

Viral shedding and antibody response in 37 patients with MERS-coronavirus infection

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40-word summary:

A study of viral load, viral shedding, and immune response in 37 cases of MERS-coronavirus infection. Virus was not eliminated from the respiratory tract upon development of neutralizing serum antibodies. Vaccination strategies should not be restricted to eliciting neutralizing antibodies.

Abstract

Background

The MERS coronavirus causes isolated cases and outbreaks of severe respiratory disease. Essential features of the natural history of disease are poorly understood.

Methods

We studied 37 adult patients infected with MERS-CoV for viral load in the lower and upper respiratory tract (LRT and URT), blood, stool, and urine. Antibodies and serum neutralizing activities were determined over the course of disease.

Results

199 LRT samples collected during the 3 weeks following diagnosis yielded virus RNA in 93% of tests. Average (maximum) viral loads were 5×10^6 (6×10^{10}) copies per mL. Viral loads (positive detection frequencies) in 84 URT samples were 1.9×10^4 cop/mL (47.6%). 33% of all 108 sera tested yielded viral RNA. Only 14.6% of stool and 2.4% of urine samples yielded viral RNA. All seroconversions occurred during the first 2 weeks after diagnosis, which corresponds to the 2nd and 3rd week after symptoms onset. IgM detection provided no advantage in sensitivity over IgG detection. All surviving patients, but only slightly more than half of all fatal cases, produced IgG and neutralizing antibodies. The levels of IgG and neutralizing antibodies were weakly and inversely correlated with LRT viral loads. Presence of antibodies did not lead to the elimination of virus from LRT.

Conclusions

The timing and intensity of respiratory viral shedding in MERS patients closely matches that of Severe Acute Respiratory Syndrome (SARS) patients. Blood viral RNA does not seem to be infectious. Extra-pulmonary loci of virus replication seem possible.

Neutralizing antibodies do not suffice to clear the infection.

Background

The Middle East Respiratory Syndrome Coronavirus (MERS-CoV) was first isolated in 2012 in Saudi Arabia [1]. Since 2012, at least 1,595 laboratory-confirmed cases of MERS-CoV infection, mostly with respiratory tract illness, have been reported. 571 of these were fatal [2]. Known cases and outbreaks have been linked to countries in the Arabian peninsula [3]. Large nosocomial outbreaks, such as in Jeddah/KSA in 2014 and the Republic of Korea in 2015, have demonstrated the potential of the virus to spread in health care settings [4-6].

Due to the sporadic nature of the disease, with cases and small outbreaks distributed over a wide geographic area, investigation of the natural history of infection has been limited. Except individual case descriptions, chronological data summarizing the main viral diagnostic parameters, such as viral load or antibodies, are lacking. Better knowledge of the kinetics of viral shedding from different body regions could help prevent nosocomial transmission and inform clinical management. Knowledge of serological features, such as the kinetics of antibody production, could guide decisions regarding diagnostic protocols and provide essential information regarding immunity and virus elimination. Quantitative data, such as viral loads and antibody titers, could enable comparisons with related diseases. In particular with SARS, for which studies of natural history were conducted in the aftermath of the 2002-2003 epidemic [7].

Materials and Methods

Patients and samples

Patients were seen during a hospital-associated outbreak between March 5th and May 1st, 2014. There was no prospective planning of statistical power. Patients were selected

for MERS-CoV testing by RT-PCR based on general clinical condition, oxygen saturation, and their need for invasive or non-invasive ventilation. Samples of patients who tested positive were taken at least daily, starting from 0 to 7 days after initial submission of samples for MERS-CoV diagnosis. The day of the first sample tested positive in RT-PCR was defined as the day of diagnosis. The mean delay between first positive sampling and return of laboratory results was 3 days. Stored samples, for retrospective analysis, were not available.

Specimens were taken from tracheal secretions via suction catheters, from the throat and the eyes using sterile swabs, and from urine and stool via sterile containers.

Baseline information on enrolled patients is provided in **Supplementary Table S1**.

