JCM
Journals.ASM.org

Real-Time Reverse Transcription-PCR Assay Panel for Middle East Respiratory Syndrome Coronavirus

Xiaoyan Lu,[a] Brett Whitaker,[a] Senthil Kumar K. Sakthivel,[a] Shifaq Kamili,[a] Laura E. Rose,[b] Luis Lowe,[b] Emad Mohareb,[c] Emad M. Elassal,[c] Tarek Al-sanouri,[d] Aktham Haddadin,[d] Dean D. Erdman[a]

Division of Viral Diseases[a] and Division of Preparedness and Emerging Infections,[b] Centers for Disease Control and Prevention, Atlanta, Georgia, USA; U.S. Naval Medical Research Unit 3, Cairo, Egypt[c]; Central Public Health Laboratory, Ministry of Health, Amman, Jordan[d]

A new human coronavirus (CoV), subsequently named Middle East respiratory syndrome (MERS)-CoV, was first reported in Saudi Arabia in September 2012. In response, we developed two real-time reverse transcription-PCR (rRT-PCR) assays targeting the MERS-CoV nucleocapsid (N) gene and evaluated these assays as a panel with a previously published assay targeting the region upstream of the MERS-CoV envelope gene (upE) for the detection and confirmation of MERS-CoV infection. All assays detected ≤10 copies/reaction of quantified RNA transcripts, with a linear dynamic range of 8 log units and 1.3×10^{-3} 50% tissue culture infective doses ($TCID_{50}$)/ml of cultured MERS-CoV per reaction. All assays performed comparably with respiratory, serum, and stool specimens spiked with cultured virus. No false-positive amplifications were obtained with other human coronaviruses or common respiratory viral pathogens or with 336 diverse clinical specimens from non-MERS-CoV cases; specimens from two confirmed MERS-CoV cases were positive with all assay signatures. In June 2012, the U.S. Food and Drug Administration authorized emergency use of the rRT-PCR assay panel as an *in vitro* diagnostic test for MERS-CoV. A kit consisting of the three assay signatures and a positive control was assembled and distributed to public health laboratories in the United States and internationally to support MERS-CoV surveillance and public health responses.

On 20 September 2012, a report appeared on ProMED-mail (http://www.promedmail.org/direct.php?id=20120920.1302733) of a novel human coronavirus (CoV) isolated several months earlier from a hospitalized patient in Saudi Arabia who had died of severe respiratory complications (1). Like the severe acute respiratory syndrome (SARS)-CoV, this new virus was most closely related to known bat coronaviruses but was genetically distinct, being classified phylogenetically in the group 2C coronavirus clade (2).

This virus was subsequently named the Middle East respiratory syndrome (MERS)-CoV because of its geographic predilection (3), and the genomic sequence obtained from this isolate was used to develop real-time reverse transcription (rRT)-PCR assays that were released on the Eurosurveillance website on 27 September 2012 (4). These assays, targeting regions upstream of the envelope gene (upE) for specimen screening and open reading frames (ORFs) 1b and later 1a (5) for test confirmation, have been used extensively to investigate the emergence of this new virus. As of 4 October 2013, 136 laboratory-confirmed cases of MERS-CoV infection, including 58 deaths, have been reported from 8 countries in the Middle East and Europe, primarily using these assays (http://www.who.int/csr/don/2013_10_04/en/index.html).

On 25 September 2012, Christian Drosten at the University of Bonn Medical Center kindly provided the U.S. Centers for Disease Control and Prevention (CDC) with sequence data for the MERS-CoV nucleocapsid (N) protein gene in advance of publication. Based on this sequence, the CDC quickly developed several rRT-PCR assays targeting the N gene to support the public health response to MERS-CoV. This report describes the validation of these assays and presents comprehensive data on the performance of the published upE assay using multiple specimen types.

(Some data from this study were presented at the 29th Clinical Virology Symposium, Daytona Beach, FL, 28 April to 1 May 2013.)

MATERIALS AND METHODS

Viruses and clinical specimens. MERS-CoV strain Jordan-N3/NCV (2012905864/VeroP1) was kindly provided by U.S. Naval Medical Research Unit 3 (NAMRU-3) (Cairo, Egypt), with permission from the Jordan Ministry of Health (MOH). Other high-titer respiratory virus stocks and virus-positive and -negative clinical specimens used for assay specificity studies were available from CDC collections. Extracts from pooled nasal wash specimens predicted to contain diverse human microbiological flora from 20 consenting healthy new military recruits were kindly provided by Lisa Lott, Eagle Applied Sciences (San Antonio, TX).

A total of 336 diverse fresh or frozen clinical specimens collected between April 2011 and April 2013 from 321 persons who had severe acute respiratory illness (SARI) and either were resident in or had a history of travel to the Middle East were available for testing. Of these, 280 were combined nasopharyngeal (NP)/oropharyngeal (OP) swab specimens collected in viral transport medium from hospitalized Jordanian children <2 years of age (15), with most of the remaining specimens being from adults. A bronchoalveolar lavage fluid sample and a serum specimen collected by the Jordan MOH Central Public Health Laboratory staff from two fatal SARI cases from a MERS-CoV pneumonia outbreak cluster at a Jordanian hospital in April 2012, and independently confirmed as positive for MERS-CoV by culture and/or sequencing by NAMRU-3, were also available for testing.

MERS-CoV culture. On receipt of the virus at the CDC, Vero E6 cell monolayers were inoculated and observed daily for cytopathic effect (CPE). At 3 to 4+ CPE, the cell culture lysate was recovered, divided into

Received 13 September 2013 Returned for modification 10 October 2013
Accepted 15 October 2013
Published ahead of print 23 October 2013
Editor: A. J. McAdam
Address correspondence to Dean D. Erdman, dde1@cdc.gov.

doi:10.1128/JCM.02533-13

TABLE 1 MERS-CoV rRT-PCR assay primer/probe sequences

Assay signature	Assay use	Genome target	Genome location	Primer or probe[a]	Sequence (5′ to 3′)	50× working concentration (μM)
upE[b]	Specimen screening	Noncoding region upstream of envelope gene	27458–27475[c]	Forward primer	GCAACGCGCGATTCAGTT	12.5
			27549–27530[c]	Reverse primer	GCCTCTACACGGGACCCATA	25
			27477–27502[c]	Probe	CTCTTCACATAATCGCCCCGAGCTCG	5
N2	Specimen screening	Nucleocapsid gene	29424–29442[c]	Forward primer	GGCACTGAGGACCCACGTT	12.5
			29498–29477[c]	Reverse primer	TTGCGACATACCCATAAAAGCA	12.5
			29445–29471[c]	Probe	CCCCAAATTGCTGAGCTTGCTCCTACA	5
N3	Specimen confirmation	Nucleocapsid gene	28748–28771[c]	Forward primer	GGGTGTACCTCTTAATGCCAATTC	25
			28814–28795[c]	Reverse primer	TCTGTCCTGTCTCCGCCAAT	25
			28773–28793[c]	Probe	ACCCCTGCGCAAAATGCTGGG	5
RP	Sample quality control	Human RNAse P gene	50–68[d]	Forward primer	AGATTTGGACCTGCGAGCG	40
			114–95[d]	Reverse primer	GAGCGGCTGTCTCCACAAGT	40
			71–93[d]	Probe	TTCTGACCTGAAGGCTCTGCGCG	10

