Three novel human coronaviruses have caused different worldwide outbreaks that had variable disease severity and geographic distribution: severe acute respiratory syndrome coronavirus (SARS-CoV) during 2003; Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) during 2012; and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which caused coronavirus disease starting in 2019 (1). Understanding the immune response to coronavirus infections is crucial for vaccine development and disease prevention (2). Recurrent MERS-CoV infection has not been described in humans. However, longitudinal studies in seropositive camels detected recurrent infections and intermittent shedding of RNA (3).

A limited number of studies have evaluated the longevity of MERS antibody responses. Payne et al. described persistence of MERS-CoV neutralizing antibodies for >34 months postinfection in 6 (86%) of 7 survivors (4). Choe et al. showed that patients who had severe disease had robust MERS-CoV neutralizing antibody titers for 1 year, and patients who had mild disease had waning antibody response over time (5). We assessed antibody responses in 48 MERS survivors who had variable disease severity and duration ≤6 years postinfection.

The Study
We recruited 48 MERS survivors from 5 hospitals in Jeddah and Riyadh, Saudi Arabia. All participants who agreed to participate provided consent. The study was approved by the institutional research boards of the hospitals involved. All MERS cases were diagnosed on the basis of positive reverse transcription PCR results. Disease severity was divided into 3 categories: mild infection (asymptomatic and upper respiratory tract infection), moderate infection (pneumonia not requiring intubation and ventilation), and severe infection (pneumonia requiring intubation and ventilation in the intensive care unit). Blood samples were collected for serologic testing from survivors in various hospitals at a single time point, except for 1 patient (case-patient 45; Table) who provided samples at 4 and 6 years postinfection. On the basis of date of

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diagnosis, MERS-CoV antibody responses were measured 2–6 years postinfection.

An ELISA was performed for 45/49 samples. Microneutralization assays were performed for 43/49 samples in China and 6/49 samples in Saudi Arabia. A total of 43/49 samples were collected 2–5 years postinfection, and 6/49 samples were collected 6 years postinfection. A commercial MERS-CoV SIELISA Kit (Euroimmun, https://www.euroimmun.com) was used to measure human IgG titers against the MERS-CoV spike protein as described (6). Samples with an optical density \( \geq 1.1 \) were considered positive, those \( <0.8 \) negative, and those \( 0.8–1.1 \) borderline. A MERS-CoV focus reduction neutralization test (modified microneutralization assay) and a MERS-CoV microneutralization test were performed.