Institutional review board approval was obtained from the Research Ethics Committee at Prince Sultan Military Medical City.

MERS-CoV RT-PCR testing

Real-time RT-PCR was performed on RNA extracts using the upE and ORF1A target genes as described in [8, 9]. Raw RNA concentrations were transformed to absolute viral loads by conversion factors, according to sample type (**Supplementary Table S2**).

MERS-CoV isolation in cell culture

Virus isolation, with increased sensitivity via the use of CaCo2 human colon carcinoma cells, was performed as described in [10].

MERS CoV serology

Recombinant enzyme-linked immunosorbent assay (ELISA)

A recombinant ELISA assay (Anti-MERS-CoV ELISA IgG, Euroimmun, Lübeck/Germany) was based on soluble MERS-CoV spike protein S1 domain expressed in HEK-293T cells [11]. The test was conducted as described previously [12, 13]. Sera were tested twice and the arithmetic mean of the two measurements was used.

IgM immunofluorescence assay (IgM-IFA)

Detection of IgM antibodies was done using immunofluorescence slides carrying Vero cells infected with full MERS-CoV, as described in Corman et al. [9]. These were converted into a homogenous reagent format by an *in vitro* diagnostics manufacturer (Anti-MERS-CoV-IIFT; Euroimmun). All sera were depleted of IgG antibodies using Eurosorb (Euroimmun) reagent according to manufacturer instructions.

Serum neutralization assay

A MERS-CoV microneutralization test (NT) was performed as described in [13-15]. Predilution before setting up log₂-dilution series was 1:10, defining 1:20 as the lowest possible significant titer for categorizing a sample as positive.

Statistical analyses

Statistical analyses were done with the help of the SPSS software (version 22). In all cases, correlation analyses and preliminary multiple regression analyses were conducted to exclude confounding due to patient age or disease duration.

Results

Patients' characteristics

To determine kinetic virological parameters in MERS-CoV infection, we followed 37 hospitalized patients. Mean age was 63 years (range 24-90 years). 73% of patients were male. MERS-CoV infection had been established in all cases by RT-PCR. 65% of all patients died during the course of study.

Sequencing of full or partial genomes from 35 of the study patients revealed the existence of at least 6 closely-related virus lineages (**Supplementary Figure S1** and **Supplementary Table S1**). Some sequences had already been seen in an earlier study [5]. Patients belonged to at least 3 nosocomial transmission clusters. Three cases could not be associated with clusters.

At time of positive diagnosis, patients had spent 11 days in hospital on average, with a maximum of 108 days. Only 20 of the 37 patients had been hospitalized for less than a week. Because of the unresolved timing of transmission events in nosocomial clusters and the existence of co-morbidities in most patients, it was impossible to determine the day of onset of symptoms in the majority of patients. Unambiguous knowledge of the day of onset of symptoms was available for only 9 patients. Mean and median duration between symptom onset and admission was 3 days (range, 0-8 days). In these 9 cases, mean and median duration between onset and diagnosis was 8 days (range, 1-16 days). The mean age of the 9 cases was not significantly different from the mean age of all patients under study. To provide a common point of reference in the clinical course of all

patients, the day of diagnosis (day of first RT-PCR positive sample) was defined as day zero in the subsequent analyses.

823 specimens from the 37 patients were tested, including 661 tests for viral load in 6 different sample categories (**Supplementary Table S2**). Because of the variable latency between diagnosis and enrolment, clinical samples were not evenly distributed over patients' courses of disease (**Supplementary Figure S2**).

Cross-sectional virus RNA detection and courses of viral load

Absolute viral RNA concentrations and positive proportion of samples were determined in 661 samples. Data are illustrated in **Figure 1** and **Supplementary Table S2**. LRT samples had the highest viral loads, up to 6.3×10^{10} copies/mL (mean 5.01×10^6). Average viral loads in all other sample types were significantly lower (two-tailed T-test, $p < 0.0001$ for all comparisons). Virus isolation trials using the six stool samples with the highest RNA concentration had negative outcomes.

