

## ORIGINAL ARTICLE

# A phylogenetically distinct Middle East respiratory syndrome coronavirus detected in a dromedary calf from a closed dairy herd in Dubai with rising seroprevalence with age

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Middle East respiratory syndrome coronavirus (MERS-CoV) was detected by monoclonal antibody-based nucleocapsid protein-capture enzyme-linked immunosorbent assay (ELISA), RNA detection, and viral culture from the nasal sample of a 1-month-old dromedary calf in Dubai with sudden death. Whole genome phylogeny showed that this MERS-CoV strain did not cluster with the other MERS-CoV strains from Dubai that we reported recently. Instead, it formed a unique branch more closely related to other MERS-CoV strains from patients in Qatar and Hafr-Al-Batin in Saudi Arabia, as well as the MERS-CoV strains from patients in the recent Korean outbreak, in which the index patient acquired the infection during travel in the eastern part of the Arabian Peninsula. Non-synonymous mutations, resulting in 11 unique amino acid differences, were observed between the MERS-CoV genome from the present study and all the other available MERS-CoV genomes. Among these 11 unique amino acid differences, four were found in ORF1ab, three were found in the S1 domain of the spike protein, and one each was found in the proteins encoded by ORF4b, ORF5, envelope gene, and ORF8. MERS-CoV detection for all other 254 dromedaries in this closed dairy herd was negative by nucleocapsid protein-capture ELISA and RNA detection. MERS-CoV IgG sero-positivity gradually increased in dromedary calves with increasing age, with positivity rates of 75% at zero to three months, 79% at four months, 89% at five to six months, and 90% at seven to twelve months. The development of a rapid antigen detection kit for instantaneous diagnosis is warranted.

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## INTRODUCTION

Since its first appearance in 2012, the Middle East respiratory syndrome (MERS) has affected more than 25 countries in four continents, with more than 1300 cases and a frightening fatality rate of greater than 30%.<sup>1</sup> A novel lineage C betacoronavirus the MERS coronavirus (MERS-CoV), has been confirmed to be the etiological agent.<sup>2,3</sup> The subsequent detection of MERS-CoV and its antibodies in dromedaries in various countries in the Middle East has led to the hypothesis that these animals may be the reservoir for MERS-CoV.<sup>4–13</sup> Furthermore, the discovery of other closely related lineage C betacoronaviruses both before and after the beginning of the MERS epidemic in various bat species and hedgehogs,<sup>14–18</sup> as well as the demonstration of the ability of the spike protein of *Tyloaestheris* bat CoV HKU4 to bind to dipeptidyl peptidase 4 (DPP4), the receptor of MERS-CoV, have also suggested that these animals may be the hosts for the ancestor of MERS-CoV.<sup>19–22</sup>

Although MERS-CoV can be cultured and antibody detection methods are available, laboratory diagnosis and epidemiology studies of MERS-CoV infections are mainly achieved by real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR), using the RNA-dependent RNA polymerase, the region upstream of the envelope gene or the nucleocapsid (N) gene of MERS-CoV as the target.<sup>1,23–27</sup> However, the cost of real-time qRT-PCR is still high and such expertise may not be available, particularly in some clinical microbiology laboratories in the Middle East where most of the cases were identified. Because enzyme-linked immunosorbent assay (ELISA) for antigen detection offers an inexpensive and user-friendly approach for laboratory diagnosis and epidemiological studies of respiratory viral infections, including severe acute respiratory syndrome,<sup>28,29</sup> we recently developed a monoclonal antibody-based capture ELISA for the detection of MERS-CoV N protein.<sup>30</sup> In this study, we describe the application of N protein ELISA, real-time

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qRT-PCR, isothermal amplification, virus isolation, and antibody detection in a cross-sectional epidemiological study of a dromedary dairy herd in Dubai after sudden death and the detection of MERS-CoV from a dromedary calf within the herd.

## MATERIALS AND METHODS

### Animals and samples collection

The 1-month-old female dromedary calf with sudden death belonged to a closed dairy herd reared for milk production, which roamed a small desert area in Dubai, the United Arab Emirates. In addition to the calf that succumbed, samples were collected from the remaining 254 dromedaries in the herd, comprising 133 adult female dromedaries and 121 dromedary calves. There were no adult male dromedaries in the herd and mating was human-assisted.

