Kinetics of Serologic Responses to MERS Coronavirus Infection in Humans, South Korea

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We investigated the kinetics of serologic responses to Middle East respiratory syndrome coronavirus (MERS-CoV) infection by using virus neutralization and MERS-CoV S1 IgG ELISA tests. In most patients, robust antibody responses developed by the third week of illness. Delayed antibody responses with the neutralization test were associated with more severe disease.

Knowledge of the kinetics and clinical correlates of serologic responses to Middle East respiratory syndrome coronavirus (MERS-CoV) infection is essential for diagnosing the disease, interpreting seroepidemiologic data to define prevalence and risk factors for infection, understanding pathogenesis, and assessing a potential role for passive immunotherapy. To address this knowledge gap, we investigated serologic responses to MERS-CoV in 17 patients.

The Study

During May–June 2015, an outbreak of MERS-CoV in South Korea resulted in 186 infections and 36 deaths (*1*–*3*); the outbreak strain was a clade B MERS-CoV closely related to viruses circulating in the Middle East (*1*). Seventeen patients with reverse transcription PCR–confirmed MERS-CoV infections were included in this study; the patients were hospitalized at Seoul National University (SNU) Hospital or SNU Boramae Medical Center in Seoul, South Korea, or at SNU Bundang Hospital, in Bundang, South Korea. We investigated early serologic responses; thus, patients who were transferred to these facilities ≥14 days after illness onset were excluded from study.

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Patients' demographic and clinical profiles are shown in online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/21/12/15-1421-Techapp1.pdf). Of the 17 patients, 9 had severe disease (4 required mechanical ventilation, 4 required supplemental oxygen; 1 died) and 8 had mild disease. Serial serum samples were collected and analyzed. The study was approved by the SNU Institutional Review Board.

Antibody to MERS-CoV was detected by using the plaque reduction neutralization test (PRNT) and MERS-CoV S1 IgG ELISA (EUROIMMUN, Lübeck, Germany) (*4*,*5*) (online Technical Appendix). MERS-CoV EMC was used for the PRNT assay; a 50% PRNT endpoint ($PRNT_{50}$) was used because it was more sensitive than the 90% PRNT cutoff in detecting mild infections (*6*). The ELISA was based on the recombinant spike S1 region of strain EMC because that region is sufficiently divergent between different coronavirus species and expected to lead to less cross-reaction (*4*).

Overall, serologic responses were robust and were detected in most patients by week 3 of illness (Figure). Of the 12 patients who had serum samples tested beyond day 18 of illness, 9 had $PRNT_{50}$ titers >1:320 by day 21 and 2 more had titers >1:320 by day 28. Patient L, a 56-year-old woman with no underlying disease, had weakly positive $PRNT_{50}$ (1:20) and borderline ELISA responses (optical density ratio 1.0), even at day 32 of illness. A chest radiograph showed she had lung infiltrates, but she was not oxygen-dependent and was not administered antiviral drugs or corticosteroids; her recovery was uneventful.

Antibody responses in patient A, a 38-year-old man, were delayed up to 16–18 days after illness onset (Figure). He required mechanical ventilation, and on illness day 14, he was given convalescent-phase plasma (200 mL; antibody titer unknown) from the outbreak index patient's wife (*1*). The next day, antibody responses were undetectable in the patient's serum by PRNT or ELISA. By day 18, he had a $PRNT_{50}$ antibody titer of 1:10 and a negative ELISA response; strong antibody responses developed from day 21 onwards. We hypothesize that the data from the first 21 days of illness represent his own serologic response, unaffected by the passive transfusion with convalescent-phase plasma on day 14; thus, these data were included in the analysis. Patient A was given a second infusion of

