Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Clinical Virology 64 (2015) 83–87

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Reliable typing of *MERS-CoV* variants with a small genome fragment

Saskia L. Smits[a,b], V. Stalin Raj[a], Suzan D. Pas[a], Chantal B.E.M. Reusken[a], Khaled Mohran[c,d], Elmoubasher A.B.A. Farag[e], Hamad E. Al-Romaihi[e], Mohd M. AlHajri[e], Bart L. Haagmans[a], Marion P. Koopmans[a,f,*]

[a] *Department of Viroscience, Erasmus Medical Center, P.O. Box 2040, 3000 CA Rotterdam, Netherlands*
[b] *ViroClinics BioSciences BV, Marconistraat 16, 3029 AK Rotterdam, Netherlands*
[c] *Ministry of the Environment, Doha, Qatar*
[d] *Biotechnology Research Department, Animal Health Research Institute, Agricultural Research Center, Egypt*
[e] *Supreme Council of Health, Doha, Qatar*
[f] *Virology Division, Centre for Infectious Diseases Research, Diagnostics and Screening, National Institute for Public Health and the Environment, Bilthoven 3720BA, Netherlands*

ARTICLE INFO

Article history:
Received 19 August 2014
Received in revised form 6 November 2014
Accepted 8 December 2014

Keywords:
MERS-CoV
Diversity
Camel
Human
Type
Surveillance

ABSTRACT

Background: Middle East Respiratory Syndrome coronavirus (*MERS-CoV*) is an emerging pathogen that causes lower respiratory tract infection in humans. Camels are the likely animal source for zoonotic infection, although exact transmission modes remain to be determined. Human-to-human transmission occurs sporadically. The wide geographic distribution of *MERS-CoV* among dromedary camels and ongoing transmissions to humans provides concern for the evolution of a *MERS-CoV* variant with efficient human-to-human transmission capabilities. Phylogenetic analysis of *MERS-CoV* has occurred by analysis of full-length genomes or multiple concatenated genome fragments, which is time-consuming, costly and limited to high viral load samples.
Objective: To develop a simple, reliable *MERS-CoV* variant typing assay to facilitate monitoring of *MERS-CoV* diversity in animals and humans.
Study Design: Phylogenetic analysis of presently known full-length *MERS-CoV* genomes was performed to identify genomic regions with sufficient phylogenetic content to allow reliable *MERS-CoV* variant typing. RT-PCR assays targeting these regions were designed and optimized.
Results: A reverse-transcription PCR assay for *MERS-CoV* targeting a 615 bp spike fragment provides a phylogenetic clustering of *MERS-CoV* variants comparable to that of full-length genomes. The detection limit corresponds to a cycle treshold value of ~35 with standard upE real time PCR assays on RNA isolated from *MERS-CoV* EMC. Nasal swabs from RT-PCR positive camels (Ct values 12.9–32.2) yielded reliable sequence information in 14 samples.
Conclusions: We developed a simple, reliable *MERS-CoV* variant typing assay which is crucial in monitoring *MERS-CoV* circulation in real time with relatively little investment on location.

1. Background

Middle East Respiratory Syndrome coronavirus (*MERS-CoV; Family Coronaviridae*) may cause severe lower respiratory tract infection in humans [1,2]. Camels are considered the likely animal source for zoonotic infection; sporadic human-to-human transmission does occur, but is considered to be inefficient based on currently available data [3–10]. Until recently, new *MERS-CoV* infections in humans were reported at a steady low rate reaching ~200 confirmed human cases early 2014. In March and April 2014, however, a surge of *MERS-CoV* infections occurred mainly in hospitals around Jeddah, Kingdom of Saudi Arabia (KSA) and United Arab Emirates (UAE). This increased case load was in part attributed to an increase in community cases, but mostly to transmission within hospitals, with no evidence for evolution of a *MERS-CoV* variant with more efficient human-to-human transmission capabilities (WHO;http://www.who.int/csr/disease/coronavirus_infections/MERS_CoV_Update_09_May_2014.pdf?ua=1). The ongoing occurrence of new cases, however, and the finding that *MERS-CoV*

* Corresponding author at: Department of Viroscience, Erasmus Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, the Netherlands. Tel.: +31 10 7044515; fax: +31 10 7044760.
E-mail address: m.koopmans@erasmusmc.nl (M.P. Koopmans).

http://dx.doi.org/10.1016/j.jcv.2014.12.006

Table 1
Viral genomes used in this study.