### Capture ELISA for detection of MERS-CoV N protein

MERS-CoV N protein ELISA on nasal swabs was performed as previously described.<sup>30</sup> Briefly, microplates (Sigma-Aldrich, St. Louis, MO, USA) were pre-coated with the first anti-MERS-CoV-recombinant N protein monoclonal antibody 1F6 and incubated at 37 °C overnight with a blocking reagent (phosphate-buffered saline with 2% sucrose, 0.2% casein-Na, and 2% gelatin). Fifty microliters of viral lysis buffer was added to each coated well and 50 µL of each nasal sample was then added to the wells in duplicate. The plate was shaken for 2 min and incubated at 37 °C for 30 min. After the wells were washed five times, 100 µL of the second anti-MERS-CoV-recombinant N protein monoclonal antibody 7C4 conjugated with horseradish peroxidase was added and the plate was incubated at 37 °C for 30 min. After five washes, detection was carried out by adding 100 µL of tetramethylbenzidine per well, incubating the samples for 10 min, and adding 50 µL of 0.2 M H<sub>2</sub>SO<sub>4</sub>. The optical density (OD) 450/630 nm was measured with a microplate reader.

### Real-time qRT-PCR screening assay

MERS-CoV nucleic acid detection on the nasal swabs and milk was performed using a real-time qRT-PCR assay targeting the region upstream of the envelope gene and by isothermal amplification using a Genie instrument (OptiGene Limited, Horsham, UK). For real-time qRT-PCR assays, a 25-µL reaction was set up containing 5 µL of RNA, 12.5 µL of 2× reaction buffer provided with the Superscript III one-step RT-PCR system with Invitrogen Platinum Taq Polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA); containing 0.4 mM of each dNTP and 3.2 mM magnesium sulfate, 1 µL of reverse transcriptase/Taq mixture from the kit, 0.4 µL of a 50 mM magnesium sulfate solution (Thermo Fisher Scientific, Inc.), 1 µg of non-acetylated bovine serum albumin (Sigma-Aldrich), 400 nM concentrations of primer upE-Fwd (GCA ACG CGC GAT TCA GTT) and primer upE-Rev (GCC TCT ACA CGG GAC CCA TA), as well as a 200 nM concentration of probe upE-Prb (6-carboxyfluorescein (FAM))-CTC TTC ACA TAA TCG CCC CGA GCT CG-6-carboxy-N,N,N,N'-tetramethylrhodamine (TAMRA)). The thermal cycling protocol was 55 °C for 20 min, followed by 95 °C for 3 min and then 45 cycles of 95 °C for 15 s and 58 °C for 30 s.

### Viral culture

MERS-CoV isolates from nasal swabs were grown on African green monkey kidney (Vero) cells.<sup>2,31,32</sup> Samples were inoculated in Vero cells at a multiplicity of infection of 1.0 for 1 h. Unattached viruses were removed by washing the cells twice in serum-free Gibco minimum essential medium (Thermo Fisher Scientific, Inc.). The

monolayer cells were maintained in minimum essential medium with 1% fetal calf serum. All infected cell lines were incubated at 37 °C for 5 days. Cytopathic effects were examined on days 1, 3, and 5 by inverted light microscopy.

### MERS-CoV IgG antibody detection

All serum samples were tested for MERS-CoV IgG antibodies using Euroimmun ELISA (Euroimmun AG, Lübeck, Germany). Samples were applied at a 1:101 dilution and incubated in the wells of microtiter strips coated with the purified spike protein subdomain S1 antigen of MERS-CoV. A ratio of >1.0 was considered to be positive.