Almost half of all sera showed detectable viral loads during the first week after diagnosis (25 of 51 sera tested). Virus isolation from 20 viraemic serum samples (10 with, and 10 without neutralizing antibodies) failed, in spite of a highly optimized protocol [10].

There was an inverse correlation between *in vitro* serum neutralization activity and viremia in 45 sera (Pearson's $R = -0.31$, $p < 0.03$). However, viral RNA and neutralizing antibodies were co-detected in several cases, suggesting that the detected viral RNA may only in part represent infectious virions (**Figure 2A**). Concentrations of RNA in serum did not correlate significantly with those in LRT samples collected on the same day

(n=31 pairs of serum and LRT samples; Spearman's correlation, $p=0.08$). Data are shown in **Figure 2B**.

Distributions of average LRT viral load per patient were summarized in three subsequent time windows, as shown in **Figure 3**. In particular, during the first five days after diagnosis, average viral loads were not normally distributed but had a skewed distribution with a preponderance of patients with high viral load. Of note, the 17 patients in the two highest viral load categories (right-most columns in the top panel in **Figure 3**) did not show a significantly increased proportion of fatal outcomes (chi-squared test, $p=0.12$).

The average viral load during the first week after diagnosis was 5×10^7 copies/mL in fatal cases and 3.9×10^6 copies/mL in survivors (2-tailed T-test, $p < 0.007$). Divergence of viral loads between survivors and fatal cases was more pronounced in the second week (1.6×10^5 and 7.8×10^6 copies/mL, respectively, $p < 0.0006$).

Time course of antibody production

Serological courses could be followed for 35 patients. Almost half of these (n=17) were already reactive (via ELISA) on the day of diagnosis. Among 27 patients with complete serological follow-up during the first week after diagnosis, 89% (n=24) had antibodies by end of the week in both ELISA and neutralization tests. 18 of these patients tested positive for IgM by IFA (titers $>1:10$) by end of the week. Only one of the IgM-positive patients did not have a concomitant positive ELISA result by end of the week. All of the 12 patients with 2 weeks of serological follow-up seroconverted (ELISA and

neutralization tests). Eleven of these 12 patients developed IgM detectable by IFA. Antibody kinetics averaged over all samples and tests are summarized in **Figure 4**.

Information on outcome was available for 34 patients with serological follow-up. All 12 patients who survived their infection showed seroconversion (ELISA and NT tests) during the first week. All developed IgM antibodies concomitantly. Among 22 fatal cases, 14 showed seroconversion by ELISA prior to death, the latest seroconversion occurring by day 11 post-diagnosis. Twelve of 22 fatal cases developed neutralizing antibodies and 11 developed detectable IgM. Antibody levels (ELISA OD and log₂ NT titers) during the first week after diagnosis were not significantly different between surviving and fatal cases (2-tailed T-test, p=0.8). During the second week after diagnosis, the average ELISA OD values in survivors were significantly higher than in fatal cases (2.9 vs. 2.1, 2-tailed T-test, p<0.02). Also, average NT titers were higher in survivors during week 2, but with less significant discrimination (2^{7.5} vs. 2^{5.4} in survivors and fatal cases, 2-tailed T-test, p<0.06).

Correlation of ELISA antibodies, NT titers, and viral loads

ELISA OD values and log₂ NT titers were compared against log₁₀ viral loads in LRT samples. From 30 patients, ELISA and viral load data were available based on matched serum and respiratory tract samples taken on the same days (198 matched data pairs, covering days 0 to 17 post-diagnosis). Because of workload and biosafety, the number of NT assays had to be restricted. However, combined ELISA, NT, and viral load data were available from 26 patients, with 91 matched datasets that covered days 1-17 after diagnosis. **Supplementary Figure 3** summarizes the distribution of matched samples over time. Pearson's test identified significant linear correlation between antibodies and

\log_{10} viral loads [ELISA, $R=-0.6$; NT, $R=-0.51$; $p<0.001$ in both analyses]. However, plots of ELISA and NT antibody results in matched sample pairs yielded no evidence of mutually exclusive occurrence of virus and antibodies (**Figures 5A and B**).