Group name	Sequences	Accession number
RIYADH_9_2013	RIYADH_9_2013	KJ156869
	RIYADH_2_2012	KF600652
AL-HASA_15_2013	AL-HASA_19_2013	KF600632
	BURAIDAH_1_2013	KF600630
	AL-HASA_2_2013	KF186566
	AL-HASA_21_2013	KF600634
	AL-HASA_25_2013	KJ156866
	AL-HASA_17_2013	KF600647
	AL-HASA_15_2013	KF600645
	AL-HASA_16_2013	KF600644
	AL-HASA_12_2013	KF600627
	AL-HASA_3_2013	KF186565
	AL-HASA_4_2013	KF186564
	AL-HASA_18_2013	KF600651
	AL-HASA_1_2013	KF186567
	AL-HASA_15_2013	KF600645
FRA/UAE_2012	KFU_HKU1_2013	KJ650297
	KFU_HKU19DAM2013	KJ650296
	KFU_HKU13_2013	KJ650295
	FRA/UAE_2012	KF745068
HAFR-AL-BATIN_6_2013	HAFR-AL-BATIN_6_2013	KJ156874
	HAFR-AL-BATIN_2_2013	KJ156910
	HAFR-AL-BATIN_1_2013	KF600628
RIYADH_5_2013	RIYADH_5_2013	KJ156944
	RIYADH_4_2013	KJ156952
	TAIF_1_2013	KJ156949
	JEDDAH_1_2013	KJ556336
	WADI-AD-DAWASIR_1_2013	KJ156881
QATAR_3_2013	QATAR_3_2013	KF961221
	QATAR_4_2013	KF961222
RIYADH_1_2012	RIYADH_1_2012	KF600612
	BISHA_1_2012	KF600620
RIYADH_3_2013	RIYADH_3_2013	KF600613
Egypt_Camel_NRCE-HKU205_2014	Egypt_Camel_NRCE-HKU205_2014	KJ477102
ENGLAND/QATAR_2012	ENGLAND/QATAR_2012	KC667074
EMC_2012	EMC_2012	JX869059
RIYADH_14_2013	RIYADH_14_2013	KJ156934
Munich/AbuDhabi_2013	Munich/AbuDhabi_2013	KF192507
Qatar_Camel_2_2014	Qatar_Camel_2_2014	KJ650098
JORDAN/N3_2012	JORDAN/N3_2012	KC776174
	Not in variant analysis	
	KSA_CAMEL_363_2013	KJ713298
	KSA_CAMEL_376_2013	KJ713299
	KSA_Camel_378_2013	KJ713296
	KSA_CAMEL_503_2013	KJ713297
	KSA_CAMEL_505_2013	KJ713295
	Jeddah_Camel_1_2013	KF917527
	Jeddah_Human_1_2013	KF958702
	Florida/USA_2_Saudi Arabia_2014	KJ829365
	Indiana/USA-1_Saudi Arabia_2014	KJ813439

is endemic in dromedary camels in a wide geographic region [3,10,11], stresses the need for surveillance of strain diversity, to help unravel the epidemiology of this newly identified pathogen, and to provide a reference for studies into *MERS-CoV* evolution.

All currently sequenced human and camel *MERS-CoV* genomes share >99% nucleotide identity across the ~30 kb genome. Phylogenetic analysis has occurred mainly by analysing full-length genomes or multiple concatenated genome fragments, to provide reliable phylogenetic information [5,12–14]. However, full length genome sequencing capacity is not widely available, and requires relatively high viral load, leading to limited success when trying to sequence animal or human samples [5].

2. Objectives

An accurate typing of *MERS-CoV* variants, preferably with a simple assay encompassing a short region of the *MERS-CoV* genome, is crucial in monitoring the *MERS-CoV* outbreak in real time with relatively little investments on location. In this study, we describe the development of a *MERS-CoV* variant typing assay, which can be used in monitoring *MERS-CoV* circulation, especially when more information on virus type is required rapidly from a large number of viruses from animals/humans.

