ORIGINAL ARTICLE

Transmission of MERS-Coronavirus in Household Contacts

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ABSTRACT

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BACKGROUND

Strategies to contain the Middle East respiratory syndrome coronavirus (MERS-CoV) depend on knowledge of the rate of human-to-human transmission, including subclinical infections. A lack of serologic tools has hindered targeted studies of transmission.

METHODS

We studied 26 index patients with MERS-CoV infection and their 280 household contacts. The median time from the onset of symptoms in index patients to the latest blood sampling in contact patients was 17.5 days (range, 5 to 216; mean, 34.4). Probable cases of secondary transmission were identified on the basis of reactivity in two reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assays with independent RNA extraction from throat swabs or reactivity on enzyme-linked immunosorbent assay against MERS-CoV S1 antigen, supported by reactivity on recombinant S-protein immunofluorescence and demonstration of neutralization of more than 50% of the infectious virus seed dose on plaque-reduction neutralization testing.

RESULTS

Among the 280 household contacts of the 26 index patients, there were 12 probable cases of secondary transmission (4%; 95% confidence interval, 2 to 7). Of these cases, 7 were identified by means of RT-PCR, all in samples obtained within 14 days after the onset of symptoms in index patients, and 5 were identified by means of serologic analysis, all in samples obtained 13 days or more after symptom onset in index patients. Probable cases of secondary transmission occurred in 6 of 26 clusters (23%). Serologic results in contacts who were sampled 13 days or more after exposure were similar to overall study results for combined RT-PCR and serologic testing.

CONCLUSIONS

The rate of secondary transmission among household contacts of patients with MERS-CoV infection is low. Our data provide insight into the rate of subclinical transmission of MERS-CoV in the home.

THE MIDDLE EAST RESPIRATORY SYN-
drome coronavirus (MERS-CoV) was first
isolated in 2012 from a patient with fatal
pneumonia in Jeddah, Saudi Arabia.[1] From 2012
through July 2014, at least 834 laboratory-con-
firmed cases of MERS-CoV infection associated
mostly with respiratory tract illness were report-
ed to the World Health Organization; of these
cases, 288 were fatal. Known cases have been
directly or indirectly linked to countries in the
Arabian peninsula.[2] The epidemiologic features
of MERS remain poorly defined. Studies that
have modeled the reproductive rate of MERS-CoV
in humans have been based on notified, clinically
apparent cases and auxiliary measurements, such
as the viral evolutionary rate.[3,4] However, these
studies have emphasized the need to reevaluate
estimates of reproductive rates through labora-
tory-based investigations of the actual rate of
transmission, including silent and subclinical
infections. Any unnoticed transmission of the
virus in the population could explain why newly
identified index cases often cannot be linked to
zoonotic exposure. However, to date, the lack of
serologic tools has hindered in-depth investiga-
tion of rates of secondary transmission.

To determine the rate of silent or subclinical
secondary infection after exposure to primary
cases of MERS-CoV infection, we performed a
cross-sectional laboratory investigation of 280
household contacts of 26 index patients, with ad-
ditional follow-up serologic analysis in 44 con-
tacts. All the contacts were tested for the pres-
ence and quantity of viral RNA in throat-swab
samples and for antibodies in blood with the use
of a staged serologic algorithm that is based on
results from enzyme-linked immunosorbent
assays (ELISAs), immunofluorescence assays, and
plaque-reduction neutralization testing (PRNT).

METHODS

PATIENTS

Household contact clusters were associated with
the 26 index patients in whom MERS-CoV infec-
tion was diagnosed from June 4, 2013, to Novem-
ber 5, 2013, with one cluster per index patient.
The median date on which clusters were sampled
was September 8, 2013. Diagnoses in index pa-
tients were based on hospitalization with bilateral
pneumonia and detection of MERS-CoV in respi-

ratory samples on reverse-transcriptase–polymer-
ase chain reaction (RT-PCR). All household con-
tacts of index patients (who were defined as
persons living in the same house, regardless of
the degree of relation to the index patient) were
obliged to participate in the investigation per de-
cree of the Ministry of Health as part of a public
health response. Clusters occurred in Riyadh
(16 clusters), Eastern Province (3), Al-Hasa (2),
Hafr Al-Batin (2), Jubail (2), and Jeddah (1).

MERS-COV RT-PCR ASSAY

We performed RT-PCR assays on RNA that was
extracted from upper respiratory tract swabs, with
screening for the MERS-CoV genomic region up-
stream of the envelope gene (upE) and within
open reading frame (ORF) 1a, as described pre-
viously.[5,6] Confirmation of upE results by ORF1a
detection involved the reextraction of RNA from
the original samples.

FULL-VIRUS IMMUNOFLUORESCENCE ASSAY

Immunofluorescence assays were performed with
slides carrying Vero cells infected with full
MERS-CoV, as described previously,[6] and were
manufactured into a homogeneous reagent for-
mat by an in vitro diagnostics company for im-
proved lot-to-lot consistency (MERS IIFT, Euro-
immun). The serum predilution was 1:100.

RECOMBINANT IMMUNOFLUORESCENCE ASSAY

We performed recombinant immunofluorescence
assays to determine the specific reactivity
against recombinant spike proteins in VeroB4
cells, as described previously.[6] The screening di-
lution was 1:40. Because the CoV spike protein
constitutes the most specific and immunogenic
antigen in CoV antibody assays,[7] open reading
frames for full spike proteins were cloned from
human coronaviruses (HCoV) HCoV-229E, HCoV-
NL63, HCoV-OC43, and HCoV-HKU1, as well as
for the severe acute respiratory syndrome corona-
virus (SARS-CoV).[6,8] Cells for all slides were
seeded on chamber slides and transfected in par-
allel with equal amounts of the respective ex-
pression plasmids.[6,8] To compare titers for
MERS-CoV with those for different HCoVs, im-
munofluorescence assays were performed by the
same staff member on the same day. Control se-
rum samples for HCoV recombinant immuno-
fluorescence assays were obtained from patients

with recent RT-PCR–confirmed HCoV-229E, HCoV-NL63, HCoV-OC43, or HCoV-HKU1 infection, as seen in routine diagnostic testing. The methods for obtaining control serum samples for testing for the presence of MERS-CoV and SARS-CoV have been described previously.[6,8-10]

IGM IMMUNOFLUORESCENCE ASSAY

Immunofluorescence assay was used to determine the presence of IgM antibodies. All serum samples were depleted of IgG antibodies with the use of Eurosorb reagent (Euroimmun), according to the protocol. The screening dilution was 1:40. Secondary detection was performed with the use of fluorescein isothiocyanate (FITC)–conjugated anti-human IgM antibodies.