Discussion

We studied quantitative viral excretion and serum antibody kinetics of a substantial group of hospitalized patients infected with MERS-CoV. The time of diagnosis chosen as a chronological reference point represents the time when an infection is suspected in hospitalized patients, or when outpatients report to hospitals due to worsening symptoms. Even though the day of onset was unknown in many of the studied cases, the presented virological courses represent the typical situation encountered during MERS treatment in hospital settings.

By providing absolute quantitative measures of virus excretion, we can for the first time compare results between MERS and SARS - a disease that is now thought to have involved higher pandemic potential than MERS [16]. In our patients and elsewhere, the LRT was found to be the main source of MERS-CoV excretion [17]. Unfortunately, there are few data on LRT virus excretion for SARS-CoV, because endotracheal sampling was widely discouraged during SARS outbreaks to avoid nosocomial risks. We have shown, in one of the few available studies, that SARS-CoV was excreted from the LRT at mean concentrations of $1.2-2.8 \times 10^6$ copies per mL, reaching a maximum of 10^{10} copies per mL [18]. That study was conducted in a similar clinical context (a treatment center in Singapore) with similar timing of samples and clinical courses. Determination of viral load was performed by the same laboratory, using equivalent calibrators and conversion factors. From this comparison we can conclude that average and peak LRT viral loads in

MERS are equal to those in SARS [18]. More data are available for URT viral loads in SARS patients. Peak URT RNA concentrations can reach up to 5×10^5 copies per sample between days 7 to 10 after onset [18, 19]. The corresponding number for MERS (peaking at 4.1×10^6 copies per sample), is equivalent or higher. Also, the 47.6% proportion positive for MERS in URT exceeded the 38% proportion positive in 98 URT samples for SARS patients in Hong Kong [19].

The timing of excretion is more difficult to compare, as many patients in studies on SARS were outpatients who entered hospital because of their SARS infection, while our MERS patients were mostly inpatients [20]. In SARS cases that occurred before Hong Kong authorities started active community contact tracing, the average time from symptom onset to admission was 4 to 7 days [20]. Because case definitions and diagnostics were well established during SARS in Hong Kong, diagnostic samples would have been taken immediately upon admission. This can be aligned with the timing in our study based on a sub-cohort of patients for whom an onset of symptoms could be reliably determined. In these patients, the time from onset of symptoms to the initiation of laboratory diagnostics (8 days) was similar to that of early Hong Kong SARS cases. The shedding peak in SARS patients occurred after ca. 10-12 days from symptom onset, which is very similar to the shedding maximum observed in our study, under the assumption that the day of first diagnosis in our MERS cases plausibly falls around the 8th day of symptoms (i.e., direct after admission) [21, 22]. In summary, neither the virus concentration nor the timing of respiratory shedding provide an explanation for differences in transmissibility between MERS-CoV and SARS-CoV. Experimental data suggest a higher sensitivity of MERS-CoV to respiratory epithelium-associated type I interferon, which

might provide a plausible explanation for its lower transmissibility in comparison with SARS-CoV [23]. Many other explanations are possible, however.