[a] Probes were labeled at the 5′ end with the reporter molecule 6-carboxyfluorescein (6-FAM) and at the 3′ end with Black Hole Quencher 1 (BHQ1) (Biosearch Technologies Inc., Novato, CA).
[b] Primer/probe sequences from a report by Corman et al. (4).
[c] Nucleotide numbering was based on human betacoronavirus 2c EMC/2012 strain (GenBank accession number JX869059.2).
[d] Nucleotide numbering was based on human RNase P (RP) mRNA (GenBank accession number NM_006413.4).

aliquots of small volumes, and stored at −70°C or below. The titers of this stock virus were determined, and the 50% tissue culture infective dose ($TCID_{50}$) was calculated using standard methods (stock titer, 1.3×10^4 $TCID_{50}$/ml). Stock virus used in the spiking experiments described below was inactivated by gamma irradiation, and the sequence was confirmed over the rRT-PCR signature regions.

Sample processing and nucleic acid extraction. For sputum samples or other lower respiratory tract specimens too viscous for downstream nucleic acid extraction, the sample was added to an equal volume of 500 mM freshly prepared No-Weigh dithiothreitol (catalog no. 20291; Pierce) and incubated at room temperature, with intermittent mixing, for 30 min or until the sample was sufficiently liquefied for processing. For stool specimens, 10% suspensions were prepared by adding 100 μl of liquid stool or a pea-sized amount of solid stool to 900 μl of phosphate-buffered saline (pH 7.4; Gibco), pulse vortex mixing the mixture for 30 s, and centrifuging the mixture at 4,000 × *g* for 10 min at 4°C. The clarified supernatant was then carefully removed for extraction. Total nucleic acid extractions were performed on 200 μl of sample using the NucliSens easyMAG system (bioMérieux, Durham, NC), following the manufacturer's default instrument settings, and 100-μl elution volumes were collected. For some comparison studies (see below), simultaneous extractions were also performed with the MagNA Pure Compact system, using nucleic acid isolation kit I (Roche Applied Science). Extracts were either tested immediately or stored at −70°C or below until use.

Primers and probes. Multiple primer/probe sets targeting regions in the 3′, middle, and 5′ regions of the N gene sequence (GenBank accession no. JX869059.2) were designed using Primer Express software, version 3.0 (Applied Biosystems, Foster City, CA). Primer/probe sets were predicted to specifically amplify MERS-CoV with no major combined homologies with other coronaviruses or human microflora on BLASTn analysis that would potentially yield false-positive test results. All primers and probes were synthesized by standard phosphoramidite chemical techniques at the CDC Biotechnology Core Facility. Hydrolysis probes were labeled at the 5′ end with 6-carboxyfluorescein (6-FAM) and at the 3′ end with Black Hole Quencher 1 (BHQ1) (Biosearch Technologies, Inc., Novato, CA). Optimal primer/probe concentrations were determined by checkerboard titrations. Primers/probes with the highest amplification efficiencies with RNA transcripts (see below) were retained for further study (Table 1).

***In vitro* RNA transcripts and viral template control.** Single-stranded DNA oligonucleotides covering the amplified region of each rRT-PCR signature and containing a 5′ T7 RNA polymerase promoter sequence (TAATACGACTCACTATAGGG) were synthesized. The oligonucleotides were amplified using the 5′ T7 promoter sequence as the forward primer, with the corresponding rRT-PCR reverse primer for each signature (Table 1). Amplification products were transcribed using a MEGAshortscript high-yield transcription kit (Invitrogen/Life Technologies). The RNA transcripts were purified using a MEGAclear kit (Invitrogen/Life Technologies) and were quantified by UV spectroscopy. A MERS-CoV viral template control (VTC) was prepared by combining the 3 signature templates with human genomic DNA (Promega) and then drying the mixture into a visible pellet with Pellet Paint Co-Precipitate (EMD Millipore) to create a thermostable product.

Real-time RT-PCR assay. The rRT-PCR assay was performed using the Invitrogen SuperScript III Platinum One-Step quantitative RT-PCR system (Life Technologies). Each 25-μl reaction mixture contained 12.5 μl of 2× master mix, 0.5 μl of SuperScript III reverse transcriptase/Platinum *Taq* DNA polymerase, 0.5 μl of probe, 0.5 μl each of the forward and reverse primers, 5.5 μl of nuclease-free water, and 5 μl of nucleic acid extract. Amplification was carried out in 96-well plates on an Applied Biosystems 7500 Fast Dx real-time PCR instrument (Life Technologies). Thermocycling conditions consisted of 30 min at 50°C for reverse transcription, 2 min at 95°C for activation of the Platinum *Taq* DNA polymerase, and 45 cycles of 15 s at 95°C and 1 min at 55°C. Each run included one viral template control and at least two no-template controls (NTCs) for the sample extraction and reaction set-up steps. A positive test result was defined as a well-defined exponential fluorescence curve that crossed the threshold within 45 cycles. Positive viral template control (VTC) and no-template control (NTC) samples were included in all runs to monitor assay performance. All specimens were tested for the human RNase P (RP) gene by rRT-PCR to monitor nucleic acid extraction efficiency and the presence of PCR inhibitors.