RECOMBINANT ELISA

A recombinant ELISA assay was based on soluble MERS-CoV spike protein S1 domain expressed in HEK-293T cells.[11] This test was developed with the use of samples obtained from small groups of humans and camels in preliminary studies.[7,12] The dilution of human serum samples in this test was 1:100. Additional technical details are provided in Section 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org.

NEUTRALIZATION TESTING

PRNT was performed as described previously.[8] The entry dilution in log_2-dilution series was 1:10. Serum dilutions causing plaque reductions of 90% ($PRNT_{90}$) and 50% ($PRNT_{50}$) were recorded as titers.

DIAGNOSTIC ALGORITHM TO IDENTIFY SECONDARY TRANSMISSION

We used the above-mentioned RT-PCR method to screen for MERS-CoV in throat swabs. Serum samples were initially screened for antibodies by means of an ELISA. Supportive serologic testing was performed in two stages. Stage 1 seropositivity was defined as reactivity on both an ELISA and a recombinant immunofluorescence assay. Stage 2 seropositivity was defined as stage 1 seropositivity plus reactivity on PRNT. Patients who had dual positive RT-PCR results or positive stage 2 serologic results were determined to have probable cases of secondary transmission. A flow chart summarizing the diagnostic algorithm and overall study outcome is provided in Figure 1.

RESULTS

STUDY POPULATION

The household clusters we studied included a mean of 11 contacts (range, 2 to 21). The median age of the 26 index patients was 55 years (range, 2 to 83; mean, 54.6); 17 of the patients were male. Of these patients, 24 had coexisting illnesses, 24 required treatment in an intensive care unit, and 18 died. Index patients had stayed at home with symptoms before hospital admission for an average of 6 days (range, 0 to 21). The median age of the 280 household contacts was 29 years (range, 2 to 77; mean, 30.7); 48% of these contacts were female. A total of 59 household contacts were 16 years of age or younger.

The median interval from the onset of symptoms in index patients to the collection of the latest blood sample from household contacts was 17.5 days (range, 5 to 216; mean, 34.4). Information with respect to coexisting illnesses was recorded for 178 household contacts; of these contacts, 12% had coexisting illnesses (Table S1 in the Supplementary Appendix).

LABORATORY TESTING

Throat swabs from all household contacts were initially tested for MERS-CoV RNA by means of RT-PCR. Of the 280 contacts, 7 (2%) who lived in three clusters had dual positive results on RT-PCR (Fig. 1A). Virus RNA levels in swabs obtained from these contacts ranged from fewer than 500 copies to 80,000 copies per swab sample, with a median level of 2700 copies per sample (Table 1). Only 1 contact with positive results on RT-PCR had mild symptoms at the time of sampling, and 2 contacts had had contact with camels, which have been identified as possible reservoirs for MERS-CoV.[12]

Serologic testing was based on a staged algorithm (Fig. 1B). The algorithm was established after comparative testing of primary serum samples from all 280 household contacts, with one sample per person (Section 3 in the Supplementary Appendix). These combined studies suggested that a recombinant immunofluorescence assay would be an appropriate first-stage diagnostic test for seropositivity for ELISA-based screening results. A total of 6 household contacts had positive ELISA results and positive results on recombinant immunofluorescence assay, fulfilling the
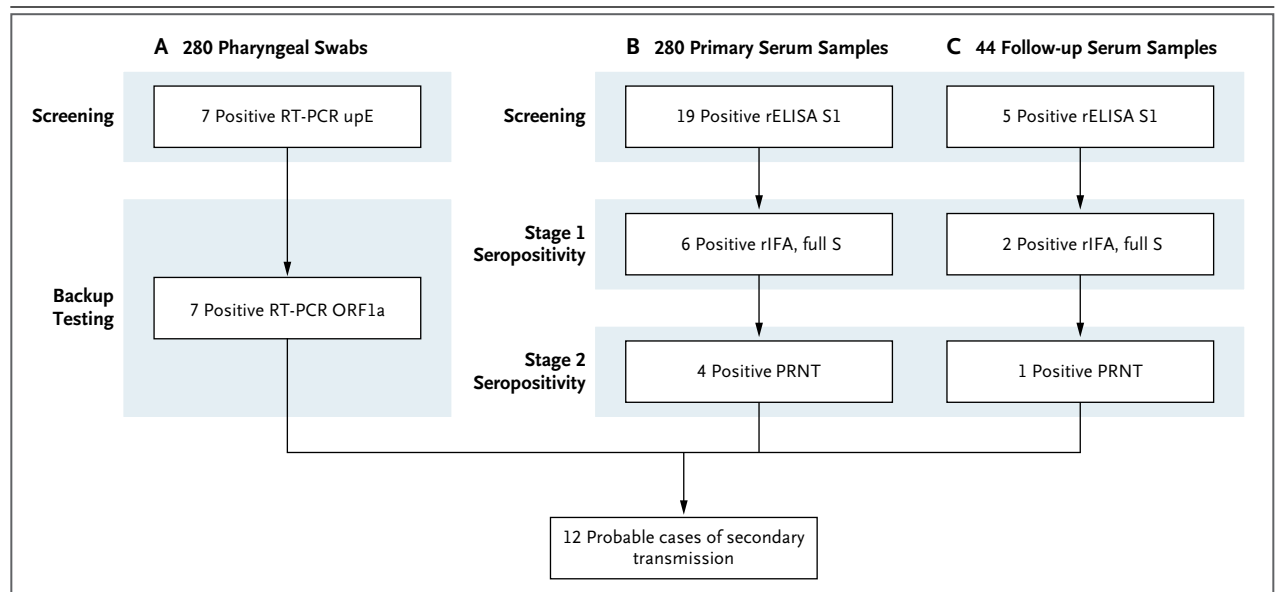
Figure 1. Diagnostic Testing Performed in the Study.