### Complete genome sequencing and analysis

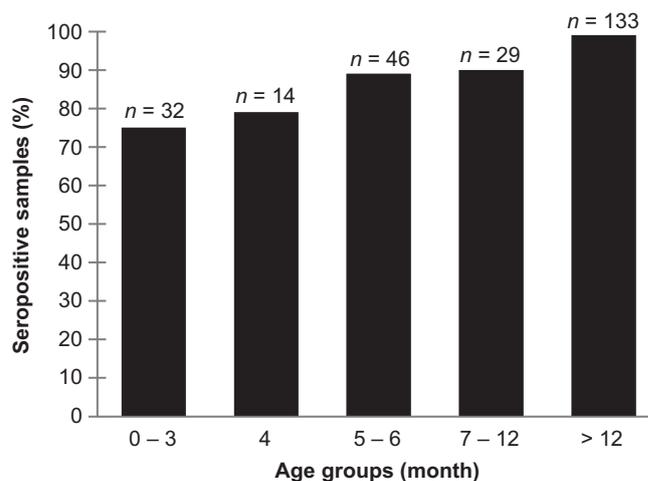
The complete genome of the MERS-CoV isolate was amplified and sequenced as described previously.<sup>33–36</sup> RNA extracted from a nasal specimen was used as the template. Sequences were assembled and edited to produce final sequences. For phylogenetic analysis, a maximum likelihood phylogenetic tree with 1000 bootstraps was constructed using PhyML v3.0, based on complete concatenated coding sequences of 68 available human MERS-CoV and 18 camel MERS-CoV genomes. A general time-reversible model of nucleotide substitution with estimated base frequencies, a proportion of invariant sites, and a gamma distribution of rates across sites was applied to conduct maximum likelihood analysis.

### Unique mutations in the genome

The nucleotide sequences of the genomes and the deduced amino acid sequences of the MERS-CoV sequenced in this study were compared to 68 available human MERS-CoV and 18 camel MERS-CoV genomes. The nucleotide and amino acid sequences of each coding sequence were aligned using MAFFT version 7.<sup>37</sup> Unique mutations resulting in amino acid substitutions at the coding sequences were revealed.

### Nucleotide sequence accession numbers

The nucleotide sequence of the genome of the camel MERS-CoV in this study has been submitted to the GenBank sequence database under accession NO KT751244.



**Figure 1** Sero-positivity of the 254 dromedaries in the herd tested in the present study.

## RESULTS

### Necropsy, capture ELISA for detection of the MERS-CoV N protein, real-time qRT-PCR screening assay and viral culture

Necropsy of the dromedary calf with sudden death showed that the cause of death was cryptosporidiosis and colisepticemia. The nasal sample, but not trachea, lung, and tonsil samples collected from the dromedary calf were positive for the N protein (OD 3.712, OD of negative control 0.065), real-time qRT-PCR, and virus isolation. In contrast, the nasal swabs of the other 254 dromedaries as well as the milk produced by the 133 female adult dromedaries were all negative for the N protein, real-time qRT-PCR, and virus isolation.