RESULTS

Signatures analyzed. As noted above, multiple primer/probe sets were designed to target the MERS-CoV N gene sequence provided in advance of publication and were evaluated for optimal perfor-

TABLE 2 MERS-CoV rRT-PCR assay primer/probe sequence identity with published MERS-CoV genome sequences

Strain	Country of origin	Specimen collection date (mo/day/yr)	Sample type	GenBank accession no.	Primer/probe nt sequence identity (%)[a]				Reference no.
					upE	N1	N2	N3	
HCoV-EMC	Saudi Arabia	6/13/2012	Isolate	JX869059.2	100	100	100	100	2
Jordan-N3/2012	Jordan	4/?/2012[b]	Isolate	KC776174.1	100	100	100	100	
England 1[c]	Qatar?[b]	9/11/2012	Lower respiratory tract	KC164505.2	100	6-nt deletion	100	100	14
England/Qatar/2012[c]	Qatar?[b]	9/19/2012	Sputum	KC667074.1	100	6-nt deletion	100	100	14
England 2	Saudi Arabia?[b]	2/10/2013	Unknown	HPA website	100	100	100	100	
Munich	United Arab Emirates	3/22/2013	Unknown	KF192507.1	100	100	100	100	
Al-Hasa_1_2013	Saudi Arabia	5/9/2013	Isolate	KF186567.1	100	100	100	100	
Al-Hasa_2_2013	Saudi Arabia	4/21/2013	Isolate	KF186566.1	100	100	100	100	
Al-Hasa_3_2013	Saudi Arabia	4/22/2013	Isolate	KF186565.1	100	100	100	100	
Al-Hasa_4_2013	Saudi Arabia	5/1/2013	Isolate	KF186564.1	100	100	100	100	

[a] nt, nucleotide.
[b] ?, not definite.
[c] From the same patient.

mance with RNA transcripts. Three candidate signatures that gave the best performance, designated N1, N2, and N3, were selected for further study. However, genomic sequences obtained from clinical specimens from a Qatari patient receiving care in London, England, in September 2012 (England 1, GenBank no. KC164505.2; England/Qatar/2012, GenBank accession no. KC667074.1), which later appeared in GenBank, revealed a 6-nucleotide deletion at the 3′ end of the forward primer of N1 that would predict assay failure (Table 2). Although this deletion has not been identified among more recently published MERS-CoV genomes, the N1 signature was withdrawn from further consideration.

Analytical sensitivity. (i) Limits of detection with MERS-CoV RNA transcripts. Serial 2-fold dilutions of each quantified RNA transcript were prepared in 10 mM Tris-EDTA buffer containing 50 ng/μl yeast tRNA (Invitrogen/Life Technologies) and were tested with each assay signature in 24-fold replicates. The highest dilution of transcript at which all replicates were positive was defined as the limit of detection (LoD) for each assay. The LoD values for all assay signatures ranged from 5 to 10 RNA transcript copies/reaction (Table 3). Linear amplification was achieved over a 8-log dynamic range, from 5 to 5×10^7 copies per reaction for N assays and from 10 to 1×10^8 copies for the upE assay, with calculated efficiency values of 99.5 to 102% (Fig. 1).

(ii) Limits of detection with MERS-CoV genomic RNA. Serial 10-fold dilutions of MERS-CoV RNA extracted from a lysate of stock cultured virus were prepared in buffer as described above and were tested in triplicate with each assay signature (Table 4).

TABLE 3 MERS-CoV rRT-PCR assay limits of detection with RNA transcripts

Predicted no. of copies/reaction	No. of positive tests/no. of transcript replicates (%)		
	upE	N2	N3
20	24/24 (100)	24/24 (100)	24/24 (100)
10	24/24 (100)[a]	24/24 (100)	24/24 (100)
5	18/24 (75)	24/24 (100)[a]	24/24 (100)[a]
2.5	14/24 (58)	21/24 (87.5)	22/24 (91.7)
1.25	8/24 (33)	14/24 (58.3)	16/24 (66.7)

[a] Highest dilution at which 100% of replicates were positive.

The LoD was approximately 1.3×10^{-3} $TCID_{50}$/ml, or 2.6×10^{-5} $TCID_{50}$ per reaction (5.0 μl/reaction), for all assay signatures.

(iii) Limits of detection with MERS-CoV spiked in different clinical matrices. Serial 10-fold dilutions of MERS-CoV were spiked in different specimen matrices constructed from pooled human clinical samples, as follows: serum samples, including lipemic and hemolytic samples (10 samples); 10 NP/OP swabs in universal transport medium (Diagnostic Hybrids); 10 sputum samples; and 15 samples of 10% stool suspensions, as described above. The LoD values for all assay signatures ranged from 1.3×10^{-2} to 1.3×10^{-3} $TCID_{50}$/ml across all sample matrices (Table 5). Similar results were obtained in direct comparisons between the NucliSENS easyMAG and MagNA Pure Compact extraction systems for NP/OP swab, serum, and sputum specimens (data not shown). However, the MagNA Pure Compact system was 1 to 2 log units less sensitive with stool specimens, and these results were replicated using a second instrument and different lots of nucleic acid isolation kit I cartridges.

Analytical specificity. (i) Reactivity with different MERS-CoV strains (*in silico* prediction). In addition to demonstrating reactivity of the rRT-PCR assay with the MERS-CoV strain Jordan-N3/NCV, primer/probe sequences were evaluated against an additional 8 genome sequences obtained from 7 patients between June 2012 and May 2013, available in GenBank, as well as a Health Protection Agency (HPA) website entry (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317138176202) (Table 2). Primer/probe sequences for all signatures were 100% identical to all published virus strains.

(ii) Cross-reactivity with other respiratory viral pathogens and human microbial flora. The specificity of the MERS-CoV rRT-PCR assay was evaluated with purified nucleic acids obtained from a diverse collection of other respiratory virus isolates or positive clinical specimens, including human CoV 229E, OC43, NL63, and HKU1 and SARS (Table 6). In addition to the respiratory and stool specimens described below, pooled nasal wash fluid specimens prepared from 20 healthy adults to represent diverse microbial respiratory flora were also tested. No false-positive test results were obtained with any clinical sample.