Throat swabs (column A) and serum samples (column B) were obtained from 280 household contacts of 26 index patients with MERS-CoV infection. A total of 44 household contacts provided a second serum sample for follow-up (column C). Screening involved testing of all throat swabs by means of a reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay and testing of all serum samples by means of recombinant enzyme-linked immunosorbent assay (rELISA) with the use of the S1 domain of the MERS-CoV spike protein. RT-PCR backup testing was based on a second RT-PCR assay, including re-extraction from the original samples. Eluted RNA was screened for the MERS-CoV genomic region *upstream of the envelope gene* (upE) and within open reading frame (ORF) 1a. Testing for stage 1 seropositivity involved the use of recombinant immunofluorescence assay (rIFA) with the use of the full MERS-CoV spike protein (full S) for all serum samples with a positive result on ELISA screening. Testing for stage 2 seropositivity was performed on serum samples with stage 1 seropositivity by means of plaque-reduction neutralization testing (PRNT) with plaque reduction of 50% as the criterion for neutralizing activity. Among the 4 samples with positive results on PRNT in column B, 2 were also determined to be positive on IgM testing. In the analysis of the 44 follow-up serum samples for stage 1 seropositivity, one case of MERS-CoV infection had already been confirmed in the primary serum sample, and one case was identified as a new seroconversion.

definition of stage 1 seropositivity. These household contacts included none of those with positive results on RT-PCR. Serum from the RT-PCR–positive contacts had been obtained 4 to 14 days after the onset of symptoms in the index patients, which suggested that these contacts were still in the period before seroconversion.

We asked all the household contacts for permission to obtain a second blood sample after 2 to 6 months for additional testing. Only 44 contacts in nine clusters agreed to provide a second sample. Of these contacts, 5 had positive results on ELISA; of these 5 contacts, 2 were seropositive on recombinant immunofluorescence assays (stage 1 seropositivity) (Fig. 1C). One of these 2 contacts had stage 1 seropositivity on the basis of his first serum sample, and the other (Contact 180) underwent seroconversion sometime between the first and second serum sample (Table S6 in the Supplementary Appendix).

Stage 2 seropositivity was tested on the basis of the above-mentioned PRNT cutoffs ($PRNT_{90}$ and $PRNT_{50}$). $PRNT_{90}$ titers were positive in an index patient (who was used as a control in the analysis of household contacts) and in samples obtained from 2 of 7 household contacts with stage 1 seropositivity. Three additional samples had positive $PRNT_{50}$ titers (Table 2). To correlate serum neutralizing activities with specific antibody titers, we performed differential recombinant immunofluorescence assays against all common HCoVs in all samples with confirmed stage 1 seropositivity (Table 2). In the serum pair for Contact 180, results on differential recombinant immunofluorescence assay indicated a significant rise in titer against MERS-CoV but not against any of the other HCoVs tested. It was concluded that Contact 180 represented a specific MERS-CoV seroconversion. Since this seroconversion was detected on $PRNT_{50}$ but not

Table 1. Laboratory Data and History of 12 Household Contacts Identified as Having Probable Secondary Infection with MERS-CoV.*

Contact No.	Age	Sex	Latency Period†	Serologic Analysis	Results of RT-PCR Assay‡	Symptoms	Coexisting Illnesses	Animal Contact
	yr		days		RNA copies/sample			
52	28	F	14	ELISA, 0.11 (negative); IFA, negative	Positive§	None	Unknown	None
278	7	F	4	ELISA, 0.06 (negative); IFA, negative	38,500	None	None	Camels
279	15	M	4	ELISA, 0.10 (negative); IFA, negative	2700	None	None	Camels
280	26	M	4	ELISA, 0.07 (negative); IFA, negative	38,500	None	None	None
257	74	F	8	ELISA, 0.06 (negative); IFA, negative	80,000	Pharyngitis¶	None	None
258	3	F	8	ELISA, 0.05 (negative); IFA, negative	<500	None	None	None
259	18	M	8	ELISA, 0.07 (negative); IFA, negative	<500	None	None	None
99	37	M	19	ELISA, 1.82 (reactive); IFA, 1:1280; PRNT, 1:160; IgM, 1:40	Negative	None	None	None
104	24	M	19	ELISA, 1.84 (reactive); IFA, 1:2560; PRNT, 1:160; IgM, 1:40	Negative	None	None	None
102	31	M	19	ELISA, 0.45 (reactive); IFA, 1:320; PRNT, 1:40; IgM, negative	Negative	None	Unknown	None
180‖	26	F	23	ELISA, 0.9 (reactive); IFA, 1:1280; PRNT, 1:40; IgM, negative	Negative	None	Unknown	None
193	31	M	13	ELISA,1.57; (reactive); IFA, 1:40; PRNT, 1:40; IgM, negative	Negative	None	None	None

* Secondary infection was defined as dual positivity on RT-PCR assay or stage 2 seropositivity. ELISA denotes enzyme-linked immunosorbent assay, F female, IFA immunofluorescence assay, M male, and PRNT plaque-reduction neutralization testing.

† The latency period was the number of days between the onset of symptoms in the index patient and sampling of the household contact.

‡ The result was based on positive outcome of two independent RT-PCR assays in the upE and ORF1a target genes after reextraction from the original sample. The approximate concentration is based on the upE target gene assay.

§ This sample was tested in a local laboratory by means of RT-PCR assays in the upE and ORF1a target genes without recording of values for the threshold cycle (RNA concentration unknown). The original sample was not retrievable.

¶ This symptom was recorded on the day of sampling. Respiratory failure and death occurred in this contact soon after testing.

‖ For this household contact, laboratory results are based on a second follow-up serum sample.

$PRNT_{90}$, we chose $PRNT_{50}$ as a criterion to define stage 2 seropositivity in our diagnostic algorithm. Application of this criterion resulted in the identification of 5 household contacts with stage 2 seropositivity.

Since it is unknown whether the development of neutralizing antibodies as measured on PRNT can lag behind the development of reactivity on ELISA and immunofluorescence assay, we evaluated IgM antibodies as an additional potential indicator of recent infection. However, IgM titers were detected in serum samples that had positive $PRNT_{90}$ titers but not in samples with isolated $PRNT_{50}$ titers, which suggests that testing for IgM antibodies in an immunofluorescence assay–based format provides low sensitivity overall (Table 2). Table 3 summarizes the final study results, including the results of testing of second serum samples when they were available.

DISCUSSION

We report the results of a targeted investigation of silent and mild infections in the 280 household contacts of 26 index patients in whom MERS-CoV infection had been confirmed on RT-PCR assay with corroborating clinical findings. Seven household contacts carried MERS-CoV in their upper respiratory tract, as shown on RT-PCR assays. Pharyngeal RNA concentrations were low in most contacts, a finding that matches observations in a study on SARS in Taiwan in which asymptomatic or mildly symptomatic health care workers were shown to carry low pharyngeal levels of viral RNA after exposure to patients infected with SARS-CoV.[13] Our findings suggest that young persons without coexisting illnesses may be able to carry low levels of MERS-CoV RNA without obvious symptoms.