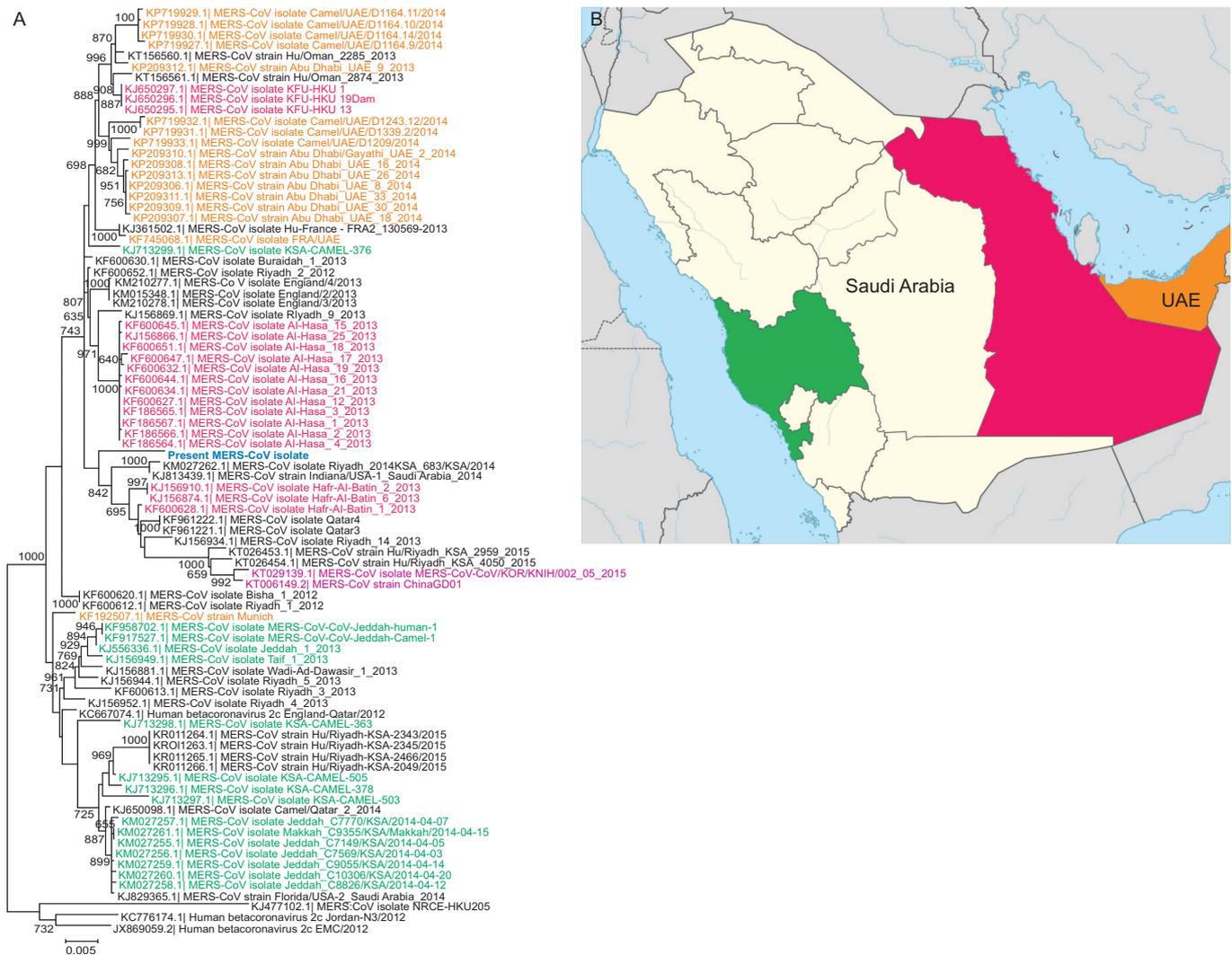
### MERS-CoV IgG antibody detection

MERS-CoV IgG was positive in the sera of 132 (99%) adult dromedaries and 102 (84%) dromedary calves. This IgG positivity showed a

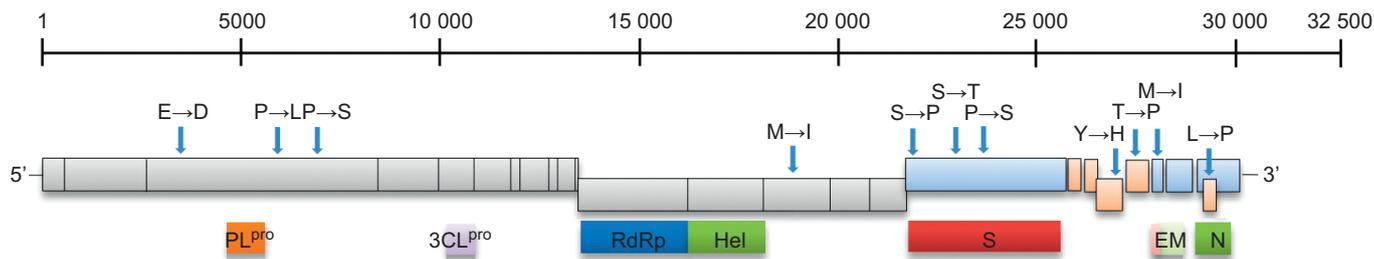
gradual increase in dromedary calves with increasing age, with positivity rates of 75% in dromedary calves of age 0–3 months, 79% at 4 months, 89% at 5–6 months, and 90% at 7–12 months (Figure 1).

### Complete genome sequencing and phylogenetic analysis

Complete genome sequencing of the MERS-CoV isolated from the nasal swab of the dromedary calf with sudden death, as well as phylogenetic analysis, showed that this MERS-CoV strain did not cluster with the other MERS-CoV strains from Dubai that we reported recently (Figure 2).<sup>38</sup> Instead, it formed a unique branch more closely related to other MERS-CoV strains from patients in Qatar and Hafr-Al-Batin in Saudi Arabia. Notably, the MERS-CoV strains from patients in the recent Korean outbreak, in which the index patient acquired infection during travel in the eastern part of the Arabian Peninsula, also belonged to this cluster (Figure 2).