3. Study design

3.1. In silico analysis

Full or near full-length *MERS-CoV* genomes encompassing nucleotides 215–29770 (numbering corresponding to *MERS-CoV* EMC genome JX869059) were aligned with MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) (Table 1). To remove the redundancy from the dataset, FastGroupII analysis (http://fastgroup.sdsu.edu/fg_tools.htm) was performed grouping all currently available viral genomes based on nucleotide composition, resulting in 15 groups (Table 1). One viral genome from each group was taken as representative in subsequent analyses. A summary of nucleotide positions that vary across the

genomes was created using BioEdit v7.2.0 [15] and the number of nucleotide positions with variations was plotted over 1000 nucleotide windows. PhyML trees were generated using Seaview 4 software with the approximate likelihood ratio test based on a Shimodaira–Hasegawa-like procedure which used general time reversible as substitution model. Nearest neighbor interchange, subtree pruning, and regrafting-based tree search algorithms were used to estimate tree topologies, as described previously [5].

3.2. MERS-CoV typing RT-PCR

Total nucleic acids were isolated using an automated MagNAPure 96 extraction with the total nucleic acid isolation kit (Roche, Mannheim, Germany) as described previously [13]. The partial S2 domain of *MERS-CoV* spike (corresponding to nucleotides 23781–24395 of *MERS-CoV* EMC genome JX869059) was amplified with the OneStep RT-PCR Kit (Qiagen) using 1x QIAGEN OneStep RT-PCR Buffer, 400 μM of each dNTP, 2 μl QIAGEN OneStep RT-PCR Enzyme Mix and 0.6 μM of primers VS804 (5′-TCAGGTTGATCAACTTAATAGT-3′) and VS805 (5′-TTGAGTAATGCCAACACCGTT-3′) in a volume of 25 μl; 30 min 50 °C, 15 min 95 °C, 40 cycles of 0.5 min 94 °C, 0.5 min 50 °C, 1 min 72 °C, and a final extension of 10 min 72 °C. A nested PCR was performed using 1x PCR Buffer, 2 mM MgCl2, 200 μM of each dNTP, 20 μM primers VS804 and VS805 and 2.5 units HotStarTaq DNA polymerase (Qiagen) in a volume of 50 μl; 15 min 95 °C, 40 cycles of 0.5 min 94 °C, 0.5 min 50 °C, 1 min 72 °C, and a final extension of 10 min 72 °C. Amplicons were sequenced directly on both strands with the BigDye Terminator version 3.1 cycle sequencing kit on an ABI PRISM 3100 genetic analyser (Applied Biosystems).

3.3. Samples

The detection limit of the assay was determined on RNA isolated from 10x dilutions of cell culture derived *MERS-CoV* EMC_2012 (JX869059), as described above. Virus stocks were prepared as described previously [16]. RNA was isolated from serial 10-fold dilutions of *MERS-CoV* EMC_2012 [13]. Serial 10-fold dilutions of this RNA were amplified in parallel with the *MERS-CoV* variant typing assay described above and the upE and N gene real time PCR assays [17,18]. The sensitivity of the *MERS-CoV* variant typing assay was expressed as cycle threshold value based on the upE real time PCR assay.

On May 13 and 15, 2014, the first two *MERS-CoV* infected patients in the Netherlands who became infected upon travel to Saudi Arabia were reported to WHO; throat swabs from these patients were available [19].

In February and April 2014, nasal swabs were taken from dromedary camels of different age and sex from a slaughterhouse in Doha, Qatar [13], which were available for this study.