Table 2. Differential Serologic and Neutralization Analyses for MERS and Other Coronaviruses in 7 Household Contacts with Stage 1 Seropositivity and 1 Index Patient.*

Cluster	Contact No.	ELISA	IgG Titer on Recombinant IFA						IgM Titer on Recombinant IFA	PRNT MERS†	
			MERS	SARS	OC43	HKU1	229E	NL63	MERS	PRNT$_{90}$	PRNT$_{50}$
F	Index patient	4.05	5120	160	1,280	1,280	1280	320	160	40	160
J	99	1.82	1280	Negative	2,560	1,280	640	160	40	80	160
J	102	0.45	320	1280	5,120	5,120	640	1280	Negative	<20	40
J	104	1.83	2560	640	20,480	20,480	1280	2560	40	40	160
RIII	180‡	0.05	Negative	Negative	2,560	640	1280	640	Negative	<20	<20
	180§	0.90	1280	Negative	1,280	320	640	320	Negative	<20	40
RIII	187‡	0.31	80	Negative	1,280	1,280	640	1280	Negative	<20	<20
	187§	0.29	160	Negative	1,280	640	640	640	Negative	<20	<20
RIV	193	1.57	40	160	320	640	1280	640	Negative	<20	40
TII	274	0.46	160	Negative	2,560	1,280	160	160	Negative	<20	<20

* SARS denotes severe acute respiratory syndrome.
† Stage 2 seropositivity was tested by means of PRNT with cutoff values of 90% (PRNT$_{90}$) and 50% (PRNT$_{50}$). A titer of less than 1:20 indicates a negative result.
‡ This sample was obtained on day 23.
§ This sample was obtained on day 178 during follow-up.

Asymptomatic, RT-PCR–positive health care workers were identified in a recent outbreak of MERS in Jeddah.

However, positive RT-PCR findings in persons with subclinical infection who are tested soon after exposure should be complemented by serologic analysis. In three earlier cross-sectional serologic studies, none of the 3024 participants showed evidence of previous infection with MERS-CoV.[1,14,15] In our study, the detection of antibodies was most likely due to our focus on household contacts of index patients. Differences in assay sensitivity may play an additional role. Our staged serologic algorithm is based on earlier experience with current assays or their technical precursors[6,8,12,14,16-20] and was developed as much as possible by a comparison of methods in the study samples available. Although validation of this algorithm will be necessary, our results suggest that ELISA is an appropriate screening assay and that recombinant immunofluorescence assay is an appropriate first-stage confirmatory test, owing to its higher specificity without an apparent substantial lack of sensitivity. Differential serologic testing by means of recombinant immunofluorescence assay cannot be used to rule out MERS-CoV infection, since anti–MERS-CoV titers did not always predominate in probable cases of secondary transmission. It is unknown whether MERS-CoV infection may cause a boost in preexisting titers against related HCoVs acquired earlier in life, such as is seen with many other infectious diseases. There are conserved regions between S proteins in MERS-CoV and those in various HCoVs that might explain the existence of cross-reactive serum antibodies (Fig. S2 in the Supplementary Appendix). Testing for stage 2 seropositivity will therefore have to rely on PRNT as long as no better information regarding antibody cross-reactivity between MERS and HCoVs is available. We consider PRNT to be the most specific test because of its reliance on functionality of measured antibodies, but we cannot rule out cross-reactivity for results obtained.

In our study population, we encountered household contacts in whom serum neutralizing activity became apparent only when we used a 50% reduction in the test virus as a more sensitive diagnostic criterion than 90% reduction. This suggests that neutralizing antibodies are produced at low levels and are potentially short-lived after mild or asymptomatic infection, which puts the predictive value of cross-sectional serologic studies in perspective if such studies are not specifically targeted at persons with recent exposure.

Table 3. Summary of Serologic Results in Household Contacts, According to Timing of Sampling. *

Variable	Time from Onset of Symptoms in Index Patient until Sampling of Contact			
	Any Time	<2 Wk	2–3 Wk	>3 Wk†
No. of household contacts	280	127	45	108
No. of clusters	26	10	3	13
Positive results — no. (%)				
RT-PCR	7 (2)	7 (6)	0	0
ELISA	20 (7)	8 (6)	3 (7)	11 (10)
IFA	7 (2)	1 (1)	3 (7)	3 (3)
IFA plus PRNT	5 (2)	1 (1)	3 (7)	1 (1)
Household contacts with possible or probable secondary infection — no. (%)				
Possible on basis of RT-PCR or stage 1 seropositivity	14 (5)	8 (6)‡	3 (7)	3 (3)
Positive RT-PCR assay	7 (2)	7 (6)	0	0
Stage 1 seropositivity	7 (2)	1 (1)	3 (7)	3 (3)
Probable on basis of dual positive RT-PCR assays or stage 2 seropositivity	12 (4)	8 (6)	3 (7)	1 (1)
Dual positive RT-PCR assays	7 (2)	7 (6)	0	0
Stage 2 seropositivity	5 (2)	1 (1)	3 (7)	1 (1)
Clusters of household contacts with possible or probable secondary infection — no. (%)				
Possible on basis of RT-PCR or stage 1 seropositivity‡	6 (23)	4 (40)	1 (33)	2 (15)
Positive RT-PCR assay	3 (12)	3 (30)	0	0
Stage 1 seropositivity	4 (15)	1 (10)	1 (33)	2 (15)
Probable on basis of dual positive RT-PCR assays or stage 2 seropositivity	6 (23)	4 (40)	1 (33)	1 (8)
Dual positive RT-PCR assays	3 (12)	3 (30)	0	0
Stage 2 seropositivity	3 (12)	1 (10)	1 (33)	1 (8)

* Percentages may not sum to the overall total in a category because of rounding.
† For household contacts who provided serum samples more than 3 weeks after exposure, the indicated test results are based on second samples if they were available.
‡ One cluster included a contact with positive results on RT-PCR and another with positive serologic results.

An important limitation of our study was the low rate of household contacts from whom we could obtain a second blood sample. Although the first serologic investigation was obligatory, the participation in follow-up investigations was voluntary. The sentiment in Saudi Arabia at the time that we conducted the study was dominated by stigmatization of patients with MERS and discrimination against affected families. There was a widespread belief that medical care facilities act as sources of infection, triggered by media reports focusing on hospital outbreaks, such as that in Al-Hasa.[21] For the combination of these reasons, household contacts would widely refuse to participate in any research activity connected with MERS.