**Figure 2** (A) Maximum likelihood phylogeny based on complete concatenated coding sequences of 68 available human MERS-CoV and 18 camel MERS-CoV genomes. A general time-reversible model of nucleotide substitution with estimated base frequencies, a proportion of invariant sites, and a gamma distribution of rates across sites was applied to conduct maximum likelihood analysis. Bootstrap values (1000 replicates) are shown next to the branches. A scale bar indicates the number of nucleotide substitutions per site. The MERS-CoV strain from the present study is shown in blue, those from eastern and western Saudi Arabia are in magenta and green, respectively, other strains from the United Arab Emirates are in orange, and those from the recent Korean outbreak are in purple. (B) Map of the Arabian Peninsula, with the colors of different geographical regions corresponding to those in panel A.



**Figure 3** Map of unique mutations in the camel MERS-CoV genome, with the nucleotide positions shown on the scale above it. Blue arrows indicate unique mutations observed in the MERS-CoV genome. Amino acid changes are shown above the arrows.

### Unique mutations in the genome

Using the available complete genomes of MERS-CoV as references, non-synonymous mutations, resulting in 11 unique amino acid differences, were observed between the MERS-CoV genome from the present study and all the other available MERS-CoV genomes (Figure 3). Among these 11 unique amino acid differences, four were found in ORF1ab (E1059D, P1951L, P2269S, and M6117I), three were found in the S1 domain of the spike protein (S28P, S459T, and P710S), and one each was found in the proteins encoded by ORF4b, ORF5, the envelope gene and ORF8.

### DISCUSSION

We report the first cross-sectional epidemiology study of MERS-CoV in a closed dromedary dairy herd using antigen, nucleic acid, and antibody detection and virus isolation. Similarly to results from previous reports, MERS-CoV was detected in a dromedary calf.<sup>39,40</sup> This dromedary calf with MERS-CoV did not show any respiratory clinical signs and had died of cryptosporidiosis and colisepticemia. This is in line with our observation that other dromedary calves with sudden death and MERS-CoV detected have also died of various diseases, such as salmonellosis, clostridial enterotoxemia, and isoporiosis instead of pneumonia, and MERS-CoV was detected in the animals only incidentally (unpublished data). Notably, the MERS-CoV strain isolated in the present study clustered with other patient strains from east Saudi Arabia and Qatar, including the one from a Korean patient who acquired infection in this area. This is in contrast to the MERS-CoV strains reported in our previous study in Dubai,<sup>38</sup> which formed another cluster not closely related to the present strain. This indicated that more than one strain of MERS-CoV is circulating in Dubai. Notably, among the three amino acid changes located at the S1 domain of the spike protein as a result of non-synonymous mutations, none are located at the receptor-binding subdomain, suggesting that the camel MERS-CoV is still likely to bind human receptor DPP4. The significance of these mutations is unclear and needs to be investigated further. The dromedaries in this study belonged to a closed herd that did not have contact with other herds because they were browsing in a confined area. It is believed that the virus could be circulating in the herd, which was probably infected in Saudi Arabia several years ago when the dams were calves. Alternatively, the deceased calf may have been infected from an unknown external source.

The present data showed increasing sero-positivity with advancing age in dromedary calves. It has been reported that the prevalence of MERS-CoV antibodies is significantly higher in dromedary adults than in calves.<sup>4,5,9–12</sup> In this study, we further demonstrated that the seroprevalence in dromedary calves increased gradually as their ages increased, with a sero-positivity of almost 100% in adult dromedaries. This is in line with the observation that MERS-CoV infections occur almost exclusively in dromedary calves after their maternal antibodies

have disappeared. In fact, we have tested samples from the nose, trachea, tonsil, lung, uterus, and placenta of more than 100 adult female dromedaries dispatched to our veterinary laboratory for necropsy, and all have shown negative results (unpublished data).

The high specificity of the N protein ELISA is confirmed in this field study. Recently, we reported the development of a monoclonal antibody-based capture ELISA for MERS-CoV N protein and evaluated its specificity using nasopharyngeal specimens from patients with various common respiratory virus infections, including the four human CoVs, and its sensitivity using simulated nasopharyngeal specimens.<sup>30</sup> We argue that the ELISA may be used in animal field studies. In the present study, results of the N protein ELISA showed a concordance with those obtained from nucleic acid detection using dromedary nasal samples, indicating that the ELISA was highly specific. It is notable that there is one amino acid difference (L178V) between the N protein sequence of the MERS-CoV isolate of the present study and the original MERS-CoV isolate we used to generate the monoclonal antibodies for the N protein ELISA. This N protein ELISA would probably be useful in laboratories in the Middle East and North Africa, where resources and expertise may be limited. Further refinements of the test and the development of a rapid antigen detection kit for rapid diagnoses are warranted.