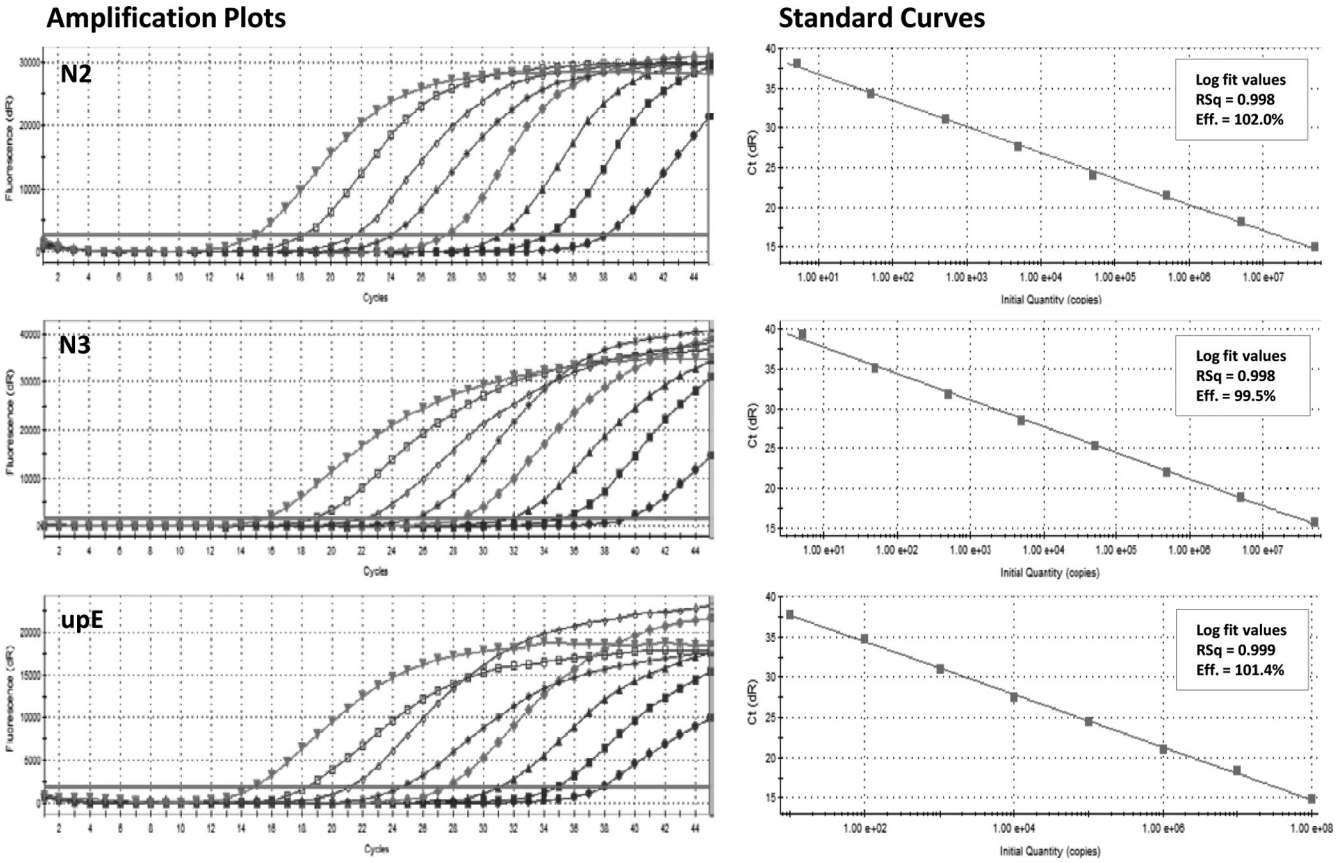

FIG 1 Plots of serial 10-fold dilutions ranging from 5 to 5×10^7 copies/reaction for the N2 and N3 RNA transcripts and 10 to 10^8 copies/reaction for the upE RNA transcripts analyzed by the MERS-CoV rRT-PCR assays. Plot inserts show calculated linear correlation coefficients (R^2) and amplification efficiencies for each assay.

Clinical studies. (i) Performance of rRT-PCR assay with authentic human clinical specimens tested during retrospective and prospective MERS-CoV surveillance. rRT-PCR results for clinical specimens obtained from persons hospitalized with SARI are shown in Table 7. Two specimens (1 bronchoalveolar lavage fluid specimen and 1 serum specimen) collected respectively from two SARI cases previously confirmed to be positive for MERS-CoV infection were positive with the three assay signatures. Of the 336 diverse clinical specimens from 321 other persons with SARI, all were negative with the corresponding assays. Assuming that all patients other than those associated with the Jordanian MERS-CoV outbreak cluster were not infected with MERS-CoV, the assay panel sensitivity, specificity, and overall agreement values were 100% (95% confidence interval [CI], 19.8% to 100%), 100% (95% CI, 98.6% to 100%), and 100% (95% CI, 98.9% to 100%), respectively.

(ii) Performance of rRT-PCR assay with contrived serum and stool specimens. To obtain additional performance data with other potentially high-value specimen types, 70 serum specimens and 70 stool specimens were obtained from the same numbers of individuals with SARI and gastroenteric illness, respectively. For each specimen type, 10 randomly selected samples were spiked

TABLE 4 MERS-CoV rRT-PCR assay limits of detection with culture-extracted MERS-CoV RNA

Virus quantity ($TCID_{50}$/ml)	upE[a]				N2				N3			
	C_T			No. positive/ no. tested	C_T			No. positive/ no. tested	C_T			No. positive/ no. tested
	Test 1	Test 2	Test 3		Test 1	Test 2	Test 3		Test 1	Test 2	Test 3	
1.3×10^{-0}	27.35	27.46	27.39	3/3	27.88	27.98	28.07	3/3	25.98	25.95	25.81	3/3
1.3×10^{-1}	31.06	31.13	31.29	3/3	31.68	31.64	31.77	3/3	29.67	29.38	29.40	3/3
1.3×10^{-2}	33.81	34.55	34.67	3/3	35.87	34.62	35.70	3/3	32.81	33.06	33.23	3/3
1.3×10^{-3}	37.03	39.01	38.94	3/3	38.74	40.01	38.11	3/3	36.60	37.28	35.82	3/3
1.3×10^{-4}	39.07	Neg	Neg	1/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3
1.3×10^{-5}	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3
1.3×10^{-6}	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3

[a] C_T, threshold cycle; Neg, negative.

