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Infectious Middle East Respiratory Syndrome Coronavirus Excretion and Serotype Variability Based on Live Virus Isolates from Patients in Saudi Arabia

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The newly emerged Middle East respiratory syndrome coronavirus (MERS-CoV) has infected at least 1,082 people, including 439 fatalities. So far, no empirical virus isolation study has been done to elucidate infectious virus secretion or serotype variability. Here, we used 51 respiratory samples from 32 patients with confirmed MERS-CoV infection for virus isolation in Vero B4 and Caco-2 cells. We found Caco-2 cells to significantly enhance isolation success over routinely used Vero cells. Isolation success correlated with viral RNA concentration and time after diagnosis as well as with the amount of IgA antibodies secreted in respiratory samples used for isolation. Results from plaque reduction neutralization assays using a representative range of serum samples and virus isolates suggested that all circulating human MERS-CoV strains represent one single serotype. The choice of prototype strain is not likely to influence the success of candidate MERS-CoV vaccines. However, vaccine formulations should be evaluated for their potential to induce IgA.

Middle East respiratory syndrome (MERS) is an acute respiratory disease first identified in September 2012 in a patient from Jeddah, Kingdom of Saudi Arabia (KSA) (1). It is caused by the MERS coronavirus (MERS-CoV). In most cases, infections have directly or indirectly been traced to the Arabian Peninsula. At least 1,082 human cases are known, including 439 fatalities (2). Clinical symptoms include fever, diarrhea, and mild to severe respiratory symptoms (3). Despite the low rate of transmission in the community, hospital outbreaks can be dramatic and cause a huge secondary burden on health care systems (3, 4). Data on the infectivity of virus excreted from different body compartments are needed to improve hospital infection control. The few available studies on virus excretion have been limited in size and have relied on reverse transcriptase PCR (RT-PCR) (5, 6). However, measuring viral RNA concentration can only provide a surrogate for infectious virus excretion because viral infectivity cannot be measured by pure quantification of viral genomes. Infectivity is additionally determined by cellular and humoral components of the body compartment from which the virus is excreted, such as IgA antibodies. Direct measurement of infectious virus excretion is best accomplished by live virus isolation in cell culture. Systematic virus isolation studies can provide important additional information, such as the serotype variability among isolates. Knowledge of viral serotype variability is crucial to determine if antibodies derived from a previous MERS-CoV infection or a potential vaccine can protect from reinfection. The currently circulating viruses are all highly similar to each other in their spike protein, against which most neutralizing antibodies are directed (7, 8). However, there are a number of other surface proteins that might be targeted by neutralizing antibodies, which is best determined empirically. Here, we aimed to study viral infectivity and IgA excretion as well as serotype variability in a sufficiently large number of patients with acute or recent MERS-CoV infection.

MATERIALS AND METHODS

Patients. Patients under study were diagnosed with MERS between February and June 2014 at the Prince Sultan Military Medical City (Riyadh, Kingdom of Saudi Arabia). Patient age was 24 to 90 years with a median age of 66 years. Seventy-five percent of patients were male. These patients were part of a larger observational, single-center trial aimed at the determination of virological parameters during MERS-CoV infection (4). A regimen to collect, store, and transport original clinical samples under continuous cold chain conditions (storage at −80°C, shipment in dry ice transport containers received intact) was implemented to facilitate a systematic study of virus isolation. A total of 51 samples from 32 patients were subjected to virus isolation. From a cross-sectional population-wide serosurvey in KSA, three serum samples with clear anamnestic MERS-CoV infection were used (9).