The detection of MERS-CoV in serum is another similarity with SARS. Up to 79% of serum samples were found to contain SARS-CoV RNA during the first week of illness, and around 50% during the second week [24-26]. These numbers match our observations for MERS. Free viraemia seems unlikely as a cause of nosocomial transmission of MERS, as no infectious virus was isolated from serum. There is evidence of SARS-CoV replicating in PBMC, macrophages, and dendritic cells, albeit at low levels [27-30]. In the present study, the absence of correlation of serum viral load with LRT viral load points to potential extra-pulmonary replication. Viremia despite the presence of neutralizing antibodies indicates a body region that is not accessible to neutralizing antibodies but which releases virus into the blood. SARS-CoV has been shown to replicate in several extra-pulmonary organs without evidence of tissue damage [27]. One organ implicated in MERS is the kidney. Kidney failure has been reported in many cases, and MERS-CoV was originally isolated in kidney cells which express DPP4, the MERS-CoV entry receptor [11]. However, earlier healthcare-associated outbreaks have been centered near dialysis centers and nephrology departments, and have affected metabolically compromised patients who are predisposed to kidney failure when suffering from systemic disease affecting blood pressure and circulation. While SARS-CoV patients showed viral RNA detection rates up to 50% in urine [7, 22], we rarely found urine-associated MERS-CoV RNA in this study. As in SARS, kidney failure in MERS patients might well be explained by severe inflammatory reaction combined with the administration of potentially nephrotoxic drugs during intensive care [31].

Nevertheless, it will be highly important to conduct post mortem examinations, particularly of the kidney, of patients who die during acute MERS-CoV infection.

A clear difference from SARS was the detection of viral RNA in stool. In SARS, the RNA prevalence in stool samples was so high that testing of stool was proposed as a reliable and sensitive way to routinely diagnose the disease [7, 22]. Active replication in the gut with live virus isolation has been demonstrated [32]. For MERS, we found stool-associated RNA in only 14.6% samples, with rather low RNA concentration and had no success in isolating infectious virus. Based on these data, fecal excretion may not have played a relevant role for nosocomial spread of MERS-CoV among the patients under study.

As in SARS, MERS-CoV nosocomial transmission was repeatedly ascribed to the potential of some patients to act as super-shedders or super-spreaders [6, 20]. Our analysis of viral loads particularly in the early acute phase of disease, supports the existence of a limited number of patients with extraordinarily high viral loads. As these patients were not more likely to die of the infection, they might not have had more severe symptoms, and thus might have been able to engage in social contact despite their disease.

The course of MERS antibody development resembles that of SARS. Patients infected with SARS seroconverted during weeks 2 and 3 after onset [7]. Most of the patients studied here had already seroconverted during the first week after diagnosis, which putatively represents the second week after onset. As in SARS, IgM was not detected earlier than IgG, which limits its diagnostic utility, in particular when considering that IgM against more prevalent HCoVs may cross-react with MERS-CoV [12, 33]. With

current methodology, IgM testing should be restricted to cases that require proof of recent and overcome MERS-CoV infection.

Information on the prognostic value of antibody response in SARS is less clear. In the present study on MERS, 36% (ELISA) and 45% (NT) of fatal cases failed to mount an antibody response prior to death. However, differences became apparent only in the second week after diagnosis, pointing to only a weak protective effect against lung disease. The development of antibodies in serum was not followed by a rapid elimination of viral RNA from the lung. Neutralizing antibodies normally include IgA secreted in respiratory fluids and saliva. We have recently shown that anti-MERS-CoV IgA is indeed secreted in respiratory fluids [10], suggesting that the development of IgA comes too late to confer timely reduction of viral replication in infected mucosa. Based on these data, vaccines against MERS-CoV should be designed so as to include and enhance cellular immune responses.

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Declaration of interests

We declare no competing interests.

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Figure legends

Figure 1

Viral loads in MERS patients. Mean viral loads in positive-testing samples per day and specimen type. Maximum and minimum viral loads are shown as purple and cyan lines, respectively. Error bars represent standard derivations. Sample numbers and proportion of positive samples are summarized in Supplementary Figure S2.

Figure 2

Correlation of serum viral RNA detection with neutralizing antibodies and viral RNA concentration in respiratory samples. A, neutralizing antibodies; B, viral RNA concentration in LRT samples. Columns in both panels show serum viral load. Empty spaces represent serum samples that tested negative for viral RNA.

Figure 3

Distribution of RNA viral loads in LRT samples in three time windows. Columns show viral loads for each patient averaged over the time windows indicated to the right of each panel. Curves represent ideal normal distributions based on sample means and variance.

Figure 4

Kinetics of antibody production

The red line shows mean IgG- titer, represented as OD ratios obtained from S1-ELISA .