TABLE 5 MERS-CoV rRT-PCR assay performance with virus-spiked specimen pools

Virus amount ($TCID_{50}$/ml)	Specimen	upE[a] C_T Test 1	upE C_T Test 2	upE C_T Test 3	upE No. positive/ no. tested	N2 C_T Test 1	N2 C_T Test 2	N2 C_T Test 3	N2 No. positive/ no. tested	N3 C_T Test 1	N3 C_T Test 2	N3 C_T Test 3	N3 No. positive/ no. tested	RP C_T Test 1	RP C_T Test 2	RP C_T Test 3	RP No. positive/ no. tested
1.3×10^{0}	NP/OP	26.08	26.35	26.32	3/3	25.88	25.92	25.86	3/3	23.38	23.50	23.51	3/3	27.62	27.66	27.72	3/3
	Sputum	26.62	26.72	26.75	3/3	26.07	26.13	26.15	3/3	23.69	23.74	23.76	3/3	23.43	23.56	23.61	3/3
	Serum	26.81	26.71	27.07	3/3	26.55	26.46	26.48	3/3	25.71	25.66	25.65	3/3	31.53	31.02	31.04	3/3
	Stool	27.24	27.67	28.05	3/3	28.26	28.34	28.45	3/3	25.13	25.40	25.56	3/3	37.36	38.15	36.74	3/3
1.3×10^{-1}	NP/OP	27.90	27.86	27.85	3/3	27.35	27.24	27.49	3/3	25.00	25.16	24.93	3/3	27.58	27.62	27.69	3/3
	Sputum	29.13	29.05	29.11	3/3	28.19	28.24	28.23	3/3	25.86	25.83	25.90	3/3	23.02	23.22	23.22	3/3
	Serum	30.18	30.76	30.75	3/3	30.11	30.26	30.23	3/3	29.30	29.26	29.37	3/3	31.19	31.04	31.42	3/3
	Stool	30.86	31.99	31.62	3/3	32.84	32.12	32.12	3/3	29.47	29.93	29.90	3/3	38.00	38.12	37.47	3/3
1.3×10^{-2}	NP/OP	34.04	34.51	34.25	3/3	34.31	33.98	34.14	3/3	31.95	31.92	31.90	3/3	27.84	27.88	27.75	3/3
	Sputum	35.29	35.59	34.88	3/3	35.35	34.67	35.52	3/3	32.57	32.69	32.27	3/3	23.28	23.28	23.28	3/3
	Serum	33.62	33.67	33.54	3/3	33.03	33.00	33.33	3/3	32.70	32.43	32.18	3/3	31.29	30.98	31.19	3/3
	Stool	34.93	35.74	35.08	3/3	36.95	37.83	36.72	3/3	32.74	33.87	33.68	3/3	36.69	38.23	38.36	3/3
1.3×10^{-3}	NP/OP	38.70	37.87	39.36	3/3	37.50	37.11	38.10	3/3	36.57	35.43	35.41	3/3	27.87	28.01	28.03	3/3
	Sputum	38.52	38.30	Neg	2/3	37.58	37.94	37.50	3/3	35.49	35.44	34.90	3/3	23.21	23.25	23.27	3/3
	Serum	40.76	37.86	37.35	3/3	36.63	37.04	37.99	3/3	36.78	36.69	36.60	3/3	31.32	31.09	31.36	3/3
	Stool	39.49	Neg	38.45	2/3	36.95	37.83	36.72	3/3	36.41	37.52	36.57	3/3	39.47	37.20	36.88	3/3
1.3×10^{-4}	NP/OP	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	28.01	28.00	27.96	3/3
	Sputum	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	24.74	24.72	24.69	3/3
	Serum	Neg	Neg	Neg	0/3	Neg	Neg	39.72	1/3	Neg	40.44	39.58	2/3	31.40	31.28	31.65	3/3
	Stool	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	Neg	Neg	Neg	0/3	37.60	37.24	36.99	3/3

[a] Neg, negative.

with moderate (1.3×10^{-1} $TCID_{50}$/ml) and 10 with low (1.3×10^{-2} $TCID_{50}$/ml) concentrations of cultured virus and 50 were left unspiked. All samples were tested in a blinded manner. The expected test results were obtained for all samples of both specimen types (Table 8).

Reproducibility studies. Assay reproducibility was evaluated with three contrived respiratory specimens constructed from pooled NP/OP swab samples as described above and spiked with high, moderate, or low concentrations of virus. Three laboratory staff members, each on a different day and blinded to content, extracted and tested the extracts in triplicate against each assay signature. Interassay variation was acceptably low for all signatures (coefficient of variation [CV] range: upE, 2.01 to 4.62%; N2, 2.81 to 8.09%; N3, 1.96 to 5.55%; RP, 1.07 to 2.26%) (Table 9).

TABLE 6 MERS-CoV rRT-PCR assay cross-reactivity with other respiratory viruses

Virus (strain)	Source	rRT-PCR result (C_T)[a] Other respiratory viruses	MERS-CoV upE	MERS-CoV N2	MERS-CoV N3
Adenovirus C1 (Ad.71)	Laboratory strain	Pos (13.7)	Neg	Neg	Neg
Coronavirus 229E	Laboratory strain	Pos (9.8)	Neg	Neg	Neg
Coronavirus OC43	Laboratory strain	Pos (12.9)	Neg	Neg	Neg
Coronavirus SARS (Urbani)	Laboratory strain	Pos (19.2)	Neg	Neg	Neg
Coronavirus HKU1	Clinical specimen	Pos (20.6)	Neg	Neg	Neg
Coronavirus NL63	Clinical specimen	Pos (19.9)	Neg	Neg	Neg
Enterovirus 68	Field isolate	Pos (21.3)	Neg	Neg	Neg
Human metapneumovirus (CAN 99–81)	Laboratory strain	Pos (15.0)	Neg	Neg	Neg
Influenza A H1N1 (A/India/2012)	Field isolate	Pos (14.7)	Neg	Neg	Neg
Influenza A H3N1 (A/Texas/2012)	Field isolate	Pos (10.7)	Neg	Neg	Neg
Influenza B (B/Massachusetts/1999)	Field isolate	Pos (8.4)	Neg	Neg	Neg
Parainfluenza 1 (C35)	Laboratory strain	Pos (16.6)	Neg	Neg	Neg
Parainfluenza 2 (Greer)	Laboratory strain	Pos (16.9)	Neg	Neg	Neg
Parainfluenza 3 (C-43)	Laboratory strain	Pos (15.2)	Neg	Neg	Neg
Parainfluenza 4a (M-25)	Laboratory strain	Pos (16.7)	Neg	Neg	Neg
Parainfluenza 4b (CH 19503)	Laboratory strain	Pos (21.5)	Neg	Neg	Neg
Parechovirus 1b	Field isolate	Pos (16.0)	Neg	Neg	Neg
Respiratory syncytial virus (Long)	Laboratory strain	Pos (15.0)	Neg	Neg	Neg
Rhinovirus 1A	Laboratory strain	Pos (13.4)	Neg	Neg	Neg

[a] Pos, positive; Neg, negative.

TABLE 7 MERS-CoV rRT-PCR assay results with 338 human specimens tested during retrospective and prospective MERS-CoV surveillance

Specimen[a]	MERS-CoV confirmed cases				Other SARI cases			
	Total no.	No. positive/no. tested			Total no.	No. positive/no. tested		
		upE	N2	N3		upE	N2	N3
URT								
NP/OP swab	0				290	0/290	0/290	0/290
Nasal swab/wash	0				2	0/2	0/2	0/2
LRT								
Sputum	0				5	0/5	0/5	0/5
Bronchoalveolar lavage fluid	1	1[b]/1	1[b]/1	1[b]/1	20	0/23	0/23	0/23
Tracheal aspirate	0				3	0/3	0/3	0/3
Lung tissue	0				4	0/4	0/4	0/4
Other								
Serum	1	1[c]/1	1[c]/1	1[c]/1	3	0/3	0/3	0/3
Stool	0				7	0/7	0/7	0/7
Pleural fluid	0				1	0/1	0/1	0/1
Urine	0				1	0/1	0/1	0/1
Total	2	2/2	2/2	2/2	336	0/336	0/336	0/336

[a] URT, upper respiratory tract; LRT, lower respiratory tract.
[b] Bronchoalveolar lavage fluid specimen: upE, $C_T = 29.5$; N2, $C_T = 27.9$; N3, $C_T = 26.2$.
[c] Serum specimen: upE, $C_T = 38.35$; N2, $C_T = 34.9$; N3, $C_T = 36.1$.