| Patient ID | Age, y/sex | Time, y between serologic analysis and infection | Diagnosis | Disease or condition | Illness grade | ELISA result | ELISA titer | NT result |
|------------|------------|-----------------------------------------------|-----------|---------------------|--------------|--------------|-------------|-----------|
| 46         | 34/F       | 6                                             | AS        | Healthy             | Mild         | –            | 0.0         | <20       |
| 47         | 41/M       | 6                                             | AS        | Healthy             | Mild         | –            | 0.02        | 40        |
| 48         | 41/F       | 6                                             | PN        | Healthy             | Moderate     | –            | 0.76        | 320       |
| 45         | 42/M       | 6                                             | PN        | Healthy             | Moderate     | –            | 0.75        | 80        |
| 43         | 56/M       | 6                                             | SPN       | Healthy             | Severe       | –            | 3.0         | 80        |
| 44         | 38/F       | 6                                             | SPN       | Pregnant, thyroid disease | Severe       | +            | 2.4         | 80        |
| 4          | 52/F       | 5                                             | URTI      | HPT, thyroid disease | Mild         | –            | 0.1         | <20       |
| 2          | 43/F       | 5                                             | URTI      | Healthy             | Mild         | –            | 0.3         | 42        |
| 15         | 35/M       | 5                                             | PN        | DM, hyperlipidemia  | Moderate     | B            | 0.8         | 30        |
| 33         | 39/F       | 4                                             | URTI      | Healthy             | Mild         | –            | 0.7         | 28        |
| 7          | 49/M       | 4                                             | URTI      | DM, HPT, BA, IHD, ESRD | Mild       | +            | 1.5         | 104       |
| 34         | 42/F       | 4                                             | URTI      | HPT                 | Mild         | +            | 1.9         | 144       |
| 40         | 28/F       | 4                                             | URTI      | Healthy             | Mild         | NP           | NP          | 40        |
| 41         | 32/F       | 4                                             | URTI      | Healthy             | Mild         | NP           | NP          | 41        |
| 31         | 33/M       | 4                                             | URTI      | Healthy             | Mild         | –            | 0.5         | 34        |
| 32         | 45/F       | 4                                             | URTI      | Healthy             | Mild         | B            | 0.9         | 44        |
| 3          | 45/M       | 4                                             | PN        | Smoker              | Moderate     | +            | 1.1         | 48        |
| 5          | 61/M       | 4                                             | PN        | DM, HPT, IHD        | Moderate     | +            | 2.9         | 160       |
| 25         | 28/M       | 4                                             | PN        | Healthy             | Moderate     | +            | 2.5         | 315       |
| 42         | 47/M       | 4                                             | PN        | Healthy             | Moderate     | NP           | NP          | 351       |
| 45         | 42/M       | 4                                             | PN        | Healthy             | Moderate     | NP           | NP          | 162       |
| 29         | 58/M       | 3                                             | URTI      | Healthy             | Mild         | –            | 0.1         | 45        |
| 23         | 28/M       | 3                                             | URTI      | Healthy             | Mild         | –            | 0.1         | 42        |
| 8          | 47/M       | 3                                             | URTI      | HPT, hyperlipidemia | Mild         | +            | 2.5         | 320       |
| 18         | 55/M       | 3                                             | URTI      | DM                  | Mild         | +            | 3.4         | 648       |
| 20         | 34/M       | 3                                             | URTI      | Healthy             | Mild         | –            | 0.6         | 81        |
| 26         | 39/M       | 3                                             | URTI      | Healthy             | Mild         | –            | 0.6         | 75        |
| 35         | 63/M       | 3                                             | URTI      | DM                  | Mild         | +            | 2.5         | 501       |
| 27         | 61/M       | 3                                             | URTI      | DM, HPT             | Mild         | +            | 1.2         | 81        |
| 14         | 32/F       | 3                                             | AS         | Healthy             | Mild         | –            | 0.1         | 45        |
| 39         | 34/M       | 3                                             | URTI      | Healthy             | Mild         | +            | 1.2         | 31        |
| 9          | 36/F       | 3                                             | URTI      | Healthy             | Mild         | –            | 0.2         | 32        |
| 6          | 74/M       | 3                                             | URTI      | DM, lipid           | Mild         | –            | 0.1         | <20       |
| 10         | 46/F       | 3                                             | AS         | Healthy             | Mild         | –            | 0.1         | 20        |
| 11         | 47/F       | 3                                             | AS         | Grave’s disease     | Mild         | –            | 0.1         | 20        |
| 12         | 33/F       | 3                                             | AS         | Healthy             | Mild         | –            | 0.3         | 20        |
| 17         | 54/F       | 3                                             | URTI      | HPT, thyroid disease | Mild         | +            | 4.3         | <20       |
| 27         | 29/M       | 3                                             | URTI      | Healthy             | Mild         | –            | 0.1         | <20       |
| 30         | 41/F       | 3                                             | URTI      | Healthy             | Mild         | –            | 0.2         | <20       |
| 4          | 41/M       | 3                                             | PN         | Stroke              | Moderate     | +            | 3.4         | 446       |
| 19         | 50/M       | 3                                             | PN         | Healthy             | Moderate     | +            | 3.7         | 315       |
| 24         | 54/M       | 3                                             | PN         | DM, HPT, myocarditis | Moderate     | +            | 2.4         | 398       |
| 22         | 57/M       | 3                                             | PN         | Asthma              | Moderate     | +            | 1.9         | 41        |
| 16         | 62/F       | 3                                             | SPN        | Asthma, hyperlipidemia | Severe       | +            | 2.6         | 416       |
| 21         | 34/F       | 3                                             | SPN        | Healthy             | Severe       | +            | 2.4         | 375       |
| 28         | 38/M       | 3                                             | SPN        | Healthy             | Severe       | +            | 1.8         | 117       |
| 13         | 59/M       | 3                                             | SPN        | Healthy             | Severe       | –            | 0.1         | 20        |
| 36         | 64/M       | 2                                             | URTI      | Healthy             | Mild         | +            | 2.5         | 160       |
| 38         | 34/M       | 2                                             | URTI      | Healthy             | Mild         | –            | 0.3         | 27        |

*AS, asymptomatic; B, borderline; BA, bronchial asthma; DM, diabetes mellitus; ESRD, end-stage renal disease; HPT, hypertension; IHD, ischemic heart disease; NP, not performed; NT, neutralization test; PN, pneumonia; SPN, severe pneumonia (patients were in intensive care unit and required intubation and ventilation); URTI, upper respiratory tract infection; –, negative; +, positive.*
in certified Biosafety Level 3 laboratories in Guangzhou, China, and Jeddah, Saudi Arabia, as described (7,8). The cutoff value for a positive neutralization assay result was 1:20 (Appendix, https://wwwnc.cdc.gov/EID/article/27/5/20-4056-App1.pdf). We used reference MERS-CoV isolates (GenBank accession nos. EMC/2012 in Guangzhou and KF958702 in Jeddah).