4. Results

To identify genome regions for reliable phylogenetic analysis comparable to that of full-length genome, (near) full-length *MERS-CoV* genomes (Table 1) were aligned. Viral genomes that were 99.9% identical were grouped and one viral genome from each group was taken as representative in the analysis (Table 1) and a PhyML tree was generated (Fig. 1A). Four major *MERS-CoV* clusters can be discerned, represented by *MERS-CoV* Qatar_3_2014, England/Qatar_2012, EMC_2012, and Egypt_Camel_NRCE-HKU205_2014 (KF961221, KC667074, JX869059, KJ477102), which have been identified previously in analyses based on full-length genomes or concatenated genome fragments [5,12–14]. The number of single nucleotide polymorphisms (SNPs) across the 15 representative *MERS-CoV* genomes

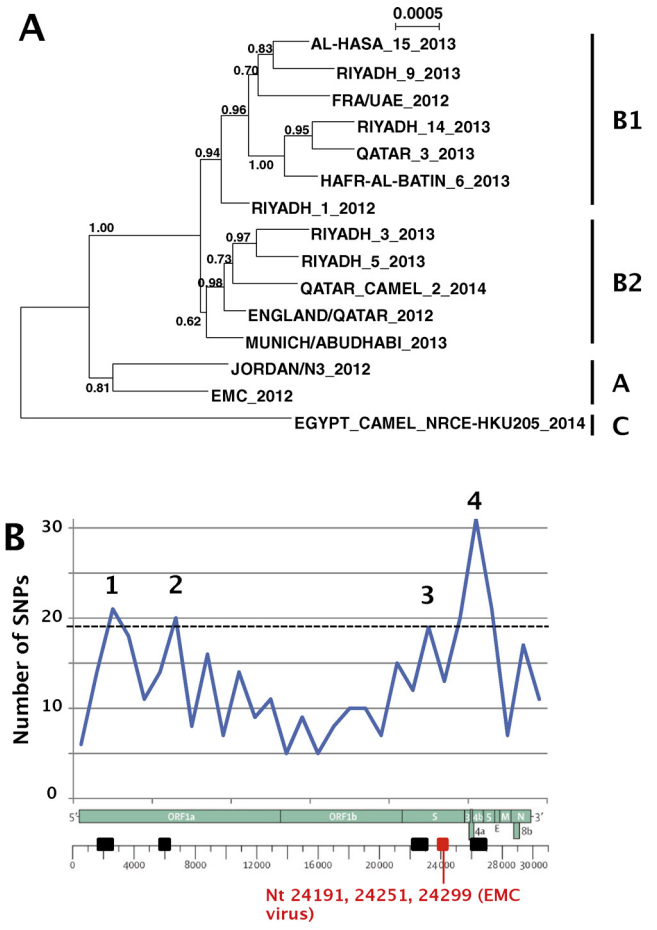

Fig. 1. Characterization of *MERS-CoV* variation. (A) PhyML tree of full-length genome sequences of 15 distinct MERS coronavirus variants based on analysis of nucleotide sequence diversity across the genome. Four major *MERS-CoV* clusters are indicated (A, B1, B2, C). (B) The number of nucleotide positions with variations over 15 representative *MERS-CoV* genomes (Supplementary Table 1) was plotted over 1000 nucleotide windows. A schematic diagram of the *MERS-CoV* genome is depicted below the graph, and four fragments with the highest number of single nucleotide polymorphisms (SNPs) are indicated with black boxes. The dotted line indicates the average number of SNPs per 1000 nt window added with 1x standard deviation. In red, nucleotide positions displaying considerable phylogenetic information regarding four identified *MERS-CoV* variant clusters and the red box indicates the 615 bp fragment of the here-described *MERS-CoV* variant typing assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was plotted over 1000 nt windows (Fig. 1B). Four genome fragments, two located in ORF1a, one in S and one in ORF4b, showed a relatively high number of SNPs (Fig. 1B) and for this reason already had been used as concatenated genome fragment for phylogenetic analysis [12]. However, phylogenetic trees created for these four fragments separately did not accurately reflect the phylogenetic positions of the currently known full-length *MERS-CoV* genomes (data not shown).

In a subsequent analysis all identified SNPs were inspected visually and regions containing SNPs with phylogenetic information regarding the previously identified four clusters of viruses were identified (Fig. 1A). The S2 domain of the spike protein contains a number of these mutations (Fig. 1B). A fragment of 615 bp, containing three of these SNPs, provided a phylogenetic tree similar to the one obtained upon full-genome analysis regarding the previously identified four *MERS-CoV* clusters (Fig. 2), whereas other *MERS-CoV* genome regions did not provide similar results. As observed for the full-length genomes, human, and camel *MERS-CoV* genomes shared >99% nucleotide identity across the 615 bp S2 domain fragment.