Test algorithm. An algorithm based on the three rRT-PCR assays was developed to guide specimen testing for MERS-CoV (Fig. 2). For routine specimen screening, N2 testing was combined with upE testing to theoretically enhance the detection of virus when present at low concentrations and to reduce the likelihood of false-negative results due to polymorphisms within the binding sites of the signature sequences. A positive test result with either or both assays would require confirmation with N3 testing to report a presumptive positive specimen result.

DISCUSSION

In response to the emergence of MERS-CoV in the Middle East and its spread to several European countries, the U.S. Health and Human Services announced on 29 May 2013 that the virus posed a significant public health threat to U.S. citizens. On 5 June 2013, the U.S. Food and Drug Administration authorized emergency use of the CDC rRT-PCR assay as an *in vitro* diagnostic test for the presumptive detection of MERS-CoV in patients with clinical signs and symptoms of MERS-CoV infection, in conjunction with clinical and epidemiological risk factors (http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm). Reagent kits were distributed by the CDC Laboratory Response Network to state public health departments and to select U.S. Department of Defense surveillance laboratories equipped to perform assays. The assay was also distributed to international public health partners in the affected region and to countries with extensive travel to and from the Middle East.

Our assay design and validation strategy were guided by several principles. First we chose to retain the upE signature designed by Corman et al. (4) due to its wide and successful use in MERS-CoV surveillance. A second signature developed by those authors to

TABLE 8 MERS-CoV rRT-PCR assay performance with individual virus-spiked serum and stool specimens

Virus quantity ($TCID_{50}$/ml)	Serum samples				Stool samples			
	Sample no.	C_T			Sample no.	C_T		
		upE	N2	N3		upE	N2	N3
1.3×10^{-1}	SE1	32.19	32.66	31.74	ST1	34.63	33.95	32.68
	SE2	37.08	37.57	36.98	ST2	31.35	31.17	29.37
	SE3	34.10	35.02	33.88	ST3	34.43	33.51	32.48
	SE4	34.48	34.36	33.63	ST4	34.40	33.74	32.94
	SE5	36.30	36.28	35.86	ST5	33.98	33.70	32.29
	SE6	32.40	32.42	31.82	ST6	34.59	34.21	32.89
	SE7	33.28	33.28	32.60	ST7	34.07	33.43	32.16
	SE8	36.33	36.33	36.33	ST8	33.96	33.07	31.84
	SE9	33.92	34.31	33.02	ST9	32.32	32.27	30.78
	SE10	32.43	32.63	31.69	ST10	34.57	33.91	32.86
		34.25 (32.19–37.08)[a]	34.49 (32.42–37.57)[a]	33.76 (31.69–36.98)[a]		33.83 (31.35–34.63)[a]	33.30 (31.17–34.21)[a]	32.03 (29.37–32.94)[a]
1.3×10^{-2}	SE11	35.56	35.93	35.78	ST11	37.08	37.10	35.89
	SE12	41.49	43.41	39.76	ST12	37.16	36.52	35.27
	SE13	36.38	37.96	38.96	ST13	37.36	36.16	35.54
	SE14	39.59	39.76	38.62	ST14	35.97	35.72	34.60
	SE15	36.47	36.12	35.75	ST15	37.76	37.10	36.50
	SE16	38.29	39.23	39.22	ST16	37.42	37.85	35.09
	SE17	38.52	38.08	38.11	ST17	36.96	37.00	36.11
	SE18	35.25	35.46	34.68	ST18	39.81	37.23	35.69
	SE19	38.18	40.11	39.23	ST19	37.53	37.39	36.00
	SE20	37.81	38.46	38.55	ST20	37.94	37.24	35.72
		37.75 (35.25–41.49)[a]	38.45 (35.46–43.41)[a]	37.87 (34.68–39.76)[a]		37.50 (35.97–39.81)[a]	36.93 (35.72–37.85)[a]	35.64 (34.60–36.50)[a]
No virus	SE21-70	Neg[b]	Neg	Neg	ST21-70	Neg	Neg	Neg

[a] Mean (range).
[b] Neg, negative.

TABLE 9 MERS-CoV rRT-PCR assay reproducibility with virus spiked into pooled respiratory specimens[a]

Technician and virus quantity ($TCID_{50}$/ml)	C_T											
	upE			N2			N3			RP		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Day 1, technician 1												
3.4×10^{1}	20.64	20.81	20.61	19.78	19.89	19.64	18.75	18.82	18.86	33.33	33.04	32.85
3.4×10^{-1}	27.14	26.79	27.14	25.96	25.94	25.87	24.89	25.19	24.89	32.49	32.93	33.06
3.4×10^{-3}	35.69	35.79	35.86	35.07	35.21	35.10	33.42	33.73	33.58	32.53	32.77	32.16
Day 2, technician 2												
3.4×10^{1}	22.11	22.09	21.61	22.88	22.89	22.74	20.25	20.49	20.50	32.39	31.88	32.67
3.4×10^{-1}	28.22	27.96	28.62	29.48	29.19	29.42	27.42	27.22	27.21	32.28	33.47	33.87
3.4×10^{-3}	37.59	35.32	35.30	37.63	36.74	37.02	34.38	35.23	34.52	32.12	32.32	32.71
Day 3, technician 3												
3.4×10^{1}	20.68	20.56	20.47	19.92	19.28	19.68	19.18	19.21	19.24	32.34	32.26	32.68
3.4×10^{-1}	25.57	25.41	25.34	24.55	24.60	24.49	24.13	24.08	24.18	31.84	31.76	32.06
3.4×10^{-3}	35.75	35.19	36.08	35.62	35.07	35.14	34.62	33.62	35.02	32.12	31.65	32.33
Summary (mean ± SD [CV (%)])												
3.4×10^{1}	21.06 ± 0.68 (3.21)			20.75 ± 1.58 (7.63)			19.48 ± 0.73 (3.73)			32.60 ± 0.44 (1.35)		
3.4×10^{-1}	26.91 ± 1.24 (4.62)			26.61 ± 2.15 (8.09)			25.47 ± 1.41 (5.55)			32.64 ± 0.74 (2.26)		
3.4×10^{-3}	35.84 ± 0.72 (2.01)			35.84 ± 1.01 (2.81)			34.23 ± 0.67 (1.96)			32.30 ± 0.35 (1.07)		

[a] The pool of respiratory specimens was constructed from combined nasopharyngeal/oropharyngeal swabs obtained from 10 persons.

confirm positive upE test results, targeting the MERS-CoV 1b open reading frame (ORF), proved less sensitive than upE in comparison studies (4) and was not adopted; another assay signature, targeting ORF 1a, which was claimed to be as sensitive as upE, was later introduced (5). As an alternate testing strategy, we introduced two new signatures targeting the MERS-CoV nucleocapsid (N) gene; one assay (N2) was combined with upE testing to enhance sensitivity for specimen screening, and the second assay (N3) was reserved for positive test confirmation. Theoretically, rRT-PCR assays targeting the MERS-CoV N gene should offer enhanced diagnostic sensitivity due to the relative abundance of N gene subgenomic mRNA produced during virus replication, although we found no clear evidence of this in our study and this was not shown in practice for clinical diagnosis of SARS-CoV (6). Validation of all assay signatures was conducted with multiple specimen types, including upper and lower respiratory tract specimens, serum samples, and stool specimens, all shown to be diagnostically valuable for SARS-CoV (see below). Finally, we chose to validate the assay using instruments and reagents in common use by U.S. state and international public health laboratories, to minimize the occurrence of off-protocol use of the test.