Because of the limited number of follow-up samples and the variable timing of contact-cluster investigations, the timing of serologic testing after the onset of symptoms in the index patients might have influenced the diagnostic usefulness of the various tests that we used. Positive RT-PCR findings in our study were identified only during the first 14 days after exposure, whereas stage 2 seropositivity was identified only 13 days or more after exposure (Table 1). This matches expected patterns of laboratory results when infections are acquired around the time of or shortly after exposure (defined as the time of symptom onset in index patients). In particular, the timing of the first antibody results for stage 2 seropositivity matches the typical

timing for the development of an IgG response. If we had evaluated only household contacts who had provided serum samples 13 days or more after exposure and considered only serologic results, we would have identified cases of probable transmission in 5 of 172 contacts (3%; 95% confidence interval [CI], 1 to 7) in 4 of 17 clusters (24%). These data from limited testing are consistent with the overall study results on the basis of RT-PCR and serologic analysis (i.e., rates of secondary infection of 4% [95% CI, 2 to 7] among household contacts, in 23% of the clusters). Therefore, although RT-PCR testing may have compensated for a lack of serologic follow-up in household contacts who provided serum samples only during the first 2 weeks after exposure, we cannot determine whether we may have missed contacts who had delayed seroconversion. We also could not evaluate possible common sources of exposure for index patients and their household contacts.

Finally, it is relevant to note that in the whole study, we identified only 12 household contacts who may have been infected by 26 index cases. Although rates of secondary transmission may be higher in other situations, such as nosocomial outbreaks or social circumstances in which there may be a more closed or intimate environment, these data may inform approaches to estimating the epidemiologic reach of MERS-CoV in humans.[3,4]

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Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplementary Data

Table of content

1   Technical assessment of serological methods

The MERS-CoV S1 domain ELISA has been developed by authors M.A.M, B.M., and C.D. (University of Bonn) in collaboration with author E.L. at Euroimmun AG as a ready-to-use diagnostic reagent set. The antigen for the assay is provided in bulk by author B.J.B. The assay includes a constant serum reference (borderline-reactive human serum from a German patient with a titer against HCoV-OC43 but not MERS-CoV proven by rIFA) that is included in every run as a calibrator of the upper limit of the reference range of non-infected persons (cut-off). Results are provided in the form of optical density (OD) ratios as a relative measure for the concentration of antibodies. Results can be evaluated semi-quantitatively by calculating a ratio of the OD of the test sample versus that of the calibrator (OD ratio). All ELISA results given in this article are expressed as OD ratios.

As part of the assay assessment process the assay was tested on MERS-CoV antibody negative sera as follows: 3 patients with high antibody titers against influenza A (based on ELISA); 1 patient with high titers against human parainfluenzavirus 1 (based on ELISA); 1 patient with high titers against human respiratory syncytial virus type A (based on ELISA); 2 patients with proven titers against HCoV-OC43 (based on rIFA); one patient with acute *Mycoplasma pneumoniae* infection based on ELISA; 7 patients with acute Epstein Barr virus infection based on ELISA; 9 patients with antibody titer against SARS-CoV based on rIFA; 10 healthy European blood donors; 10 healthy children; 10 healthy pregnant women. Background intertest variability was studied using 28 determinations of reactivity in healthy blood donors. All of these control materials are being stored at Euroimmun, Lübeck/Germany with documentation according to good manufacturing practice.

To provide an impression of the signal-to-noise ratio in patients investigated in the present study, **Figure S1** shows the OD ratio values obtained with all primary serum samples except 6 samples from the first blood samplings, as well as all 44 follow-up serum samples.

2   Additional information on contact subjects

Information on underlying diseases was recorded on the basis of a standardized questionnaire for 178 contact cases (**Supplementary Table S1**).

3   Auxiliary analyses to reach a serological strategy for contact testing

The lack of reference samples to validate different serological methods requires a pragmatic approach to the interpretation of serological results. The following text describes essential analyses and considerations behind our choice of criteria for defining stages of laboratory evidence of infection.

3.1 IFA specificity and choice of IFA method for additional testing

Initial testing of 280 sera (first bleedings) yielded 19 sera reactive by ELISA and six sera reactive by rIFA. While the six rIFA-reactive samples were congruent with 19 samples reactive in ELISA, vIFA identified three additional reactive sera which where neither overlapping ELISA results nor reactive by NT or IgM. Similar results had been obtained earlier using vIFA during contact tracing studies in Germany and a cross-sectional serosurvey in Jeddah/KSA (**Table S3**[1,2]). These were interpreted as cross-reactive or nonspecific titers in earlier studies. To determine titers against common HCoV, differential rIFA was used under a protocol that involves screening of serum at dilutions 1:100 and 1:1000 instead of full Log_2 titration. The results for those three sera that tested positive only by vIFA are shown in **Table S2,** suggesting the presence of dominant titers against common HCoVs other than MERS-CoV in these sera. For reference we summarize the results from our earlier studies in **Table S3**. Overall these results document the limited specificity of vIFA. rIFA was therefore used for further studies.

3.2 Additional testing

To decide whether ELISA results should be tested by rIFA we relied on the assumption that true positive cases should have developed antibodies following recent contact with an index case. We therefore determined whether time since exposure correlated with rates of antibody results in contacts (**Table S4**). Day of onset of symptoms in index cases was defined as the first day of exposure. Data and samples for this validation were available for N = 268 contact subjects. As shown in the table, Chi-square and Fisher´s exact tests did not reject the assumption that positive results were more frequent in samples taken after >14 days post exposure, if based on ELISA only. However, this assumption was rejected by both tests if the definition of positive samples was based on a combination of ELISA and rIFA results. It should be noted that for the given set of samples rIFA tests alone would have led to the same outcome, as no sample tested positive by rIFA and negative by ELISA. However, ELISA is considerably easier to handle and compatible with high-throughput screening. Our diagnostic strategy based on our current knowledge therefore consists of a sequential approach including screening by ELISA and positive testing by rIFA.

3.3 Sensitivity of ELISA and rIFA

Because 14 sera reacted by ELISA but not by rIFA (13 in the first sampling of 280 subjects, 1 in the second sampling of 44 subjects), we determined whether these samples had concomitant titers against common HCoV. Differential rIFA was therefore done like above. The results for all 14 sera as shown in **Table S5** confirm the presence of titers against common HCoVs other than MERS-CoV in all sera. However, further studies will be necessary to determine whether antibodies against HCoV cross-react with MERS-CoV.