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- 1 Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen KY. Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. *Clin Microbiol Rev* 2015; **28**: 465–522.
- 2 Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 2012; **367**: 1814–1820.
- 3 van Boheemen S, de Graaf M, Lauber C *et al*. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *mBio* 2012; **3**: e00473-12
- 4 Alagaili AN, Briese T, Mishra N *et al*. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. *mBio* 2014; **5**: e00884–00814.
- 5 Meyer B, Muller MA, Corman VM *et al*. Antibodies against MERS coronavirus in dromedary camels, United Arab Emirates, 2003 and 2013. *Emerg Infect Dis* 2014; **20**: 552–559.

- 6 Perera RA, Wang P, Gomaa MR *et al*. 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* 2013; **18**: pii=20574.
- 7 Woo PC, Lau SK, Wernery U *et al*. Novel betacoronavirus in dromedaries of the Middle East, 2013. *Emerg Infect Dis* 2014; **20**: 560–572.
- 8 Reusken CB, Haagmans BL, Muller MA *et al*. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. *Lancet Infect Dis* 2013; **13**: 859–866.
- 9 Hemida MG, Perera RA, Al Jassim RA *et al*. Seroepidemiology of Middle East respiratory syndrome (MERS) coronavirus in Saudi Arabia (1993) and Australia (2014) and characterisation of assay specificity. *Euro Surveill* 2014; **19**: 20828.
- 10 Reusken CB, Ababneh M, Raj VS *et al*. Middle East respiratory syndrome coronavirus (MERS-CoV) serology in major livestock species in an affected region in Jordan, June to September 2013. *Euro Surveill* 2013; **18**: 20662.
- 11 Corman VM, Jores J, Meyer B *et al*. Antibodies against MERS coronavirus in dromedary camels, Kenya, 1992–2013. *Emerg Infect Dis* 2014; **20**: 1319–1322.
- 12 Hemida MG, Perera RA, Wang P *et al*. Middle East respiratory syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. *Euro Surveill* 2013; **18**: 20659.
- 13 Alagaili AN, Briese T, Mishra N *et al*. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. *mBio* 2014; **5**: e00884–00814.
- 14 Corman VM, Kallies R, Philipps H *et al*. Characterization of a novel *Betacoronavirus* related to Middle East respiratory syndrome coronavirus in European hedgehogs. *J Virol* 2014; **88**: 717–724.
- 15 Woo PC, Wang M, Lau SK *et al*. Comparative analysis of twelve genomes of three novel group 2c and group 2d coronaviruses reveals unique group and subgroup features. *J Virol* 2007; **81**: 1574–1585.
- 16 Lau SK, Li KS, Tsang AK *et al*. Genetic characterization of *Betacoronavirus* lineage C viruses in bats reveals marked sequence divergence in the spike protein of *Pipistrellus* bat coronavirus HKU5 in Japanese Pipistrelle: implications for the origin of the novel Middle East respiratory syndrome coronavirus. *J Virol* 2013; **87**: 8638–8650.
- 17 Ithete NL, Stoffberg S, Corman VM *et al*. Close relative of human Middle East respiratory syndrome coronavirus in bat, South Africa. *Emerg Infect Dis* 2013; **19**: 1697–1699.
- 18 Memish ZA, Mishra N, Olival KJ *et al*. Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. *Emerg Infect Dis* 2013; **19**: 1819–1823.
- 19 Wang Q, Qi J, Yuan Y *et al*. Bat origins of MERS-CoV supported by bat coronavirus HKU4 usage of human receptor CD26. *Cell Host Microbe* 2014; **16**: 328–337.
- 20 Corman VM, Ithete NL, Richards LR *et al*. Rooting the phylogenetic tree of Middle East respiratory syndrome coronavirus by characterization of a conspecific virus from an African bat. *J Virol* 2014; **88**: 11297–11303.
- 21 Yang Y, Du L, Liu C *et al*. Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. *Proc Natl Acad Sci USA* 2014; **111**: 12516–12521.