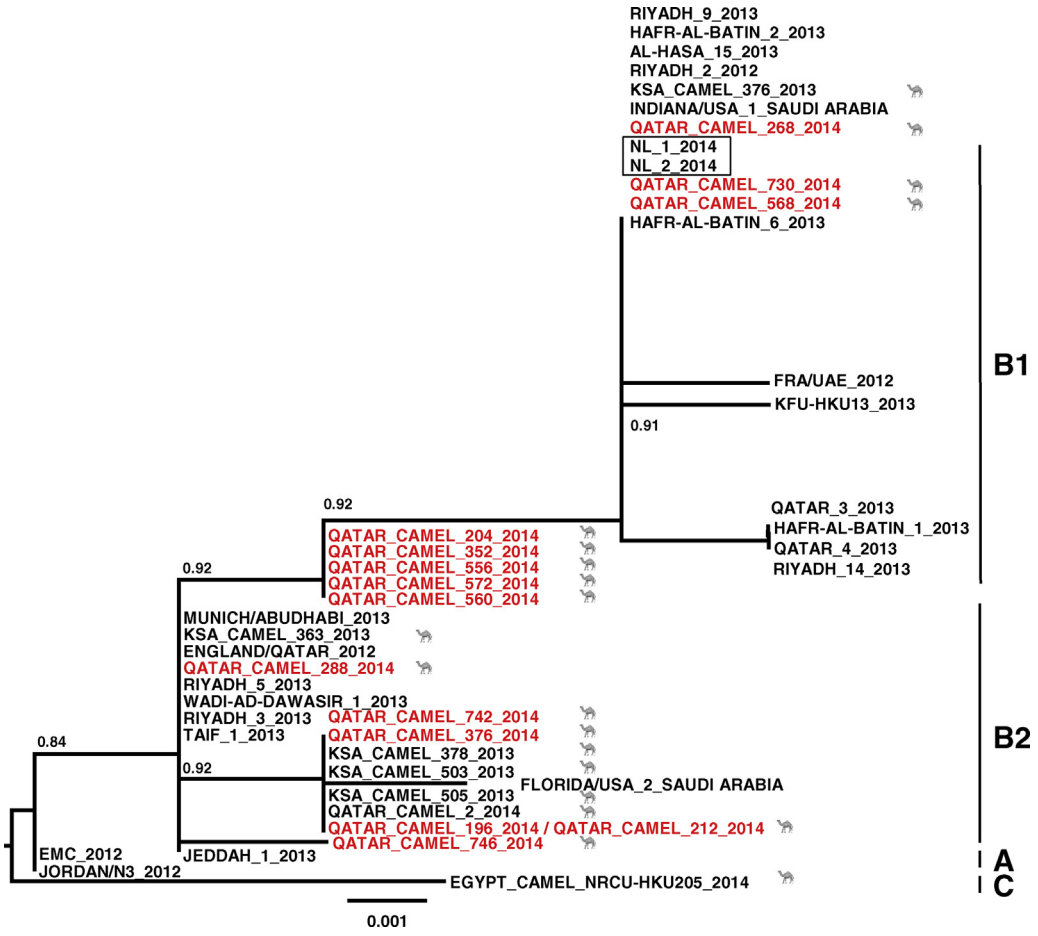

Fig. 2. Phylogenetic analysis of human and camel *MERS-CoVs* using the *MERS-CoV* variant typing assay. A PhyML tree was generated from a spike S2 domain genome fragment corresponding to nt 23781–24395 of *MERS-CoV* EMC genome (accession number JX869059) for known *MERS-CoV* genomes (Table 1) and the first two Dutch *MERS-CoV* patients (boxed). In red are newly identified camel sequences from a slaughterhouse in Qatar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MERS-CoV genomes that were released recently and not taken along in the variation analysis were typed using the 615 bp S2 domain and clustered similar to their phylogenetic positions as upon full genome analysis (Fig. 2 and Table 1), thereby validating the assay.