In spite this analysis suggested the excess of ELISA-positive samples to be a result of lack of specificity in ELISA rather than superior sensitivity over rIFA, it was difficult to compare sensitivity directly because this would require serum panels taken sequentially during seroconversion on different patients. For the purpose of this study, we could only use those serum pairs (of 44 in total) in which a positive ELISA result was obtained in the second bleeding sample. **Table S6** summarizes all five serum pairs in which this was the case. Because the ELISA used in these studies is calibrated against a reference serum, and because the OD values in all samples are below 0.5 suggesting no saturation of signal, it was assumed that ELISA ratio values are approximately proportional to titers. Consequently, it was assumed that a significant increase in titer over the observation period should involve an increase in ELISA ratio signal by >4 fold. This condition was only fulfilled by one of five serum pairs (No 180 marked with an asterisk). This serum pair showed a strong IFA titer in the second sample. Another serum pair (No 187) showed a constant titer in ELISA and IFA which is compatible with non-recent seroconversion. One serum (No 211) had a decreasing ELISA titer, matching the absence of any signal detectable by IFA. In summary, these results suggest that IFA may detect significant rises in antibody titers in contact cases even if not triggered by MERS-CoV specifically.

3.4 Technical considerations regarding neutralization assay format

Virus neutralization test (NT) is based on co-incubation of patient serum with a seed dose of virus in the range of 50 to 100 infectious units (plaque forming units) followed by subsequent infection in cell culture. In the context of this study we were interested to apply a highly sensitive version of NT. Sensitivity can be increased by reducing the virus

seed dose. However, seed dose should not be decreased below 50 infectious units to avoid variation due to poisson distribution effects. We therefore chose a version of NT which relies on plaque assay cell culture read out wherein the cell monolayers used to detect remaining infectious virus after serum co-incubation are overlaid with a diffusion barrier medium that reveals infection events caused by individual infectious virus particles contained in the seed dose. This assay format enabled us to determine the antibody titer that was capable of reducing the infectious virus seed dose by 50% and by 90%, instead of 100% reduction as in a normal NT. The 50% and 90% plaque reduction neutralization titers are referenced as $PRNT_{50}$ and $PRNT_{90}$. As the entry dilution in these titrations was 1:10, titers equal or greater than 1:20 were counted as significant. Results of comparative testing are summarized in the main text, **Table 2**.

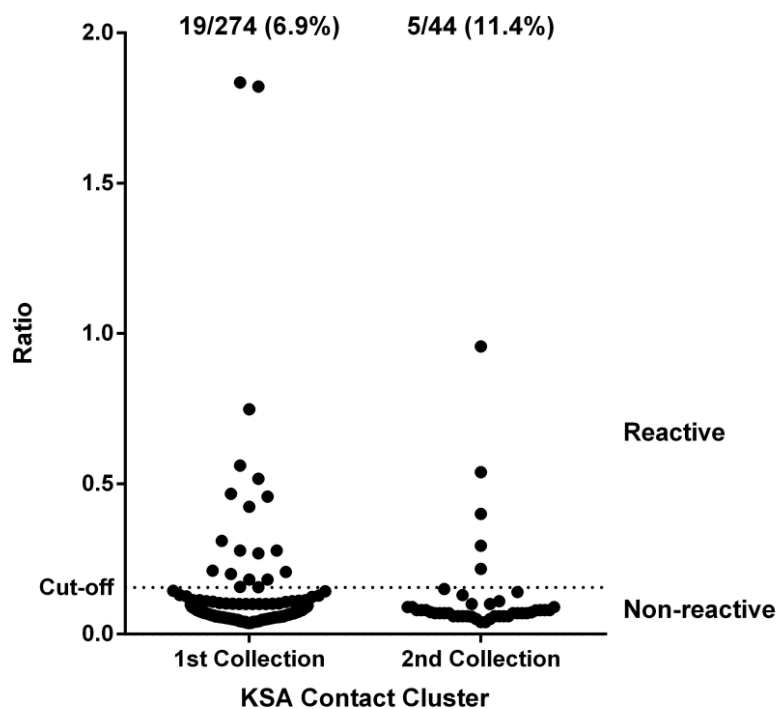## 4   Supplementary Figures

4.1   Figure S1

Figure S1. ELISA Cutoff in relation to actual OD ratios in 274 sera from contact subjects (first collection) and all 44 sera in the second collection. Sere were pre-

diluted 1:100. Optical densities (OD) were measured at 450 nm. Ratios were determined

by dividing individual OD values with a calibrator serum used in all rELISA tests.