We presented continuous variables as median and interquartile range (IQR). We used Kruskal-Wallis, Mann-Whitney, Jonckheere-Terpstra, Fisher exact, and Gamma tests to study the differences between variables. All p values were 2-tailed, and p values <0.05 were considered significant. We used SPSS Statistics 25.0 (IBM Corp., https://www.ibm.com) for all statistical analyses.

Of 49 specimens, 28 (57.1%) were collected from MERS convalescent patients at 2–3 years postinfection, 12 (24.5%) at 4 years postinfection, and 9 (18.4%) at 5–6 years postinfection. Of 49 specimens, 31 (63.3%) were collected from MERS convalescent patients who had mild disease, 12 (24.5%) from those who had moderate disease, and 6 (12.2%) from those who had severe disease (Table). We found that 38/49 specimens had neutralizing antibodies (median [IQR] titer 45 [29–161]). Of these 38 samples, 12 (31%) were negative by ELISA. Ten of these 12 samples were collected from survivors who had mild illness (Table).

The percentage of samples that had positive neutralizing antibodies was 20/28 (71.4%) at 2–3 years, 11/12 (91.7%) at 4 years, and 7/9 (77.6%) at 5–6 years postinfection (p = 0.405 for any difference and 0.349 for trend) (Table). The median (IQR) titer of neutralizing antibodies was 45 (20–319) at 2–3 years, 76 (40–162) at 4 years, and 42 (23–80) at 5–6 years postinfection (p = 0.499 for any difference and 0.755 for trend) (Figure, panel A).

Positive neutralizing antibodies were found in 21 (67.7%) of 31 survivors who had mild disease, 12 (100.0%) of 12 survivors who had moderate disease, and 5 (83.3%) of 6 survivors who had severe disease (p = 0.054 for any difference and p = 0.035 for trend) (Table). The median (IQR) titer of neutralizing antibodies was 40 (20–81) for survivors who had mild disease, 239 (56–343) for survivors who had moderate disease, and 99 (65–385) for survivors who had severe disease, respectively (p = 0.004 for any difference and p = 0.002 for trend).

Survivors who had mild, moderate, and severe disease had the following median (IQR) titers for neutralizing antibodies: 37 (20–81), 357 (110–434), and 246 (44–406) at 2–3 years postinfection (p = 0.109 for any difference and p = 0.053 for trend); 41 (34–104) and 162 (104–333) (mild or moderate disease only) at 4 years postinfection (p = 0.010); and 28 (15–42), 80 (30–320), and 80 (80–80) at 5–6 years postinfection (p = 0.130 for any difference and p = 0.065 for trend) (Figure, panel B). We found no major decrease in neutralizing antibody titers over 6 years (Figure, panel A). Survivors who had moderate and severe disease had higher titers than survivors who had mild disease over 6 years (Figure, panel B).

**Conclusions**

At 6 years postinfection, we detected antibody responses in 100% of MERS survivors who had severe disease.

**Figure.** Neutralization antibody titers in Middle East respiratory syndrome (MERS) convalescent-phase serum samples measured 2–6 years postinfection, Saudi Arabia. Three groups (patients who had mild, moderate, or severe MERS) were enrolled in this study, and serum samples were collected for neutralizing antibody detection (median focus reduction neutralization test titer) at the indicated times after recovery. The cutoff value was 1:20. Median titers of neutralizing antibody (red dots) and interquartile range (blue bars) were measured according to years postinfection (panel A) and disease severity (panel B). There was no major decrease in neutralizing antibodies over 6 years postinfection. Survivors who had moderate and severe disease had higher neutralizing antibody titers than survivors who had mild disease. Mod, moderate; Sev, severe.
or moderate disease and in 50% of survivors who had mild disease, demonstrating durability of the MERS-CoV-specific antibody response. Because we did not measure MERS-CoV-specific T lymphocyte responses, the number of MERS survivors who had detectable immune responses was probably underestimated. T-cell responses were detected in several MERS survivors who had negative antibody responses at 6 months postinfection (9). The results are consistent with those of previous studies, which the association between disease severity and decrease of antibody response in MERS survivors over time (10). Similar results were described after the SARS epidemic. SARS survivors had persistent antibody responses for 3 years postinfection, and a decrease by 6 years postinfection (11,12). However, a recent study indicated that low levels of SARS-CoV-specific antibody could be detected in some survivors at 12 years postinfection (X. Guo et al., Sun Yat-sen University, pers. comm., 2020 Jan 1).

