kinetics of anti-N and anti-S antibodies in the setting of MERS serodiagnosis requires further study. Antibodies remain detectable long after clearance of infection; neutralizing antibodies were persistently detectable in 86% of patients up to 34 months after the Jordanian MERS outbreak of 2012 (Payne *et al.*, 2016).

A wide variety of serological assays have been described for the detection of MERS-CoV-specific antibodies with variations in assay format, the antigen used and the antibody subtype detected. MERS specific IgM detection does not feature in diagnostic algorithms promulgated by the WHO or US CDC (<http://www.cdc.gov/coronavirus/mers/lab/lab-testing.html>; [http://apps.who.int/iris/bitstream/10665/176982/1/WHO\\_MERS\\_LAB\\_15.1\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/176982/1/WHO_MERS_LAB_15.1_eng.pdf)). Although IgM titers theoretically rise before IgG, experience with sera from SARS and MERS patients suggests that the time lag between the two may be too short to be of much clinical value (Woo *et al.*, 2004; Meyer *et al.*, 2014a; Wang *et al.*, 2016b). Furthermore, IgM assays are potentially prone to non-specific positivity and cross-reactivity with other coronaviruses requiring te-

dious specimen preparation and quality control (Buchholz *et al.*, 2013). The additional value of testing MERS-CoV IgM in patients presenting with acute illness requires further elucidation. In view of these factors, most of the serological assays described for MERS diagnosis either aim to detect total immunoglobulin or IgG.

A major variable in serological assays for MERS-CoV is the source of the antigen. For reference or research laboratories with BSL-3 and cell culture facilities, MERS-CoV-infected cells are the most convenient source of antigen. The cell lysate may be spotted on glass slides, microtiter plates or Western blot strips for downstream serological assays. Although convenient, such assays require a reliable means of inactivating live virus within the culture extracts. Furthermore, such assays have also been shown to cross react with other coronaviruses, as infected cells express a wide range of viral antigens, some of which are likely to be conserved across different coronavirus subgroups and even genera (Aburizaiza *et al.*, 2014). For SARS, it has been shown that Western blotting with whole virus lysates may enable us to differentiate genuine seropatterns from false-positives; however, this is a tedious procedure requiring technical expertise and well characterized control sera (Guan *et al.*, 2004).

In view of these shortcomings, recombinant antigens have been used for ELISA, immunofluorescence (IFA), Western blot, protein microarray and even pseudoparticle neutralization assays (Corman *et al.*, 2012b; Perera *et al.*, 2013; Reu-

skan *et al.*, 2013b; Chan *et al.*, 2015b; Park *et al.*, 2015a; Wang *et al.*, 2016b). Using recombinant antigens has two major advantages: firstly, biosafety during assay production is not a major concern and secondly, this method enables the selection of immunogenic and MERS-CoV-specific antigens for maximizing assay specificity and sensitivity. Both viral N and S proteins are abundantly expressed immunogenic antigens stimulating antibody production (Meyer *et al.*, 2014a). In SARS patients, there is evidence that anti-N antibodies rise before anti-S antibodies (Woo *et al.*, 2005). However, convalescent sera tend to react against both antigens with moderate to high sensitivity. The difference between the two antigens lies in their specificity. Although the N protein is easier to clone and purify (being smaller with fewer glycosylation sites), it is more conserved within coronavirus subgroups compared to the S protein, which is the major target for neutralizing antibodies (Meyer *et al.*, 2014a). Therefore, recombinant MERS-CoV N protein-based serological assays are expected to have higher rates of cross reactivity compared to anti-S detection assays. Indeed, N epitope cross reactivity may even extend across coronavirus genera: a SARS-CoV N protein-based ELISA showed a propensity to produce false positive results when tested against convalescent sera from patients recovering from HCoV-229E and even HCoV-OC43 infections (Woo *et al.*, 2005). However, there is evidence to suggest that even sections of the S2 subunit of the S protein can induce cross-reactive antibodies against different beta-