4.2   Figure S2

```
MERS        1 MIHSVFLLMFLLTPTESYVDVGPDSVKSACIEVDIQQTFFDKTWPRPLDVSKADGIIYPQGRTYSNITITYQGLFPYQGDHGDMYVYSAGHATGTTPQKL
OC43        1 -----MFLILLISLPTAFAVIGDLKCTSDTSYINDKDTGPPPISTDTVDVTNGLGTYYVLDRVYLNTTLFLNGYYPTSGST------------------
HKU1        1 ------MLLIFILPTTLAVIGDFNCTN--FAINDLNTTVPRLSEYVVDVSYGLGTYYILDRVYLNTTLFTGVFPKSGAN-------------------
229E        1 ------------------------------------------------------------------------------------------
NL63        1 ----------MKLFLILLVLPLASCFFTCNSNANLSMLQLGVPDNSSTIVTGLLPTHWFCANQSTSVYSANGFFYIDVGN-----------------
consensus   1               ..  ...   ...       ..      .. ...  ....   ..  ...

MERS      101 FVANYSQDVKQFANGFVVRIGAAANSTGTVIISPSTSATIRKIYPAFMLGSSVGNFSDGKMGRFFNHTLVLLPDGCGTLLRAFYCILEPRSGNHCPAGNS
OC43       77 -----YRNVALKGSVLSRFWKPPFLSFINGIFAKVKNTKVIKDRVMYSEFPAILIGSTFVNTSYSVVVQPRTINTSDGYYNKLQGLLEVSVCQYNVC
HKU1       74 -----FRDLSLKGTTYLSTLWYQKPFLSFNNGIFSRVKNTKLYVNKTLYSEFSTIVIGSVFINNSYTIVVQPEH----------HNGVLEITACQYTMC
229E        1 ---------------------------------------------------------------------------------------MFVL
NL63       71 -----HRSAFALHGYYDANQVYIYVTNEIGLNASVTLKICKFSRNTTFDFLSNASSFDCIVNLLFTEQLGAPLGITISG---ETVRLHLYNVTRTFYV
consensus 101        .  .  ..  .  ..   ..            ..   .    ....   .    .. .*  * ...  ...

MERS      201 YTSEATYHTPATDCSDGNVNRNASLNSFKEYFNLRNCTFMYTYNITEDEILEWFGITQTAQGVHLFSSRYVDLYGGNMSQFATLPVYDTIKYYSIIPHSI
OC43      172 EYPQTICHPNLGNHRKELWHLDTGVVSCLYKRNFTYDVNADYLYFHFYQ-EGGTFYAYFTDTGVVTKFLFNVYLGMVLSHYYVMPLTCN
HKU1      157 EYPHTICKS-KGSSRNESWHDKSEPLCLFKKNFTYNVSTDWLYFHFYQ-ERGTFYAYMADSGMPTTFLFSLSLLGTTLSHYYVLPLTCN
229E        5 LVAYASLHIAG----------------------COTTNGLNTSYSVCNGCVGYSENVFAVESGGYLPSDFAFNNWFLTTNLSSVVDGVVRS
NL63      163 PAAMKITKLSVKCYFNYSC-----------VFSVVNAILVTVNVTTHNGRVVNYTVCDDCNGYTDNIFSVQQDGRTENGPPFNNWFLLTNGSTLVDGVSRL
consensus 201    .   .   ..  .. .        .   .   ..  ...   .. .      * * ... ..... ..

MERS      301 RSIQSDRKAWAAFYVYKLQPLTFLLDFSVDGYIRRAIDCGFNDLSQLHCSYESFDVES------GVYSVSSFEAKESGSVVEQAEGVECDFSELLSG--T
OC43      260 SKVKN--GFTLEYWVTPLTSRQYLLAFNQDGIIYNAVDCMSDFMSEIKCKTQSIAPP-----TGVYPLNGYTVQPIADVYRRKLNPNCNTEAWLNDKS
HKU1      244 AISSNTDNETLQYVVTLPLSKRQYLLKFDNRGVITNAVDCSSSFFSDIQCKTKSLLFN----TGVYDLSGFTVQPVATVYHRRIPDLPFCDTDKWLNNFN
229E       74 FQPLL---LNCLLSVSCLRFTIGFVYNGTG-RGDCKGFSSDVLSDVIRYNLNFEEN----LRRGTILFKISYGVVVFYCTNNTLVSGDAHIFGTVLGN
NL63      252 YQPLR---LTCLMPVPGLKSSIGFYFNATGSDVNCNGYQHNSVVDVMRYNLNFSANSLDNLKSGVIVFKTLQYDVLFYCSNSSSGVLDTTILFGPSSQP
consensus 301       .  .* . ....   * . *      ....   ..   .. ... . .* ... .. .   ..  .  .

MERS      393 PFQVYNFKRLVFTNCNYNLTKLLSLFSVNDFTCSQISPAAIASNCYSSLILDYFSYELSMKSDLSVSSAGPISQFNYKQSFSNPTCLILATVEHN-----
OC43      352 VPSPLNWERKTFSNCNFNMSSLMSFIQADSFTCNNIDAAKIYGMCFSSITIDKFAIPNGRKVDLQLGNLGYLQSFNYRIDTTATSCQLYYNLEAANVSVS
HKU1      338 VPSPLNWERKTFSNCNFNLSTLLRIVHTDSFSCNNFDESKIYGSCFKSIVLDKFAIPNSRRSDLQLGSSGFLQSSNYKIDTTSSSCQLYYSLEAINVTIN
229E      166 FYCFVNTTIGNETTSAFVGALPKTVREFVISRTGHFYINCYRYFTLGNVEAVNENYTTAETHDFCTVALASYADVLVNVSQTSIANTLYCNSVINRLRCD
NL63      349 YYCFINSTINTTHVSTVGILPPTVREIVVARTGQFYINCKFYDLGFIEAVNFNYTTSAIDFWTVAFTFVDVLVNVSAFNIQNLTYCDSFEKLQCE
consensus 401     . .*.  ...........   ...   .. .  ... . ...  ...*... .  .....  ... .. .

MERS      488 --------------------------LTITKPLKYSYINKCSRLLSDDRTEVPQLVNANQYSPCVSIVPS----------------------------
OC43      452 RFNPSTWNKRFGFIEDSVFKPRPAGVLANHDVVYAQHCFKAPKNFCPCKLNGSCVGSGPGKNNGIGTCPAG----TNYLTCDN------------LCTPD
HKU1      438 NYNPSSWNRRYGFNN---FN-------LSSHSVVYSRYCFSVNNTTCPCAKP-SFASSCKSHKFPSASCPIG-----TNYRSCESTTVLDHTDWCRCSCLPD
229E      266 QLS---FDVPDGFYSTSPIQS---VELPVSIVSLPVVHKCTFIVLYVDFKPQSCGKKFNCYEAGVNTTLANFNEKGPLTCVDTS------HFTTKYVAV
NL63      449 HLQ---FGLQDGFYSANFLDD-----NVLPETYVALPIMYQCTDINGTATA--SFGSCYVCKFHQVNISLN---GNTSVCVRTS------HFSIRYLYN
consensus 501     .    ..          *           ..  ..  ..           ..   ...   .

MERS      533 --LAVWEDGDYYRKQLSPLEGGGYLVASG-----------------STAVAMTEQLQMGFGITVQYG----------TDTNSVCEKLEPANDTKIASQLGNC
OC43      536 PITFKATGTYKCPQTKSLVGIGEHCSGLAVKSDYCGG------GNSCTCRPQAFLGWSADSCQQGDKCNIANTILDVNSGTTCSTLQKANTDLIIGVC
HKU1      524 PITAYDP--RSCSQKKSLVGVGEHCQGFGFGVDEEKCGVLDGSYNVSCICSTDAFLGWSYDTCVSNNRCNIPSNILGNSGTTCSNLLLQNTEVFTDVC
229E      354 YANVG-----RWSASINTGNCPISFGKVNNFVKGGSVCFSLKDIPGGCAMPIVANWAMSKYYTIC--SLYVSSDGEGITGVPQPVEGVSSFMNVTLDKC
NL63      530 RVKSGSPGDSSWHIYLKSGTCPISFSKLNNFQKLKTICFSTVEVPGSGNFPLEATWHYTSYTIVG--ALYVTWSEGNSITGVPYPVSGREFSNLVLNNC
consensus 601          *    . .    .          ..                  .    ....  ....    .. . *

MERS      604 VEYSLYGVSGRGVFQNCTAVGVRQQR-FVYDAYQNLVGYYSDDG-NYYCLRACVSVPVSVIYDKETKTHATLFGSVACEHISSTMSQYSRSTRSMLKRRD
OC43      630 VNRDLYGQFGQGIFKEVNATYYNSWQNLLYDSNGNLYGFRDYITNRTDMIRSCYSGRVSAAFHANSSEPALLFRNIKCNYVFNNS--------LTRQL
HKU1      622 VDYDLYGITGQGIFKEVSAVYYNSWQNLLYDSNGNIIGFKDFVTNKTYNIPPCYAGRVSAAFHQNASSLALLYRNLKCSYVLNN----------ISL
229E      447 TKNIYDVSGVGVIRVSNDTFLNGIT--YTSISGNLLGFKDVTKGTIYSITPCNPPDQLVYQCAVVGAMLSEN--FTSYGFSN----------------
NL63      628 TKNIIYDYVGTGIIRSSNQSLAGGIT--YVSNSGNLLGFKNVSTGNIEIVTPCNQPDQVAVYQCSIIGAMTAVN--ESRYGLQN-----------------
consensus 701 . * .*...* *...        ...     ..  ....*..*     .....    ....  ..

MERS      702 STYGPLQTPVGCVLGLVN-SSLFVEDCKLPLGQSLCALP----DIPSTLTPR
OC43      720 QPINYFDSYLGCVVNAYNSTAISVQTCDLTVGSGYCVDY-----SKNRRSRG
HKU1      709 TTQPYFDSYLGCVENADNLTDYSVSSCALRMGSGFCVDYNSPSSSSRRKRR
229E      527 ------VVELPKFYASNGTYNCTDAVLTYSSFGVCADG------------S
NL63      708 ------LLQLPNFYYVSNGGNNCTTAVMTYSNFGICADG------------S
consensus 801       . ...... * .      . ..   ..*..
```