In this study, we performed ELISA and neutralizing antibody assays for all cases. Although cases of severe disease showed good concordance between the 2 assays, some cases of mild or moderate disease had a negative ELISA result and a positive neutralizing test result. Similar results were observed in camel workers who had asymptomatic MERS-CoV infections, most of whom had negative ELISA results but detectable neutralizing antibody titers (13). Negative ELISA results might reflect either insensitivity of the assay or high cutoff values established by the manufacturer to minimize the rate of false-positive results. In either instance, these results suggest that negative ELISA results should be read with caution in some settings.

A limitation of our study was the small number of cases of moderate or severe disease and a lack of serial samples for nearly all patients. It will also be useful to determine whether levels of antibody would be protective if MERS-CoV reinfection occurred. In conclusion, we showed that virus-specific neutralizing antibodies are detectable in most MERS survivors for ≥6 years, consistent with durable immunity against the virus.

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S.M.F organized data; A.N.A. designed the study, collected data, and wrote the manuscript; S.P. designed the study, analyzed data, and reviewed the manuscript; Z.A.M. investigated study sites and reviewed the manuscript; J.Z., M.A.A., E.I.A., A.D., Y.W., I.A., A.M.H., and S.A.E, performed experiments; S.A.B, S.A., W.A.A., M.A.E, F.E.E., N.E.M., L.L., and HAB investigated sites; N.A.S. and I.Q. provided samples; and A.E.S. and A.H. analyzed data.

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Appendix

Middle East Respiratory Syndrome Coronavirus Neutralizing Antibody Assays

A Middle East respiratory syndrome coronavirus (MERS-CoV) focus reduction neutralization test was adapted from a microneutralization assay and performed in a certified Biosafety Level 3 laboratory in Guangzhou, China as described (1). Plasma samples (50 µL) were serially diluted, mixed with 50 µL of MERS-CoV (100 focus-forming units) in 96-well microwell plates and incubated for 1 hour at 37°C. Mixtures were then transferred to 96-well plates seeded with Vero 81 cells (American Type Culture Collection, https://www.atcc.org) for 1 hour at 37°C to enable virus entry. Inocula were then removed before adding the overlay media (100 µL minimal essential medium [MEM]) containing 1.2% carboxymethylcellulose. The plates were then incubated at 37°C for 48 hours. Overlays were removed and cells were fixed with 4% paraformaldehyde solution for 30 min. Cells were permeabilized with 0.2% Triton X-100 and incubated with a human anti-MERS-CoV-S monoclonal antibody for 1 hour at room temperature before adding horseradish peroxidase–conjugated goat anti-human IgG (heavy plus light chain) antibody (Jackson ImmunoResearch, https://www.jacksonimmuno.com). A human monoclonal antibody (tentatively named ZMC1411, diluted to 10 mg/mL) against MERS-CoV receptor binding domain was used as the primary antibody (it was isolated, cloned from memory B cells from a convalescent-phase patient, produced, and validated in our laboratory).

Cells were further incubated at room temperature. Reactions were performed with TrueBlue Peroxidase substrates (Seracare Life Sciences Inc., https://www.seracare.com). The numbers of MERS-CoV foci were calculated by using an EliSpot Reader (Cellular Technology Ltd., https://www.pharmaceutical-technology.com).

A MERS-CoV microneutralization assay was performed as described (2) in a certified Biosafety Level 3 laboratory in Jeddah, Saudi, because of shipment difficulties to China during
the coronavirus disease pandemic lockdown. Two-fold serial dilutions of heat-inactivated serum samples prepared in Dulbecco’s modified Eagle medium starting at a 1:10 dilution were incubated with equal volumes of Dulbecco’s modified Eagle medium containing $1.58 \times 10^7$ median (50%) tissue culture infectious doses/mL of MERS CoV for 1 h at 37°C in a 5% CO2 incubator. The mixture of virus–serum was used to infect confluent Vero E6 cell monolayers in 96-well plates (in triplicate) and incubated at 37°C in a 5% CO2 incubator. Cytopathic effects were observed on a daily basis for 3 days to determine neutralizing antibody titer and stained by using crystal violet. The neutralizing antibody titer for each serum sample is reported as the reciprocal of the highest dilution that completely protected cells from cytopathic effects in 50% of the wells.

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