**Table 2** Comparison of different antibody detection assays for MERS serodiagnosis

Assay format	Variations	Antigen	Advantages	Disadvantages
IFA	<ul style="list-style-type: none"> <li>Conventional IFA</li> <li>Recombinant antigen IFA</li> </ul>	<ul style="list-style-type: none"> <li>MERS-CoV-infected cell lines</li> <li>Transfected cell lines expressing MERS N &amp; S protein</li> </ul>	<ul style="list-style-type: none"> <li>Can detect either anti-MERS-CoV IgG or IgM subclasses</li> <li>Short processing time</li> <li>Antigens are presented with accurate conformation and glycosylation</li> <li>Non-specific reactions may be identified by experienced microscopist, considered to be more specific than ELISA</li> <li>Commercial kit available</li> </ul>	<ul style="list-style-type: none"> <li>Requires experienced operators</li> <li>Potential for inter-observer variation when reading titres</li> <li>Requires different conjugates for humans and animals</li> <li>In-house design of conventional IFA assay requires BSL-3 facility</li> </ul>
ELISA	• N/A	• S1 subunit of spike protein	<ul style="list-style-type: none"> <li>Short processing time</li> <li>Straightforward assay procedure</li> <li>Large throughput</li> <li>Commercial kit available</li> </ul>	<ul style="list-style-type: none"> <li>Potential for false-positives, confirmatory assay recommended</li> <li>Requires different conjugates for humans and camels</li> </ul>
Western Blot	• N/A	• Lysate of transfected cells expressing recombinant MERS N & S proteins	<ul style="list-style-type: none"> <li>Potentially higher specificity compared to ELISA</li> </ul>	<ul style="list-style-type: none"> <li>Very limited evaluation data</li> <li>Limited role in diagnostic algorithms as more convenient assays available for screening and more specific assays available for confirmation</li> </ul>
Protein microarray	• N/A	• S1 subunit of spike protein	<ul style="list-style-type: none"> <li>Allows for detection of IgG and IgM subtypes</li> <li>One-stage, high throughput assay with minimal sample requirement</li> <li>Antigen is correctly folded and glycosylated</li> <li>Good specificity</li> </ul>	<ul style="list-style-type: none"> <li>Requires further evaluation of diagnostic sensitivity by comparison with neutralization assays</li> </ul>
Neutralization	<ul style="list-style-type: none"> <li>PRNT</li> <li>Micro-neutralization</li> <li>Pseudoparticle neutralization assay</li> </ul>	<ul style="list-style-type: none"> <li>Whole virus</li> <li>HIV/MERS-CoV pseudoparticle expressing MERS-CoV S protein</li> </ul>	<ul style="list-style-type: none"> <li>Gold-standard assay with high specificity</li> <li>Comparable sensitivity and specificity to conventional neutralization assays</li> <li>Does not require BSL-3 facility</li> <li>Objective signal detection using luminometer</li> </ul>	<ul style="list-style-type: none"> <li>Cumbersome</li> <li>Requires several days for completion</li> <li>Requires BSL-3 facility</li> <li>Needs further evaluation in clinical specimens from MERS patients, but performance data from South Korean outbreak sera promising</li> </ul>

Abbreviations: BSL, biosafety laboratory; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; IFA, immunofluorescence assay; Ig, immunoglobulin; MERS-CoV, Middle East respiratory syndrome coronavirus; N, nucleocapsid; N/A, not applicable; PRNT, plaque reduction neutralization test; S, spike.

coronaviruses (Chan *et al.*, 2013e). Further studies are required to elucidate optimally specific recombinant antigens for MERS serological tests.

Many of the classical serological assay formats have been applied to MERS with modifications in the interest of assay specificity and biosafety. The principles, advantages and disadvantages of each assay type are summarized in Table 2. Commercial IgG ELISA assays are now available and provide a fast and sensitive screening tool. Positive results by ELISA require confirmation by a more specific assay – either IFA or gold-standard neutralization assays.