Although the MERS-CoV rRT-PCR assay panel proved both sensitive and specific, the study was subject to several limitations. First, only two authentic specimens from patients with independently confirmed MRS-CoV infection were available for testing. Most data were derived from mock specimens spiked with cultured virus, which may not accurately replicate specimens obtained during natural virus infections. Also, spiked mock specimens were not subjected to the same collection, handling, and storage conditions to which authentic specimens would be subjected, which might negatively affect virus detection. Moreover, we cannot be certain that the specimens collected from other suspected MERS-CoV cases were truly negative for the virus. However, patient demographic and clinical features, evidence of infection with other respiratory pathogens, confirmed MERS-CoV seronegativity in some cases, and the self-validating negative test results obtained with all three assay signatures support this assumption. Finally, assay validation was necessarily limited to the use of specific instrumentation, reagents, and procedures. Use of different assay platforms or modifications in methodology could negatively affect assay performance.

The choice of appropriate specimen type, collection technique, and timing after the onset of symptoms is also critical for diagnostic success. Among some patients, MERS-CoV appears to be detected more often and with higher viral loads in lower respiratory tract specimens than in specimens from the upper respiratory tract (7, 8); consequently, lower respiratory tract specimens have been prioritized for collection by the WHO and the CDC (http://www.who.int/csr/disease/coronavirus_infections/update_20121221/en/). Other specimen types, such as serum/blood samples and stool specimens, may also prove valuable. Studies performed during the SARS epidemic found that SARS-CoV could be detected in serum/blood samples during the early prodromic phase of infection (9, 10) and was shed for prolonged periods at high titers in stool, facilitating detection later in the course of illness (11, 12). MERS-CoV RNA was reported to be detected in stool and urine specimens from one infected immunosuppressed patient (7), and the virus has been identified in serum samples from other patients (this study). Testing of more samples from additional patients infected with MERS-CoV, at all stages of illness, is essential to guide testing strategies. When respiratory specimens are collected late or are not available for molecular testing, serological testing may be an effective diagnostic alternative for MERS-CoV (13).

Even when optimal specimens and molecular tests are available, accurate diagnosis of MERS-CoV infection can still be chal-

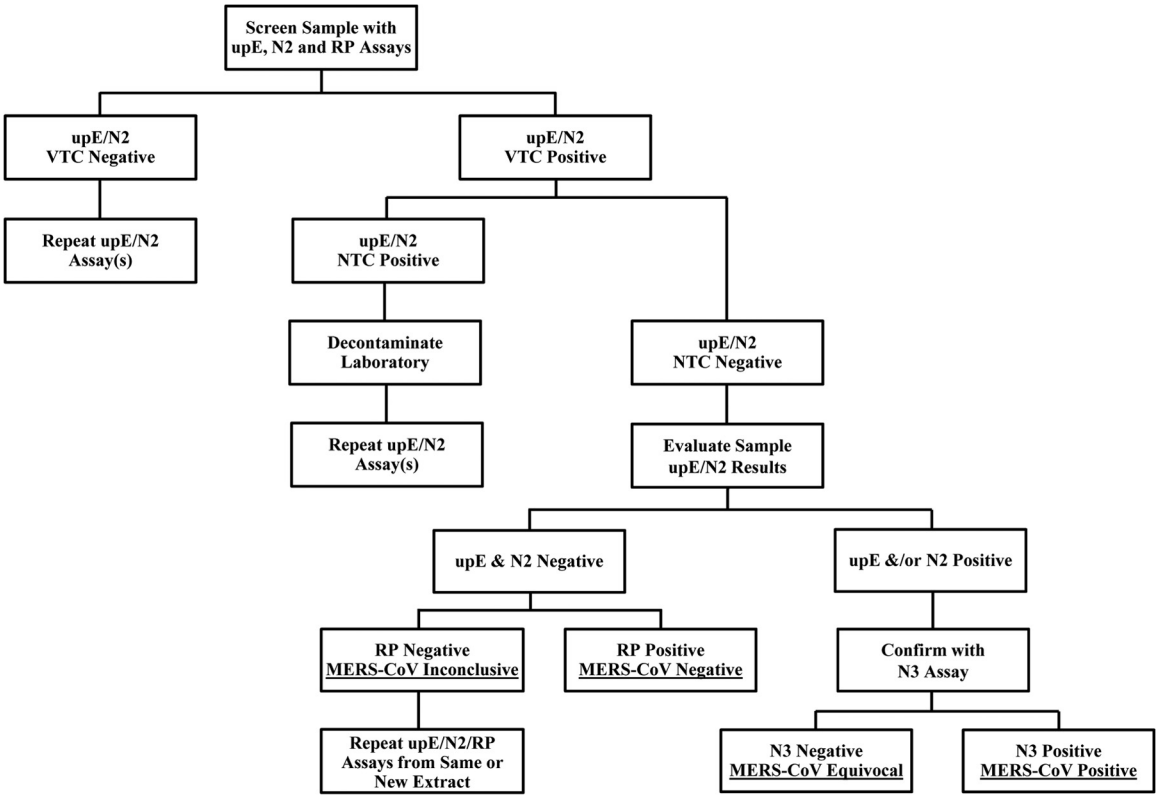

FIG 2 Algorithm for MERS-CoV rRT-PCR specimen testing and reporting of test results. For specimen screening, the N2 assay was combined with upE testing to potentially increase screening sensitivity and to reduce the possibility of false-negative results due to polymorphisms near the binding site of the oligonucleotide primers or probes. A positive test result by either or both assays requires confirmation with the N3 assay for reporting of a presumptive positive test result. VTC, viral template control; NTC, no-template control; RP, human RNase P gene.

lenging. Although rRT-PCR tests are less susceptible to amplicon contamination than are conventional RT-PCR assays, false-positive rRT-PCR results can still occur if practices designed to minimize the risk of contamination are not stringently followed. Access to multiple rRT-PCR assays targeting different regions of the MERS-CoV genome, with some assays kept in reserve for positive test result confirmation, is essential to prevent misidentifying MERS-CoV cases. It is also recommended that laboratories conducting MERS-CoV surveillance partner with the CDC or another qualified reference laboratory that can independently confirm rRT-PCR positive results by sequencing. While rRT-PCR assays are relevant for rapid diagnosis and patient management, genomic sequencing can provide public health authorities with needed confidence for response planning, can help avoid false alarms, and can provide data essential for monitoring both virus evolution and rRT-PCR assay signature integrity.