Virus isolation. Five hundred microliters Vero B4 (DSMZ-AC33) cells per well was seeded on a 24-well plate at 3×10^5 cells/ml in Dulbecco

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modified Eagle medium (DMEM) containing 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, 1% penicillin-streptomycin, and 10% fetal calf serum (FCS; all Gibco, Darmstadt, Germany) 1 day prior to infection. Caco-2 cells (ATCC HTB-37) were used at a concentration of 4×10^5 cells/ml and seeded 2 days prior to infection. All patient materials were diluted in 5 ml OptiPro serum-free medium (Gibco) to reduce viscosity and improve pipetting. Two hundred microliters diluted patient material per 24-well plate was used to inoculate cells for 1 h at 37°C. Afterward, cells were washed three times with phosphate-buffered saline (Gibco) and supplied with 700 μl fresh medium composite as described above—except for a reduced FCS content of 2%—with or without 1% amphotericin B and further incubated. Cells were checked daily for cytopathogenic effects. Upon observation of cytopathogenic effects, and otherwise every second day, 50 μl of cell culture supernatant was taken to monitor the increase of MERS-CoV RNA by real-time RT-PCR using the MERS-CoV upE assay as described previously (10). The supernatant of isolation-positive wells was harvested, centrifuged at 200 × *g* for 3 min to remove cell debris, diluted 1:2 in OptiPro (Gibco) containing 0.5% gelatin for storage, and used to infect Vero B4 cells for the production of virus stocks. All produced virus stocks were quantified by plaque titration.

Virus strains. Virus strains used for plaque reduction neutralization assay were chosen to represent 3 major clades within the MERS-CoV species. Strain Najran-351 represents the Hafr_Al_Batin_1 clade, strain Jeddah-10306 represents clade Riyadh_3, while EMC/2012 is a member of clade A. Together, these clades cover the whole variability of MERS-CoV as observed in all human cases.

Plaque titration and plaque reduction neutralization assay. Titration of MERS-CoV was done as described previously (11). Vero B4 cells (3×10^5 cells/ml) were seeded 16 h prior to infection with a serial dilution (in OptiPro) of virus-containing medium for 1 h at 37°C. After removing the inoculum, cells were overlaid with 2.4% Avicel (FMC BioPolymers, Brussels, Belgium) 1:2 diluted in 2× DMEM supplemented with 2% sodium pyruvate, 2% nonessential amino acids, 2% L-glutamine, 2% penicillin-streptomycin, and 20% FCS. Three days after infection, the overlay was discarded and cells were fixed in 6% formaldehyde and stained with a solution containing 0.2% crystal violet, 2% ethanol, and 10% formaldehyde (all from Roth, Karlsruhe, Germany).

For plaque reduction neutralization assays, 100 μl of a virus solution containing 60 to 80 PFU was incubated with 100 μl diluted patient serum for 1 h at 37°C prior to infection of Vero B4 cells as described above.

Recombinant enzyme-linked immunosorbent assay. IgA and IgG detection in the respiratory tract and serum samples was done using a recombinant enzyme-linked immunosorbent assay (recELISA; EUROIMMUN AG, Lübeck, Germany) based on the S1 subunit of the MERS-CoV spike protein purified from HEK-293T cells as described elsewhere (12). All samples were diluted 1:100 before applying 100 μl per well and incubating for 30 min at room temperature. Secondary detection was performed using either anti-human IgA or anti-human IgG antibodies conjugated with horseradish peroxidase as described in the manufacturer's instructions. Optical density (OD) was measured at 450 nm and 630 nm for background correction with the Synergy 2 multi-mode reader (BioTek, Bad Friedrichshall, Germany). Results are given either in absolute OD (IgA) or as OD ratios determined by dividing individual OD values with a calibrator serum (IgG).

RESULTS

MERS-CoV isolation from patient material. We studied clinical samples from 32 patients with confirmed MERS-CoV infection who were hospitalized in Riyadh, Kingdom of Saudi Arabia. Initial diagnostic tests were done by RT-PCR at Riyadh regional laboratory using upE and ORF1A assays as described previously (10). The clinical courses and their correlation with virological data will be described separately (V. M. Corman, submitted for publication).

From those 32 patients whose samples could be stored and shipped under continuous cold chain conditions, all appropriate respiratory samples were subjected to virus isolation attempts in Vero B4 cells that are commonly used for cultivation of MERS-CoV. Due to our own preliminary experience, we also used the human colon carcinoma cell line Caco-2 as an alternative virus isolation cell line. Out of 51 samples from 32 different patients, a total of 21 MERS-CoV isolates were obtained. As two patients each yielded 2 virus isolates due to using samples of the same patient taken at different time points, this represented viruses from 19 patients. No virus could be isolated from any of the 4 upper respiratory tract samples, while isolation success for the 47 lower respiratory tract samples was 48.6% in endotracheal aspirates and 33.3% in sputa (Fig. 1A).