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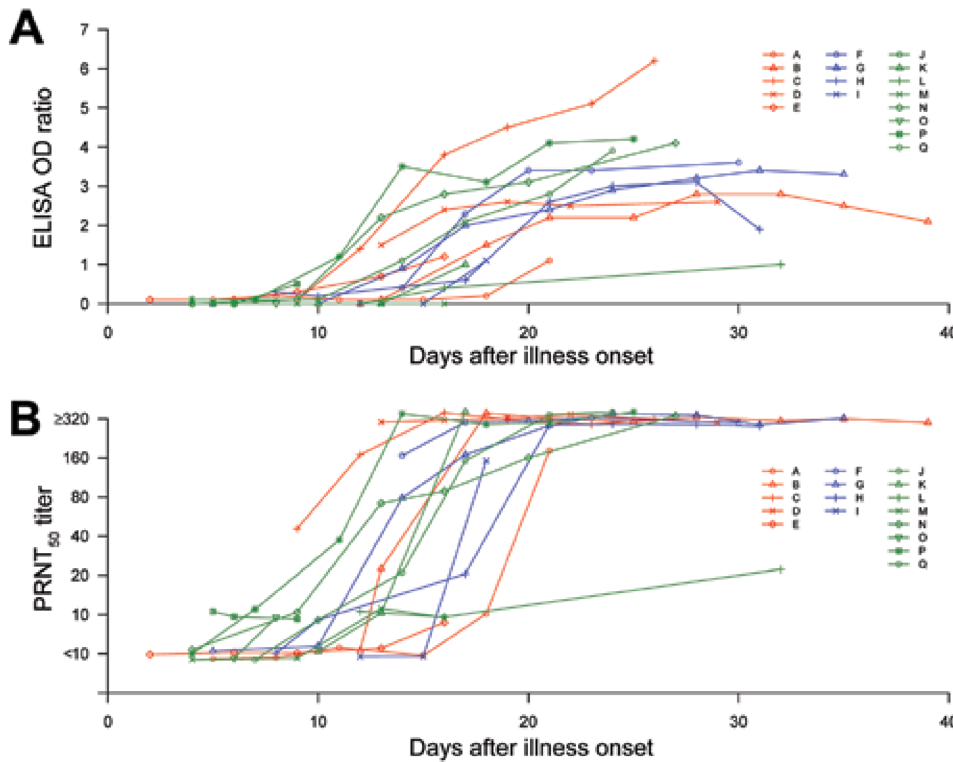

Figure. Antibody response kinetics in patients with Middle East respiratory syndrome coronavirus (MERS-CoV) infection, by days after illness onset, as determined by using a 50% endpoint plaque reduction neutralization test ($PRNT_{50}$) (A) and an S1 IgG ELISA (B). Key indicates individual patients; red inicates patients with severe illness requiring mechanical ventilation; blue indicates patients with severe illness requiring only supplemental oxygen therapy; and green indicates patients with mild illness. For better presentation, the $PRNT_{50}$ titers have been jittered vertically (random noise added to prevent overplotting) (*7*) by adding random numbers to the titers within the range of –0.2 to 0.2 at the log scale. OD, optical density.

convalescent-phase plasma on day 24, and serologic data after day 21 were excluded from analysis.

We constructed a statistical model in which age, sex, incubation period, concomitant conditions, and therapy with corticosteroids or antiviral drugs were adjusted for disease severity. We assessed how these factors were associated with the time from illness onset to commencement of the log-phase antibody response (Table 1) and the time for the antibody response to reach a titer of 1:40 ($PRNT_{50}$) or become positive in the ELISA (online Technical Appendix Table 2). An accelerated failure model was used for a more natural interpretation of the median time from illness onset to the aforementioned antibody responses (online Technical Appendix). Because the increase in antibody titers exhibited an S-shaped pattern, we assessed the rate of change in antibody response after the commencement of the exponential phase by manually removing data from the steady state, thus restricting antibody data to the log-phase response (Table 2). A linear mixed model was used to test the potential difference in the rate of increase by the above factors (online Technical Appendix). Patients with severe disease had significant delays in the commencement of $PRNT_{50}$ antibody responses (Table 1) but had a steeper slope to the antibody response once it began (Table 2). Thus, a delayed adaptive immune response may contribute to increased severity, and passive therapy with convalescent-phase immune plasma may be clinically beneficial. In avian influenza A(H7N9) virus infection of humans, earlier antibody responses and a faster rate of increasing antibody titers were associated with milder disease (*8*), but in SARS-CoV infection, earlier antibody responses were associated with an adverse outcome (*9*).