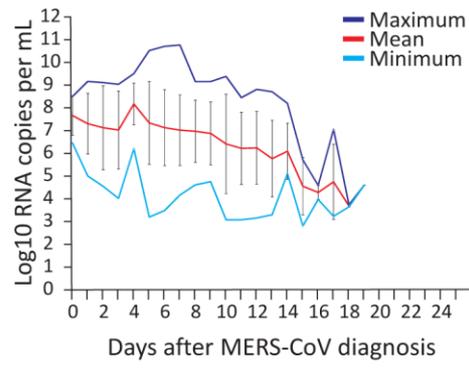
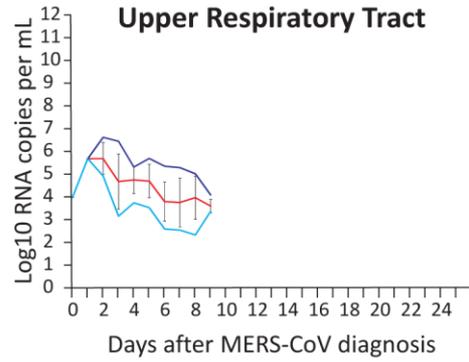
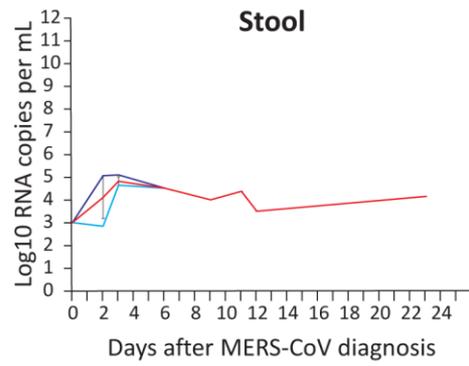
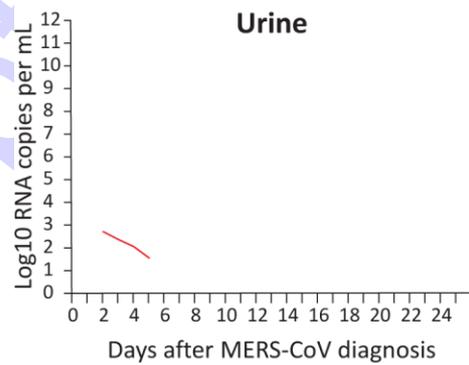
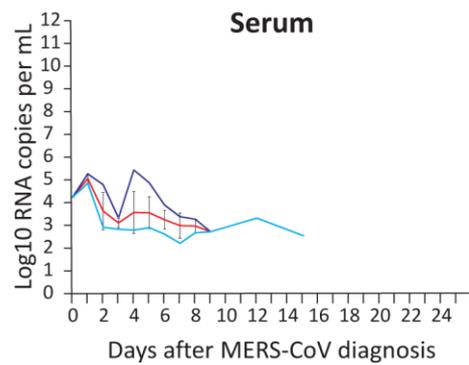
The yellow line shows mean IgM-titer from an immunofluorescence test. The cyan line

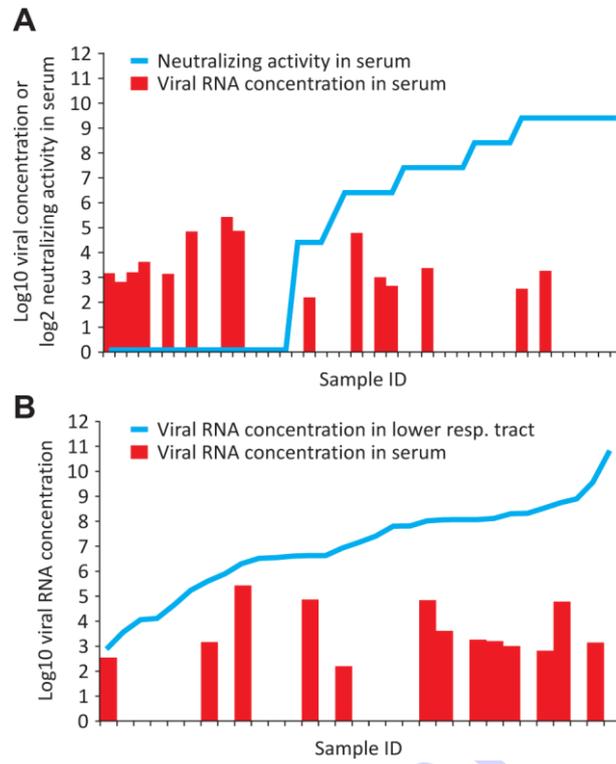
shows virus neutralization titer. Titers from each patient are averaged over successive 3-day time intervals.