A sequencing RT-PCR targeting the identified genome fragment was developed and optimized using RNA isolated from cell culture-derived *MERS-CoV*. In limiting dilution experiments, the *MERS-CoV* variant typing assay amplified the 615 bp fragment down to a cycle treshold value of ~35 as determined by diagnostic upE real time PCR assays [17,18]. The *MERS-CoV* variant typing assay was used to type RT-PCR positive nose swabs from the first two human Dutch *MERS-CoV* cases in 2014. The results showed grouping consistent with previous findings based on long sequence fragments [19] (Fig. 2). In addition, the *MERS-CoV* variant typing assay was performed on camel samples from a slaughterhouse in Qatar [13] and sequences for 14 *MERS-CoV* positive animals with cycle threshold values ranging from 12.9 to 32.2 as determined by UpE real time RT-PCR [17,18] were obtained (Fig. 2). Five different camel *MERS-CoV* variants in clusters B1 and B2 were detected, without the need for full genome sequencing.

5. Discussion

Phylogenetic analysis of representative *MERS-CoV* full-length genomes indicated that four regions in the *MERS-CoV* genome exist with a substantially higher nucleotide variation across genomes. However, phylogenetic analysis of these genome regions separately did not provide reliable phylogenetic information, in contrast to an analysis of the concatenated fragments [5,12,19]. Subsequent analyses revealed a region in the open reading frame that encodes the spike protein with a number of positions in which nucleotide variation occurs between *MERS-CoV* variants with a strong phylogenetic signal regarding previously identified clusters of viruses based on full-length *MERS-CoV* genomes. The here-described *MERS-CoV* variant typing assay based on a 615 bp spike fragment provides a crude indication of the *MERS-CoV* variant under study on the basis of four identified clusters of *MERS-CoV* variants, exemplified by *MERS-CoV* Qatar_3_2014, England/Qatar_2012, EMC_2012, and Egypt_Camel_NRCE-HKU205_2014. In silico, the assay accurately typed all currently known *MERS-CoVs* for which full-length genomes are available, including the recently released Florida/USA_2_KSA_2014 and Indiana/USA-1_KSA_2014 strains. This was confirmed by typing of samples available in our laboratory from the first two Dutch *MERS-CoV* cases that phylogenetically grouped with Indiana/USA-1_KSA_2014 and other viruses in cluster I [19] and from camels from a slaughterhouse in Doha, Qatar [13]. The observed detection limit of a cycle treshold value of ~35 allows variant typing in clinical samples obtained from humans and animals with relatively low viral loads. It enables inclusion of samples in phylogenetic analysis that would not have been included when only full length genomes would have been accepted.

This *MERS-CoV* variant typing assay, targeting a part of the *MERS-CoV* spike gene, is a relatively simple RT-PCR sequencing assay that could be performed more widely as initial screening

assay in laboratories with basic sequencing capacity. It provides accurate crude *MERS-CoV* type information, applicable in monitoring viral variants in real time. New variants identified through this initial screening could then be sent to a reference laboratory for further characterization. The continued occurrence of transmission between humans in health care and family settings is an ongoing concern as stated by the World Health Organization (http://www.who.int/csr/disease/coronavirus_infections/MERS_CoV_Update_27_March_2014.pdf?ua=1), although the outbreaks appear to be self-limiting or extinguishable with rigorous implementation of appropriate infection control guidelines at present. However, as the primary route of transmission to humans is uninterrupted, human-to-human transmissions will continue to occur. The data obtained from the *MERS-CoV* variant typing assay would aid in informing the most effective international preparedness and response, allowing ad hoc risk assessment and implementation of containment strategies if necessary.

Conflict of interest

Saskia L. Smits is part time employed by Viroclinics Biosciences BV. This does not alter our adherence to all the policies on sharing data and materials.

Bart Haagmans has a patent on *MERS-CoV* field.

Funding

This work was funded by ZonMW TOP project 91213058.

Ethical approval

All procedures were performed in compliance with relevant laws and institutional guidelines and in accordance with the Declaration of Helsinki.

Authors' contributions

All authors contributed to gathering and analysis of the information. Saskia Smits, Bart Haagmans, and Marion Koopmans drafted and revised the manuscript based on all authors contributions.