Table 1. Associations and p values for different clinical factors with time from illness onset to commencement of log phase of antibody response in $PRNT_{50}$ and S1-ELISA*

Clinical factors	Acceleration factor of time from illness onset to log phase of antibody response			
	$PRNT_{50}$ titer	p value	S1-ELISA OD ratio‡	p value
Severe disease	1.61	<0.001	1.19	0.21
Male sex†	0.90	0.52	0.90	0.48
Age ≥60 y†	0.95	0.73	1.08	0.60
Incubation period, d†	0.97	0.06	0.95	<0.001
Use of corticosteroid†	1.19	0.33	1.14	0.47
Use of antiviral drugs†	1.07	0.61	0.76	0.03
Concomitant conditions†	1.08	0.57	1.15	0.30

*Accelerated failure time models were used; acceleration factor >1 means a longer interval to commencement of antibody response. OD, optical density; $PRNT_{50}$, 50% endpoint plaque reduction neutralization test.
†Effects were adjusted for severity.
‡Increase over S1-ELISA OD ≥0.8.

Table 2. Testing potential difference in rates of change in antibody titers over day of illness during the exponential phase of the antibody response, accounting for sequential measurements taken at different days of illness and adjusted for severity*

Clinical factors	Difference in rates of change in log antibody titers			
	$PRNT_{50}$ titer	p value	S1-ELISA OD ratio	p value
Severe disease	0.09	0.01	0.08	0.07
Male sex†	0.07	0.05	0.14	0.01
Age $\geq$60 y†	0.05	0.22	−0.03	0.65
Incubation period, d†	0.01	0.16	0.02	0.004
Use of corticosteroid†	0.06	0.37	−0.04	0.58
Use of antiviral drugs†	0.06	0.10	0.05	0.35
Concomitant conditions†	0.06	0.06	0.07	0.16

*Differences in rates of change and p values were estimated by using linear mixed models; positive value indicates a faster increase in antibody titer. Given that the antibody titers exhibited an S-shaped pattern, the analysis was restricted to data for log-phase antibody responses by manually removing data from the inductive/steady-state phase. Increases in antibody titers during the log phase were compared by different factors, adjusted for disease severity, by using a linear mixed model to account for repeated measurements, assuming a linear increasing trend by days since illness onset. $PRNT_{50}$ titers were first log-transformed (with base 10). OD, optical density; $PRNT_{50}$, 50% endpoint plaque reduction neutralization test.
†Effects were adjusted for severity.

Extensive contact tracing during the outbreak enabled us to determine the date of MERS-CoV exposure and incubation periods for patients (online Technical Appendix Table 1). A longer incubation period was associated with earlier commencement of antibody responses detectable by ELISA (Table 1; online Technical Appendix Table 2) and with a steeper slope to the response once it began (Table 2). Even after adjusting for disease severity, the use of interferon and antiviral drugs was associated with earlier commencement of antibody responses detectable by ELISA (Table 1). The time to commencement of response was similar for men and women, but the slope of the response was steeper for male patients (Table 2).

Conclusions

An understanding of MERS-CoV antibody response kinetics helps in defining the window during which passive antibody therapy may be useful. In our study, this window was the first 21 days of illness for most patients. However, some patients may not develop strong antibody responses even after 4 weeks of illness, so therapy must be individualized.

Our study has some limitations. First, no MERS-CoV isolates from the study patients were available, so MERS-CoV EMC was the basis of the serologic assays we used. Strain EMC is a clade A virus, and the outbreak in South Korea was caused by a clade B virus (*1*). However, using serum from naturally infected camels, we previously showed that clade A and B viruses and genetically diverse MERS-CoVs from Egypt were serologically indistinguishable (*10*). Another study reported that isolates of MERS-CoVs circulating in Saudi Arabia in 2014 were antigenically indistinguishable from the EMC strain in neutralization tests with human convalescent-phase serum (*5*). Thus, it is unlikely that the use of MERS-CoV EMC in our study considerably affected the observed antibody titers. A second limitation was the small number of patients studied (n = 17) and that they were followed only through the acute stage of illness. Longer term follow-up is needed to define the duration of antibody responses. If MERS-CoV antibody responses wane, as has been reported with SARS (11), this is relevant for interpretation of seroepidemiologic studies and for finding convalescent-phase donors with high antibody titers for passive immunotherapy. It would be useful to investigate IgM antibody responses and antibody responses to other virus proteins, including the MERS-CoV nucleoprotein, especially in patient L, who had poor antibody responses.