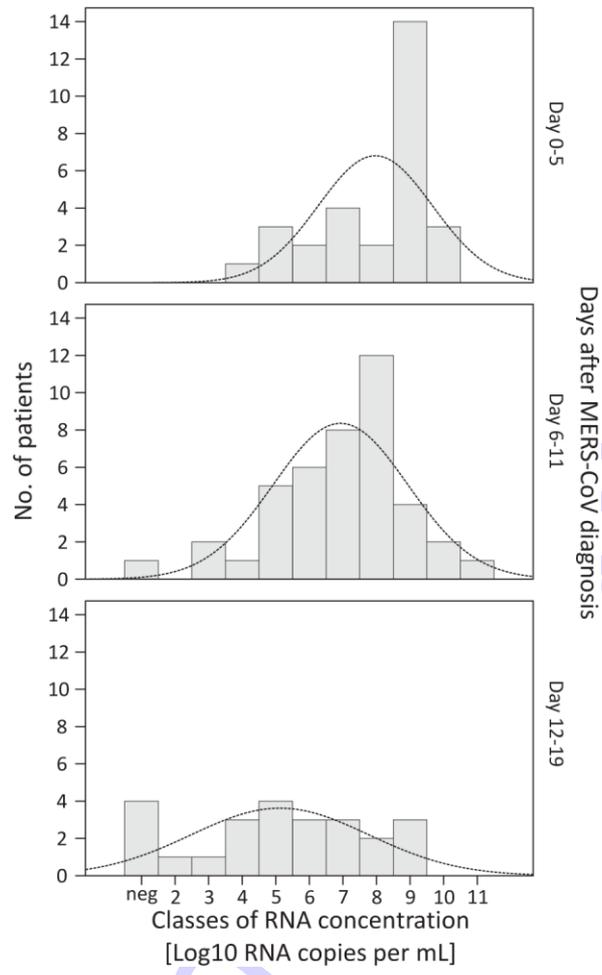
Figure 5

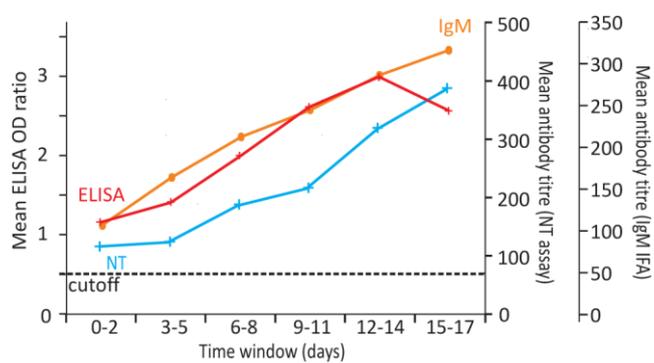
Effect of serum antibodies on LRT viral loads. This analysis is based on paired serum and LRT samples taken from the same patient on the same day. Antibodies are shown as line graphs. Viral loads in the corresponding LRT samples are shown as columns. The panels show samples sorted according to increasing levels of ELISA OD ratios (A) or neutralizing antibody titers (B). Sample numbers for this analysis are summarized in Supplementary Figure S3.

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Lower Respiratory Tract**Upper Respiratory Tract****Stool****Urine****Serum**







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