ACKNOWLEDGMENTS

We thank Christian Drosten from the University of Bonn Medical Center for providing the MERS-CoV nucleocapsid gene sequence. We also thank others at the CDC who contributed substantially to this effort, including Kanwar Bedi, Willie Betts, Michael Farrell, Matthew Marcum, Eileen Schneider, Yvonne Stifel, Nicky Sulaiman, and Azaibi Tamin.

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the CDC or the U.S. Department of Health and Human Services (DHHS). Names of specific vendors, manufacturers, or products are included for public health and informational purposes; inclusion does not imply endorsement of the vendors, manufacturers, or products by the CDC or the DHHS.

REFERENCES

1. **Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA.** 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N. Engl. J. Med. **367:**1814–1820. http://dx.doi.org/10.1056/NEJMoa1211721.
2. **van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, Osterhaus AD, Haagmans BL, Gorbalenya AE, Snijder EJ, Fouchier RA.** 2012. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. mBio **3:**e00473-12. http://dx.doi.org/10.1128/mBio.00473-12.
3. **de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, Fouchier RA, Galiano M, Gorbalenya AE, Memish ZA, Perlman S, Poon LL, Snijder EJ, Stephens GM, Woo PC, Zaki AM, Zambon M, Ziebuhr J.** 2013. Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. J. Virol. **87:**7790–7792. http://dx.doi.org/10.1128/JVI.01244-13.
4. **Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, van Boheemen S, Gopal R, Ballhause M, Bestebroer TM, Muth D, Muller MA, Drexler JF, Zambon M, Osterhaus AD, Fouchier RM, Drosten C.** 2012. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. **17:**20285. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20285.
5. **Corman VM, Muller MA, Costabel U, Timm J, Binger T, Meyer B, Kreher P, Lattwein E, Eschbach-Bludau M, Nitsche A, Bleicker T, Landt O, Schweiger B, Drexler JF, Osterhaus AD, Haagmans BL, Dittmer U, Bonin F, Wolff T, Drosten C.** 2012. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill. **17:**20334. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20334.
6. **Poon LL, Chan KH, Wong OK, Cheung TK, Ng I, Zheng B, Seto WH,**

Yuen KY, Guan Y, Peiris JS. 2004. Detection of SARS coronavirus in patients with severe acute respiratory syndrome by conventional and real-time quantitative reverse transcription-PCR assays. Clin. Chem. **50:**67–72. http://dx.doi.org/10.1373/clinchem.2003.023663.

7. **Drosten C, Seilmaier M, Corman VM, Hartmann W, Scheible G, Sack S, Guggemos W, Kallies R, Muth D, Junglen S, Muller MA, Haas W, Guberina H, Rohnisch T, Schmid-Wendtner M, Aldabbagh S, Dittmer U, Gold H, Graf P, Bonin F, Rambaut A, Wendtner CM.** 2013. Clinical features and virological analysis of a case of Middle East respiratory syndrome coronavirus infection. Lancet Infect. Dis. **13:**745–751. http://dx.doi.org/10.1016/S1473-3099(13)70154-3.
8. **Guery B, Poissy J, el Mansouf L, Sejourne C, Ettahar N, Lemaire X, Vuotto F, Goffard A, Behillil S, Enouf V, Caro V, Mailles A, Che D, Manuguerra JC, Mathieu D, Fontanet A, van der Werf S, MERS-CoV Study Group.** 2013. Clinical features and viral diagnosis of two cases of infection with Middle East respiratory syndrome coronavirus: a report of nosocomial transmission. Lancet **381:**2265–2272. http://dx.doi.org/10.1016/S0140-6736(13)60982-4.
9. **Wang H, Mao Y, Ju L, Zhang J, Liu Z, Zhou X, Li Q, Wang Y, Kim S, Zhang L.** 2004. Detection and monitoring of SARS coronavirus in the plasma and peripheral blood lymphocytes of patients with severe acute respiratory syndrome. Clin. Chem. **50:**1237–1240. http://dx.doi.org/10.1373/clinchem.2004.031237.
10. **Grant PR, Garson JA, Tedder RS, Chan PK, Tam JS, Sung JJ.** 2003. Detection of SARS coronavirus in plasma by real-time RT-PCR. N. Engl. J. Med. **349:**2468–2469. http://dx.doi.org/10.1056/NEJM200312183492522.
11. **Cheng PK, Wong DA, Tong LK, Ip SM, Lo AC, Lau CS, Yeung EY, Lim WW.** 2004. Viral shedding patterns of coronavirus in patients with probable severe acute respiratory syndrome. Lancet **363:**1699–1700. http://dx.doi.org/10.1016/S0140-6736(04)16255-7.
12. **Peiris JS, Chu CM, Cheng VC, Chan KS, Hung IF, Poon LL, Law KI, Tang BS, Hon TY, Chan CS, Chan KH, Ng JS, Zheng BJ, Ng WL, Lai RW, Guan Y, Yuen KY, HKU/UCH SARS Study Group.** 2003. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet **361:**1767–1772. http://dx.doi.org/10.1016/S0140-6736(03)13412-5.
13. **Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, Muth D, Raj VS, Vries LS, Corman VM, Drexler JF, Smits SL, El Tahir YE, De Sousa R, van Beek J, Nowotny N, van Maanen K, Hidalgo-Hermoso E, Bosch BJ, Rottier P, Osterhaus A, Gortazar-Schmidt C, Drosten C, Koopmans MP.** 2013. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infect. Dis. **13:**859–866. http://dx.doi.org/10.1016/S1473-3099(13)70164-6.
14. **Cotten M, Lam TT, Watson SJ, Palser AL, Petrova V, Grant P, Pybus OG, Rambaut A, Guan Y, Pillay D, Kellam P, Nastouli E.** 2013. Full-genome deep sequencing and phylogenetic analysis of novel human betacoronavirus. Emerg. Infect. Dis. **19:**736–742B. http://dx.doi.org/10.3201/eid1905.130057.
15. **Khuri-Bulos N, Payne DC, Lu X, Erdman D, Faouri S, Shehabi A, Johnson M, Becker MM, Denison MR, Williams JV, Halasa NB.** Novel human coronavirus not detected in children hospitalized with acute respiratory illness in Amman, Jordan, March 2010–September 2012. Clin. Microbiol. Infect., in press.