In summary, our findings showed that an early MERS-CoV antibody response was associated with reduced disease severity. Robust neutralizing and S1 ELISA IgG antibody responses were mounted by the third week of illness in most patients. However, a robust response did not occur in a few patients, and infections in such patients may be undetectable by serologic and seroepidemiologic methods.

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Dr. Park is a clinical scientist at Seoul National University Hospital. His research interest is the vaccine immunology against bacterial or viral diseases.

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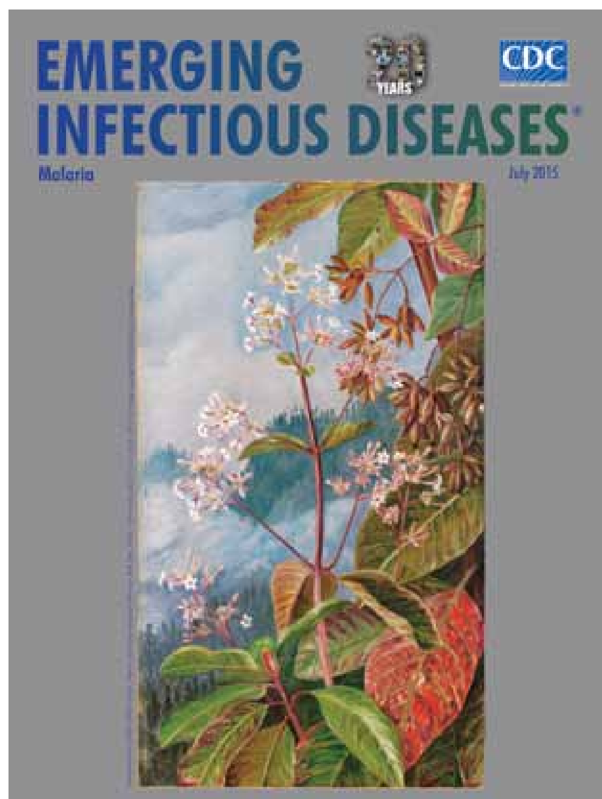

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Technical Appendix

Methods

Serologic Tests

The serum samples were heat-inactivated for 30 minutes at 56°C before testing. Sequential serum samples from the patients were analyzed for MERS-CoV antibody by plaque reduction neutralization tests (PRNT) and IgG ELISA tests. Sequential serum samples from each patient were tested in parallel.

The PRNTs were performed in a 24-well format in duplicate for each serum dilution. Two-fold serum dilutions (starting dilution of 1:10) were incubated with 40–60 PFUs of virus for 1 h at 37°C. The virus–serum mixture was added onto the Vero cells monolayer for 1hr at 37°C in a 5% CO incubator. Then, the supernatant was removed and the cells overlaid with 1% Agarose (SeaKem LE Agarose; Lonza, Switzerland) in cell culture medium (Minimum Essential Medium with 2% fetal bovine serum). After 3 days, the plates were fixed and stained. The titers were determined by defining the highest serum dilutions that resulted in ≥50% ($PRNT_{50}$) reduction in the number of plaques (*1*,*2*). Positive and negative controls and a virus back-titration were included in each assay.

The S1 ELISA EI 2604–9601G kit was purchased from EUROIMMUN AGfor detection of human IgG against MERS-CoV (http://www.euroimmun.com) and the test was done according to the manufacturer's instructions (*1*). The assay includes a calibrator which defines the upper limit of the reference range in non-infected humans and this value is defined as the cut off. The assay is made semiquantitative by calculating the ratio of the extinction of the patient

sample/ extinction of the calibrator. Ratios <0.8 is considered negative, those ≥1.1 as positive and those ≥0.8 to <1.1 regarded as borderline.

Statistical Methods

We fitted accelerated failure time models assuming a lognormal distribution to compare time from illness onset to the log phase of antibody response measured by $PRNT_{50}$ and ELISA optical density (OD) ratios, accounting for interval censoring due to time of testing. The model was also used to identify factors associated with longer time to the log phase of antibody response, including disease severity, and other factors such as sex, age, incubation period, use of steroid and antivirals and comorbid conditions adjusted for disease severity. The model can be specified as

$$Y = \log(T) = \mu + \beta' X + \sigma\varepsilon$$

where T is the duration from illness onset to commencement of antibody response, X are the factors of interest, β and σ are the intercept and scale parameters and ε is the error term. Similar analyses were conducted to compare time from illness onset to $PRNT_{50}$ titers reaching 1:40 and ELISA positive (OD ratios ≥1.1), respectively. The anti-log of the estimated coefficient β for the factor of interest is presented as the acceleration factor, which is interpreted as the multiplier on the median time length from illness onset to the commencement of different antibody responses.