- 22 Woo PC, Lau SK, Li KS, Tsang AK, Yuen KY. Genetic relatedness of the novel human group C *betacoronavirus* to *Tylonycteris* bat coronavirus HKU4 and *Pipistrellus* bat coronavirus HKU5. *Emerg Microbes Infect* 2012; **1**: e35.
- 23 Bermingham A, Chand MA, Brown CS *et al*. Severe respiratory illness caused by a novel coronavirus, in a patient transferred to the United Kingdom from the Middle East, September 2012. *Euro Surveill* 2012; **17**: 20290.
- 24 Corman VM, Eckerle I, Bleicker T *et al*. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. *Euro Surveill* 2012; **17**: 20285.
- 25 Guery B, Poissy J, el Mansouf L *et al*. Clinical features and viral diagnosis of two cases of infection with Middle East respiratory syndrome coronavirus: a report of nosocomial transmission. *Lancet* 2013; **381**: 2265–2272.
- 26 Lu X, Whitaker B, Sakthivel SK *et al*. Real-time reverse transcription-PCR assay panel for Middle East respiratory syndrome coronavirus. *J Clin Microbiol* 2014; **52**: 67–75.
- 27 Corman VM, Muller MA, Costabel U *et al*. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. *Euro Surveill* 2012; **17**: 20334.
- 28 Lau SK, Che XY, Woo PC *et al*. SARS coronavirus detection methods. *Emerg Infect Dis* 2005; **11**: 1108–1111.
- 29 Lau SK, Woo PC, Wong BH *et al*. Detection of severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein in SARS patients by enzyme-linked immunosorbent assay. *J Clin Microbiol* 2004; **42**: 2884–2889.
- 30 Chen Y, Chan K-H, Kang Y *et al*. A sensitive and specific antigen detection assay for Middle East respiratory syndrome coronavirus. *Emerg Microbes Infect* 2015; **4**: e26.
- 31 Chan KH, Chan JF, Tse H *et al*. Cross-reactive antibodies in convalescent SARS patients' sera against the emerging novel human coronavirus EMC (2012) by both immunofluorescent and neutralizing antibody tests. *J Infect* 2013; **67**: 130–140.
- 32 Chan JF, Chan KH, Choi GK *et al*. Differential cell line susceptibility to the emerging novel human betacoronavirus 2c EMC/2012: implications for disease pathogenesis and clinical manifestation. *J Infect Dis* 2013; **207**: 1743–1752.
- 33 Woo PC, Lau SK, Lam CS *et al*. Discovery of a novel bottlenose dolphin coronavirus reveals a distinct species of marine mammal coronavirus in *Gammacoronavirus*. *J Virol* 2014; **88**: 1318–1331.
- 34 Lau SK, Feng Y, Chen H *et al*. SARS coronavirus ORF8 protein is acquired from SARS-related coronavirus from greater horseshoe bats through recombination. *J Virol* 2015; **89**: 10532–10547.
- 35 Lau SK, Li KS, Tsang AK *et al*. Recent transmission of a novel *alphacoronavirus*, bat coronavirus HKU10, from *Leschenault's* rousettes to *pomona* leaf-nosed bats: first evidence of interspecies transmission of coronavirus between bats of different suborders. *J Virol* 2012; **86**: 11906–11918.
- 36 Woo PC, Lau SK, Lam CS *et al*. Discovery of seven novel mammalian and avian coronaviruses in the genus *deltacoronavirus* supports bat coronaviruses as the gene source of *alphacoronavirus* and *betacoronavirus* and avian coronaviruses as the gene source of *gammacoronavirus* and *deltacoronavirus*. *J Virol* 2012; **86**: 3995–4008.
- 37 Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013; **30**: 772–780.
- 38 Wernery U, Corman VM, Wong EY *et al*. Acute Middle East respiratory syndrome coronavirus infection in livestock Dromedaries, Dubai, 2014. *Emerg Infect Dis* 2015; **21**: 1019–1022.
- 39 Hemida MG, Chu DK, Poon LL *et al*. MERS coronavirus in dromedary camel herd, Saudi Arabia. *Emerg Infect Dis* 2014; **20**: 1231–1234.
- 40 Briese T, Mishra N, Jain K *et al*. 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* 2014; **5**: e01146–01114.



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