Detailed evaluations for many of the published assays have not been possible because of a lack of well-characterized control sera. While the specificity of most assays can be assessed using sera of patients in non-endemic regions, diagnostic sensitivity and comparison-of-methods data are still difficult to come by. In a recent study, Park *et al.* compared the plaque reduction neutralization test (PRNT), microneutralization and pseudoparticle neutralization tests using sera of 17 patients from the South Korean MERS outbreak (Park *et al.*, 2015a). They found that the different neutralization test formats had excellent correlation with each other when testing convalescent clinical specimens.

In the first ten days after symptom onset, anti-MERS antibodies are usually undetectable irrespective of assay format (Park *et al.*, 2015a, 2015b). Therefore, serology testing is not useful in acute MERS, although the WHO includes seroconversion (confirmed by neutralization) in paired sera taken at least 14 days apart as one of the diagnostic criteria for a confirmed case ([http://apps.who.int/iris/bitstream/10665/176982/1/WHO\\_MERS\\_LAB\\_15.1\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/176982/1/WHO_MERS_LAB_15.1_eng.pdf)). The role of MERS serology, therefore, is threefold: firstly, to provisionally diagnose mild or asymptomatic MERS cases who present late with only convalescent sera available; secondly, for serosurveillance of at-risk individuals either as part of an outbreak investigation or in abattoirs where exposure to zoonotic sources may have occurred (Drosten *et al.*, 2014; Muller *et al.*, 2015; Kim *et al.*, 2016a); thirdly, for seroepidemiological studies of zoonotic sources to identify affected camel herds. Indeed, demonstration of MERS-CoV neutralizing antibodies in camel sera is one of the lines of evidence for zoonotic transmission of MERS from camels to humans (Meyer *et al.*, 2014b).

In view of the deficiencies of serological assays outlined in Table 2, most authorities recommend using at least two different assays for specific serodiagnosis of MERS. The WHO recommends using either an ELISA- or IFA-based screening assay followed by confirmatory testing of positive sera using a specific neutralization assay ([http://apps.who.int/iris/bitstream/10665/176982/1/WHO\\_MERS\\_LAB\\_15.1\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/176982/1/WHO_MERS_LAB_15.1_eng.pdf)). The CDC also adopts a two-phase approach with the first test being an IgG ELISA followed by confirmatory testing using IFA (<http://www.cdc.gov/coronavirus/mers/lab/lab-testing.html>). Microneutralization is performed on ELISA-positive, IFA-indeterminate sera for final resolution.

## Conclusions and future perspectives

The experience with MERS and other recent transnational epidemics proves that emerging infectious diseases will con-

tinue to be a major challenge in the future. Rising human populations force animals and humans into ever closer proximity increasing the risk of zoonotic transmission of novel infectious diseases. Overcrowding facilitates human-to-human transmission of these infections, both in the community and in healthcare settings. High volumes of air travel enable rapid transport of infected humans to non-endemic regions leading to major outbreaks. All these features were clearly illustrated in the recent MERS epidemic. The key to combating these threats is information sharing, constant vigilance, and efficient infection control. The diagnostic laboratory plays an increasingly important role in the early detection of infected patients enabling prompt initiation of infection control measures and appropriate patient management. However, this role is a complex one requiring a battery of tests and diagnostic algorithms as highlighted in this review. The laboratory also faces several challenges in this role, related to assay validation, reagent shortages, a lack of standard materials, protocol standardization, and quality assurance. Creation of regional and global laboratory networks under the aegis of the WHO or other organizations will be crucial to overcome these difficulties. Pooling of positive control material for serological and molecular assays in biobanks would also be valuable. This is particularly important with the increasing introduction of massively multiplexed and point-of-care tests for the diagnosis of emerging infections, which is an emerging trend in the microbiology laboratory.

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