Only 9 of the 21 MERS-CoV isolates were obtained on Vero B4 cells, the cell line used for isolating the first MERS-CoV strain EMC/2012 (1). In contrast, 20 isolates were obtained in Caco-2 cells. There was only one isolate that grew exclusively in Vero B4 cells but 12 that grew exclusively in Caco-2 cells. The proportion of successful isolates was significantly superior in Caco-2 cells over Vero cells (45.5% versus 19.1%; Fisher's exact test, $P = 0.013$). The use of Caco cells resulted in a general, sample type-independent enhancement of isolation success. Four of 4 isolates from sputa and 16 of 17 isolates from endotracheal aspirates were grown in Caco cells while only 1 isolate from sputa and 8 isolates from endotracheal aspirates grew in Vero B4 cells.

Factors with potential influence on virus isolation success were analyzed, including viral load in RT-PCR, days after initial diagnosis at the time of sampling, and IgA antibody titers in respiratory samples used for virus isolation and IgG antibody titers in patients' serum samples from corresponding days. In general, viral load was significantly higher in samples that yielded an isolate than that in samples from which isolation failed (*t* test, $P < 0.0001$). There was no significant correlation between viral load and days after diagnosis (Pearson's $r = -0.038$, $P = 0.8$). The proportion of successful isolates was 66.7% at RNA concentrations above 10^7 copies/ml but only 5.9% below this value (Fig. 1B). In samples taken from patients within 5 days after diagnosis, more than half (58.6%) of samples yielded an isolate while only 22.2% of the samples yielded isolates if taken later (Fig. 1C). Because the reduced isolation success in later stages of the infection might be a result of rising antibody titers, IgA antibodies in respiratory tract samples used for virus isolation as well as IgG antibodies in sera from the same patient on the same day were determined by recombinant ELISA. The optical density values from IgA and IgG measurements correlated significantly (Fig. 1D; Pearson's $r = 0.66$, $P < 0.001$). The general levels of IgA and IgG were substantially lower in samples yielding an isolate than those of samples from which isolation failed (*t* test, $P = 0.012$ and $P < 0.001$, respectively).

MERS-CoV serotype variability. Even though it is known that the amino acid variability within the viral spike protein is extremely low between MERS-CoV strains (4), there might be other factors that determine the virus's immunogenicity that can only be evaluated using replicating virus in neutralization assays. Strains for characterization of viral serotypes were chosen to represent three major phylogenetic lineages of MERS-CoV as defined by Cotten et al. (13).

Serum samples from 3 patients with recent infection (278, 639, 1,057) and serum samples from 3 subjects with anamnestic infection (884, 4,880, 8,692) were selected. The subjects with anamnestic infec-