Figure S2 Protein sequence alignment of the Spike S1 subunits of MERS-CoV
(residues 1-747), HCoV-OC43 (residues 1-766), HKU1 (residues 1-760), HCoV-229E
(residues 1-560) and HCoV-NL63 (residues 1-741)(NCBI Reference Sequences:

AFS88936.1, NP_937950.1, YP_173238.1, NP_073551.1 and YP_003767.1, respectively).
Multiple sequence alignment was performed with ClustalO and visualized with
BoxShade. Identical and similar residues are indicated with an asterisk or dot,
respectively.

5 Supplementary Tables

5.1 Table S1. Comorbidities in 178 of 280 studied subjects

Comorbidities	Number	%
Asthma	6	3.4
Diabetes	6	3.4
Arterial hypertension	1	0.6
Arterial hypertension, Diabetes	5	2.8
Arterial hypertension, Heart disease	2	1.1
Arterial hypertension, Sickle cell anemia	2	1.1
None	156	87.6
Total	178	100

5.2 Table S2. Differential rIFA assays for three sera reactive in MERS-CoV vIFA but not rIFA

Subject ID	Serum dilution	229E-S	HKU1-S	MERS-S	NL63-S	OC43-S	SARS-S
2	1:100	reactive	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	reactive	neg.
58	1:100	reactive	reactive	neg.	neg.	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	neg.	neg.
107	1:100	reactive	reactive	neg.	neg.	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	neg.	neg.

5.3 Table S3. Summary of results of vIFA and rIFA testing in 2 earlier studies

Study	vIFA	Confirmatory assay		Reference
		rIFA	NT	
Slaughterhouse study KSA	8/356 (2.2%)	0/8	0/8	[1]
Case contact study Essen, Germany	2/85 (2.4%)	0/2	0/2	[2]

5.4 Table S4. Rates of positive results by ELISA and rIFA versus time since exposure

Time since exposure	ELISA only (N reactive/ N neg.)	Statistical test	ELISA and IFA combined* (N reactive/ N neg.)	Statistical test
<14 days	8/148	Chi2=1.5; p>0.12	1/148	Chi2=4.3; p<0.018
>14 days	10/102	Fisher´s exact, p>0.16	5/102	Fisher´s exact, p<0.048

*in the dataset, this combination of results is equal to IFA results only.

5.5 Table S5. Differential rIFAs in all 14 samples reactive by ELISA but not rIFA

Subject ID	Serum Dilution	229E-S	HKU1-S	MERS-S	NL63-S	OC43-S	SARS-S
3	1:100	reactive	neg.	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	neg.	neg.
44	1:100	neg.	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	neg.	neg.
94	1:100	reactive	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	reactive	neg.
161	1:100	reactive	reactive	neg.	neg.	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	reactive	neg.
174	1:100	reactive	reactive	neg.	neg.	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	reactive	neg.
185	1:100	reactive	reactive	neg.	reactive	reactive	neg.
	1:1000	reactive	neg.	neg.	neg.	reactive	neg.
207	1:100	reactive	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	reactive	neg.
211	1:100	reactive	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	neg.	neg.
217	1:100	pos	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	neg.	neg.
225	1:100	reactive	reactive	neg.	reactive	reactive	neg.
	1:1000	reactive	neg.	neg.	neg.	reactive	neg.
229	1:100	neg.	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	neg.	neg.
249	1:100	reactive	reactive	neg.	neg.	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	reactive	neg.
106	1:100	reactive	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	reactive	neg.
39	1:100	reactive	reactive	neg.	reactive	reactive	neg.
	1:1000	neg.	neg.	neg.	neg.	reactive	neg.

5.6   Table S6. Summary of test results selected in serum pairs

Case	Time since exposure	ELISA 1st serum	ELISA 2nd serum	IFA 1st serum	IFA 2nd serum	Latency 1st to 2nd serum (days)	NT any serum	OD ratio increase
39	13	0.14	0.21	neg	neg	73	neg	no
180*	13	0.05	0.9	neg	1280	155	neg	yes
187	23	0.31	0.29	80	160	155	neg	no
211	23	0.78	0.39	neg	neg	104	neg	no
229	11	0.28	0.53	neg	neg	120	neg	no

*>4-fold increase of ELISA ratio in paired sera. Highest titers in rIFA against HCoV-OC43 and MERS-CoV. MERS-CoV NT negative.

6   References

1.   Aburizaiza AS, Mattes FM, Azhar EI, et al. Investigation of anti-middle East respiratory syndrome antibodies in blood donors and slaughterhouse workers in jeddah and makkah, saudi arabia, fall 2012. The Journal of infectious diseases 2014;209:243-6.
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