Acknowledgment

This work was funded by ZonMW TOP project 91213058Z. This does not alter our adherence to all the policies on sharing data and materials.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2014.12.006.

References

[1] The Who Mers-Cov Research Group, State of knowledge and data gaps of *Middle East Respiratory Syndrome coronavirus* (*MERS-CoV*) in humans, PLoS Curr. (2013) 5.
[2] A.M. Zaki, S. van Boheemen, T.M. Bestebroer, A.D. Osterhaus, R.A. Fouchier, Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia, N. Engl. J. Med. 367 (2012) 1814–1820.
[3] A.N. Alagaili, T. Briese, N. Mishra, V. Kapoor, S.C. Sameroff, P.D. Burbelo, E. de Wit, V.J. Munster, L.E. Hensley, I.S. Zalmout, A. Kapoor, J.H. Epstein, W.B. Karesh, P. Daszak, O.B. Mohammed, W.I. Lipkin, *Middle East respiratory syndrome coronavirus* infection in dromedary camels in Saudi Arabia, MBio 5 (2014) e00884–00814.
[4] D.K. Chu, L.L. Poon, M.M. Gomaa, M.M. Shehata, R.A. Perera, D. Abu Zeid, A.S. El Rifay, L.Y. Siu, Y. Guan, R.J. Webby, M.A. Ali, M. Peiris, G. Kayali, MERS coronaviruses in dromedary camels, Egypt, Emerg. Infect. Dis. 20 (2014) 1049–1053.
[5] B.L. Haagmans, S.H. Al Dhahiry, C.B. Reusken, V.S. Raj, M. Galiano, R. Myers, G.J. Godeke, M. Jonges, E. Farag, A. Diab, H. Ghobashy, F. Alhajri, M. Al-Thani, S.A. Al-Marri, H.E. Al Romaihi, A. Al Khal, A. Bermingham, A.D. Osterhaus, M.M. AlHajri, M.P. Koopmans, *Middle East respiratory syndrome coronavirus* in dromedary camels: an outbreak investigation, Lancet Infect. Dis. 14 (2014) 140–145.
[6] M.G. Hemida, R.A. Perera, P. Wang, M.A. Alhammadi, L.Y. Siu, M. Li, L.L. Poon, L. Saif, A. Alnaeem, M. Peiris, Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013, Euro Surveill. 18 (2013) 20659.
[7] Z.A. Memish, M. Cotten, B. Meyer, S.J. Watson, A.J. Alsahafi, A.A. Al Rabeeah, V.M. Corman, A. Sieberg, H.Q. Makhdoom, A. Assiri, M. Al Masri, S. Aldabbagh, B.J. Bosch, M. Beer, M.A. Muller, P. Kellam, C. Drosten, Human infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013, Emerg. Infect. Dis. 20 (2014) 1012–1015.
[8] B. Meyer, M.A. Muller, V.M. Corman, C.B. Reusken, D. Ritz, G.J. Godeke, E. Lattwein, S. Kallies, A. Siemens, J. van Beek, J.F. Drexler, D. Muth, B.J. Bosch, U. Wernery, M.P. Koopmans, R. Wernery, C. Drosten, Antibodies against MERS coronavirus in dromedary camels, United Arab Emirates, 2003 and 2013, Emerg. Infect. Dis. 20 (2014) 552–559.
[9] R.A. Perera, P. Wang, M.R. Gomaa, R. El-Shesheny, A. Kandeil, O. Bagato, L.Y. Siu, M.M. Shehata, A.S. Kayed, Y. Moatasim, M. Li, L.L. Poon, Y. Guan, R.J. Webby, M.A. Ali, J.S. Peiris, G. Kayali, Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013, Euro Surveill. 18 (2013) 20574.
[10] C.B. Reusken, B.L. Haagmans, M.A. Muller, C. Gutierrez, G.J. Godeke, B. Meyer, D. Muth, V.S. Raj, L. Smits-De Vries, V.M. Corman, J.F. Drexler, S.L. Smits, Y.E. El Tahir, R. De Sousa, J. van Beek, N. Nowotny, K. van Maanen, E. Hidalgo-Hermoso, B.J. Bosch, P. Rottier, A. Osterhaus, C. Gortazar-Schmidt, C. Drosten, M.P. Koopmans, *Middle East respiratory syndrome coronavirus* neutralising serum antibodies in dromedary camels: a comparative serological study, Lancet Infect. Dis. 13 (2013) 859–866.