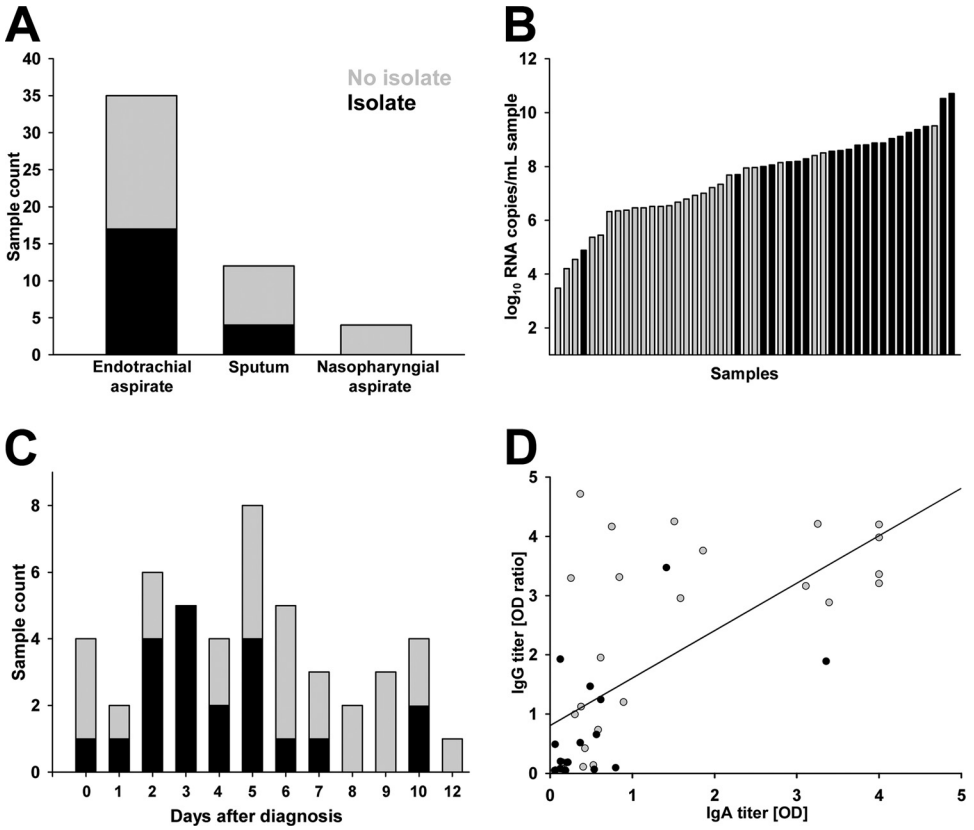

FIG 1 Parameters determining isolation success of MERS-CoV from patient material. (A) Virus isolates were only obtained from patient samples of the lower respiratory tract. Isolation success was strongly dependent on the amount of viral RNA copies in patient samples as measured by PCR (B), days after diagnosis at which samples were taken (C), and (D) the amount of IgA and IgG antibodies in the samples itself and the corresponding patients' serum samples. (D) The amount of IgA and IgG corresponded significantly across all samples (Pearson's $r = 0.66$, $P < 0.001$).

tion were not aware they had overcome MERS-CoV infection. However, they showed unambiguous serological evidence of past MERS-CoV infection in a cross-sectional population-wide serosurvey in KSA (9). Virus strain Najran-351 and serum 639 were obtained from the same patient, providing a matched pair of serum and virus against which all other combinations can be compared. There were no obvious differences in neutralization efficiency between virus strains or between serum samples (Fig. 2).

DISCUSSION

We have conducted the first study of MERS-CoV infection based on virus isolation, providing information on infectious doses in patient material as well as serotype variability of human MERS-CoV strains. We introduce a new and highly sensitive cell culture model for MERS-CoV cultivation and, for the first time, provide data on secretion of mucosal IgA antibodies against MERS-CoV.

Our data show that isolation of MERS-CoV is most successful when using samples from the lower respiratory tract. This finding is in line with the assumption that MERS-CoV mainly replicates in the lower respiratory tract where it causes severe disease (12, 14). Caco-2 cells should be preferred over other cell lines for isolation of MERS-CoV, as they have already been found to enhance isolation success for a number of known respiratory viruses (15).

Viral isolation success provided a useful correlate of infectious virus shedding. Next to a clear correlation with RNA concentration, our analyses revealed a decrease of isolation success when disease was present for a longer period of time. As there was no significant correlation between viral load and time after diagnosis for those samples tested in this study, factors other than RNA concentration might confer an additional influence on the infectivity of clinical samples. One obvious possibility to explain this observation was the presence of anti-MERS-CoV IgA antibodies in respiratory secretions after seroconversion. By adapting an ELISA for IgA detection, we could confirm that IgA secretion is quantitatively correlated with IgG production in serum and that the presence of IgA indeed influences the rate of successful virus isolation. IgA may have prognostic value if used as a routine diagnostic in MERS-CoV patients and may influence the potential for reinfection. For instance, it has been described for influenza virus that the level of IgA antibodies in respiratory secretions has an influence on infection rates and virus-associated illness (16). As the presence of mucosal IgA might have a more direct influence on the susceptibility against infection with MERS-CoV than that of serum IgG, IgA production in secretions could be included in regimens to evaluate the potency of candidate vaccines against MERS-CoV.