We also identified any of the above factors which associated with a steeper rate of increase in $PRNT_{50}$ titers and ELISA OD ratios during the log phase, adjusted for disease severity. We visually excluded data in the lag and steady-state phase and fitted linear mixed models assuming a first-order autoregressive structure to account for repeated measurements, assuming a linear increasing trend by days since illness onset.

$$Y_{ij} = \beta' X_i + b_i T_{ij} + \varepsilon$$

where Y_{ij} is the jth measurement for patient i on day T_{ij} since illness onset, X_i are the above factors of interest including days since illness onset and ε is the error term. b_i is assumed to follow a multivariate normal distribution with first-order autoregressive structure, i.e., covariances $\gamma_{ts} \propto \rho^{|t-s|}$. The estimated coefficients of the interaction term between the above factors and days since illness onset indicate the potential differences in the rate of increase in $PRNT_{50}$ titers and ELISA OD ratios. For analyses based on continuous measurements, titers were first log-transformed

(with base 10). All statistical tests were considered significant at the level of $p<0.05$ and were conducted by using R version 3.1.2 (https://www.r-project.org/).

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Technical Appendix Table 1. Demographic and clinical profiles of patients with Middle East respiratory syndrome coronavirus infection

Patient	Sex/age, y	Underlying disease	Oxygen therapy	Mechanical ventilation	Corticosteroid use	Antiviral drug use†	Outcome
A	M/38		Yes	Yes	Yes	Yes	Hospitalized (as of D77)‡
B	M/65		Yes	Yes	Yes	Yes	Hospitalized (as of D70)‡
C	M/55		Yes	Yes	No	Yes	Discharged
D	M/35	Pneumonia	Yes	Yes	Yes	Yes	Discharged
E	F/79	CHD, CKD, dementia	Yes	Yes§	Yes	Yes	Died
F	M/55	Bladder cancer DM, CPD, lung abscess	Yes	No	Yes	No	Discharged
G	M/56		Yes	No	No	Yes	Discharged
H	M/71	DM, CVA	Yes	No	No	No	Discharged
I	F/77	DM, asthma	Yes	No	No	No	Discharged
J	M/76	DM, CHD, dementia	No	No	No	No	Discharged
K	M/59	CHD	No	No	No	Yes	Discharged
L	F/56		No	No	No	No	Discharged
M	M/56	DM, CHD, CLD, pulmonary tuberculosis	No	No	No	No	Discharged
N	F/54		No	No	No	No	Discharged
O	M/46		No	No	No	No	Discharged
P	M/35		No	No	No	Yes	Discharged
Q	M/52	Liver abscess	No	No	No	Yes	Discharged

*Gray shading indicates patients with severe disease. CHD, chronic heart failure; CKD, chronic kidney disease; CLD, chronic liver disease; CPD, chronic pulmonary disease; CVA, cerebrovascular accident; DM, diabetes mellitus.
†Interferon and ribavirin +/– lopinavir/ritonavir.
‡Patient status on August 13, 2015.
§Noninvasive mechanical ventilator.

Technical Appendix Table 2. Association and p values for different clinical factors with time from illness onset to $PRNT_{50}$ titers reaching 1:40 and S1-ELISA antibody reaching positive cut off value*

	Acceleration factor of time from illness onset to reaching respective antibody level			
Clinical factors	$PRNT_{50}$ titer ≥1:40	p value	S1-ELISA positive	p value
Severe disease	1.03	0.89	0.91	0.65
Male sex†	0.76	0.24	0.88	0.54
Age ≥60 y†	1.07	0.78	0.94	0.77
Incubation period†	0.96	0.14	0.92	<001
Use of corticosteroid†	1.23	0.51	1.07	0.78
Use of antiviral drugs†	0.76	0.19	0.84	0.37
Concomitant conditions†	0.94	0.79	0.93	0.72

*Accelerated failure time models were used; acceleration factor >1 means a longer interval to reaching the threshold. $PRNT_{50}$, 50% endpoint plaque reduction neutralization test.
†Effects were adjusted for severity.