[11] T. Briese, N. Mishra, K. Jain, I.S. Zalmout, O.J. Jabado, W.B. Karesh, P. Daszak, O.B. Mohammed, A.N. Alagaili, W.I. Lipkin, *Middle East respiratory syndrome coronavirus* quasispecies that include homologues of human isolates revealed through whole-genome analysis and virus cultured from dromedary camels in Saudi Arabia, MBio 5 (2014) e01146–01114.
[12] N. Nowotny, J. Kolodziejek, *Middle East respiratory syndrome coronavirus* (*MERS-CoV*) in dromedary camels, Oman, 2013, Euro Surveill. 19 (2014) 20781.
[13] V.S. Raj, E.A.B.A. Farag, C.B.E.M. Reusken, M.M. Lamers, S.D. Pas, J. Voermans, S.L. Smits, A.D.M.E. Osterhaus, N. Al-Mawlawi, H.E. Al-Romaihi, M.M. Alhajri, A.M. El-Sayed, K.A. Mohran, H. Ghobashy, F. Alhajri, M. Al-Thani, S.A. Al-Marri, M.M. El-Maghraby, M.P.G. Koopmans, B.L. Haagmans, Isolation of MERS coronavirus from a dromedary camel, Qatar, 2014, Emerg. Infect. Dis. 20 (2014), Ahead of print.
[14] M. Cotten, S.J. Watson, P. Kellam, A.A. Al-Rabeeah, H.Q. Makhdoom, A. Assiri, J.A. Al-Tawfiq, R.F. Alhakeem, H. Madani, F.A. AlRabiah, S. Al Hajjar, W.N. Al-nassir, A. Albarrak, H. Flemban, H.H. Balkhy, S. Alsubaie, A.L. Palser, A. Gall, R. Bashford-Rogers, A. Rambaut, A.I. Zumla, Z.A. Memish, Transmission and evolution of the *Middle East respiratory syndrome coronavirus* in Saudi Arabia: a descriptive genomic study, Lancet 382 (2013) 1993–2002.
[15] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucl. Acids Symp. Ser. 41 (1999) 95–98.
[16] S. van Boheemen, M. de Graaf, C. Lauber, T.M. Bestebroer, V.S. Raj, A.M. Zaki, A.D. Osterhaus, B.L. Haagmans, A.E. Gorbalenya, E.J. Snijder, R.A. Fouchier, Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans, MBio 3 (2012) e00473–00412.
[17] V.M. Corman, I. Eckerle, T. Bleicker, A. Zaki, O. Landt, M. Eschbach-Bludau, S. van Boheemen, R. Gopal, M. Ballhause, T.M. Bestebroer, D. Muth, M.A. Muller, J.F. Drexler, M. Zambon, A.D. Osterhaus, R.M. Fouchier, C. Drosten, Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction, Euro Surveill. 17 (2012) 20285.
[18] V.M. Corman, M.A. Muller, U. Costabel, J. Timm, T. Binger, B. Meyer, P. Kreher, E. Lattwein, M. Eschbach-Bludau, A. Nitsche, T. Bleicker, O. Landt, B. Schweiger, J.F. Drexler, A.D. Osterhaus, B.L. Haagmans, U. Dittmer, F. Bonin, T. Wolff, C. Drosten, Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections, Euro Surveill. 17 (2012) 20334.
[19] Kraaij–Dirkzwager M, Timen A, Dirksen K, Gelinck L, Leyten E, Groeneveld P, Jansen C, Jonges M, Raj S, Thurkow I, van Gageldonk-Lafeber R, van der Eijk A, Koopmans M, Netherlands obotM-Coitot. 2014. *Middle East respiratory syndrome coronavirus* (*MERS-CoV*) infections in two returning travellers in the Netherlands, May 2014. Eurosurveillance 19:20817.