Using the virus strains isolated in this study, we were able to comparatively study the neutralizing ability of individual serum samples to a representative panel of MERS-CoV strains. We used a sensitive plaque neutralization assay format that identified even subtle differences in serum neutralization. The use of whole viruses instead of spike-based pseudotype assays ensured that all

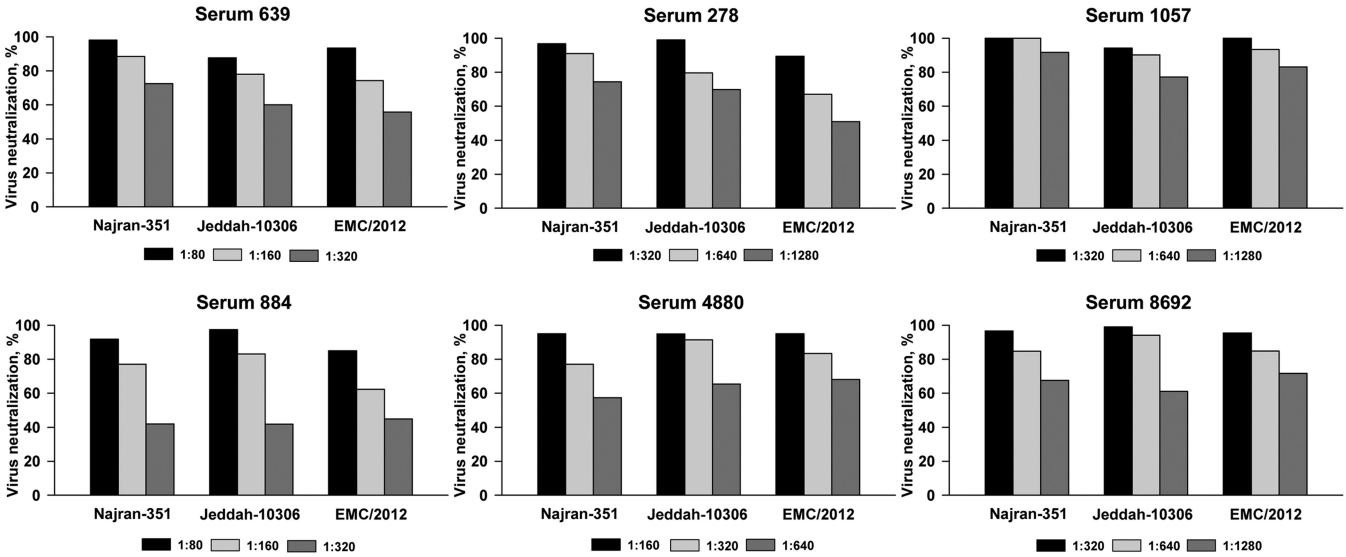

FIG 2 Plaque reduction neutralization assay of three MERS-CoV isolates. MERS-CoV isolates Najran-351, Jeddah-10306, and EMC/2012 were neutralized with 3 serum samples from recently seroconverted patients (278, 639, 1,057) and 3 serum samples from patients with anamnestic MERS-CoV infection (884, 4,880, 8692). Serum 639 was taken from the patient from which MERS-CoV Najran-351 was isolated, providing a reference for virus neutralization by a homologous serum.

viral proteins were taken into account in the test. Our studies found no relevant variation between the tested isolates, representing all circulating human MERS-CoV strains. With all serum samples, the quantitative deviations among the tested viruses' susceptibilities to serum neutralization were insufficient to define more than one distinct serotype because differences in plaque-reducing activity were less than 4-fold. A 4-fold difference would minimally be expected in different serotypes according to the common definition of significant neutralization titer differences. All of the presently circulating strains would therefore be interchangeable and equivalent for use in candidate vaccine formulations.

Taken together, this study showed that Caco-2 cells should be preferred for MERS-CoV isolation from clinical samples, IgA antibodies are produced in respiratory tract secretions and protect against MERS-CoV, and presumably all MERS-CoV variants currently circulating in the human population form only one serotype.

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