OBSERVATION

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

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ABSTRACT **Complete Middle East respiratory syndrome coronavirus (MERS-CoV) genome sequences were obtained from nasal swabs of dromedary camels sampled in the Kingdom of Saudi Arabia through direct analysis of nucleic acid extracts or following virus isolation in cell culture. Consensus dromedary MERS-CoV genome sequences were the same with either template source and identical to published human MERS-CoV sequences. However, in contrast to individual human cases, where only clonal genomic sequences are reported, detailed population analyses revealed the presence of more than one genomic variant in individual dromedaries. If humans are truly infected only with clonal virus populations, we must entertain a model for interspecies transmission of MERS-CoV wherein only specific genotypes are capable of passing bottleneck selection.**

IMPORTANCE **In most cases of Middle East respiratory syndrome (MERS), the route for human infection with the causative agent, MERS coronavirus (MERS-CoV), is unknown. Antibodies to and viral nucleic acids of MERS-CoV have been found in dromedaries, suggesting the possibility that they may serve as a reservoir or vector for human infection. However, neither whole viral genomic sequence nor infectious virus has been isolated from dromedaries or other animals in Saudi Arabia. Here, we report recovery of MERS-CoV from nasal swabs of dromedaries, demonstrate that MERS-CoV whole-genome consensus sequences from dromedaries and humans are indistinguishable, and show that dromedaries can be simultaneously infected with more than one MERS-CoV. Together with data indicating widespread dromedary infection in the Kingdom of Saudi Arabia, these findings support the plausibility of a role for dromedaries in human infection.**

Received 9 April 2014 **Accepted** 16 April 2014 **Published** 29 April 2014

Citation Briese T, Mishra N, Jain K, Zalmout IS, Jabado OJ, Karesh WB, Daszak P, Mohammed OB, Alagaili AN, Lipkin WI. 2014. 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(3): e01146-14. doi:10.1128/mBio.01146-14.

Editor Michael Katze, University of Washington

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Two hundred twelve cases of Middle East respiratory syndrome (MERS), 88 of them fatal, have been reported since April 2012 (1). Although examples of human-to-human transmission have been identified, the origin of infection with the causative agent, MERS coronavirus (MERS-CoV), is unexplained in the majority of cases (2). Serologic evidence of infection in dromedary camels (DC) and, more recently, the detection of viral nucleic acid in DC, particularly in juvenile DC, suggest the possibility that DC may serve as a reservoir or vector for human infection (3–12). However, there are as yet no published analyses of complete MERS-CoV genomic sequences or virus isolation from DC in the Kingdom of Saudi Arabia (KSA).

In a collaborative effort between the Center for Infection and Immunity in the Mailman School of Public Health at Columbia University and the Mammals Research Chair, Department of Zoology, College of Science, King Saud University, a mobile laboratory was established in Saudi Arabia to investigate the possible role of DC, other domestic animals, and wildlife in the transmission of MERS-CoV through molecular and serological analyses. In a previous publication, we reported detection of high loads of MERS-CoV nucleic acid in nasal swabs from DC (10). Here, we describe MERS-CoV complete genome sequencing, detailed phylogenetic analyses, and the recovery of live virus through culture.

Reverse transcription-PCR (RT-PCR) assays of nasal swab samples demonstrated the presence of MERS-CoV RNA in DC at a high prevalence in KSA (10). Sequence analysis of products representing three regions of the MERS-CoV genome revealed identity over approximately 3,000 nucleotides (nt) with human MERS-CoV sequences. To determine whether this identity extended across larger regions of the MERS-CoV genome, we pur-

TABLE 1 High-throughput sequencing of MERS-CoV from dromedary camels in Saudi Arabia

Sample[e]	Sequencing approach					
	Ion Torrent			Illumina		
	Random-primed cDNA (swab extract)[a]	Preamplified RT-PCR fragments (swab extract)[b]	Random-primed cDNA (culture extract)[c]	Random-primed cDNA (swab extract)[a]	Preamplified RT-PCR fragments (swab extract)[b]	Random-primed cDNA (culture extract)[c]
KSA-363n	Yes	Yes	Yes	Yes	Yes	NA
KSA-378n	Yes	NA[d]	Yes	Yes	NA	NA
KSA-376n	Yes	Yes	NA	Yes	Yes	NA
KSA-344r	Yes	Yes	NA	Yes	Yes	NA
KSA-409n	Yes	Yes	NA	Yes	Yes	NA

[a] Sequencing library prepared from random-primed cDNA that was generated from total nucleic acid extract of nasal swab sample.
[b] Sequencing library prepared from pooled overlapping RT-PCR fragments (14) amplified from total nucleic acid extract of nasal swab sample.
[c] Sequencing library prepared from random-primed cDNA that was generated from total nucleic acid extract of cell culture supernatant from infected Vero cells.
[d] NA, not applicable.
[e] n, nasal swab sample; r, rectal swab sample.

sued whole-genome sequencing using the Ion Torrent and Illumina platforms employing as the template random-primed cDNA libraries and pools of PCR products based on primers that represented published human MERS-CoV genomic sequence.

Raw Ion Torrent and Illumina data from 5 DC were assembled against MERS-CoV scaffolds available from GenBank. No platform-dependent differences were apparent; thus, sequence data were combined and used to assemble consensus sequences for each sample. The specific processing of individual samples is summarized in Table 1. Consensus full-genome sequences of MERS-CoV from DC were obtained for samples KSA-363-Taif-21, KSA-378-Taif-36, and KSA-376-Taif-34 (10). Partial genomes were obtained for samples KSA-344-Taif-2 and KSA-409-Tabuk-26.

Two additional full genomic sequences were generated entirely by overlapping direct RT-PCR amplification of random-primed cDNA generated from total nucleic acid extract of Arabian DC nasal swab samples KSA-503-Taif-45 and KSA-505-Taif-47. These samples were collected in January 2014 in Taif from a 1-year-old imported African and a 1-year-old Arabian breed of DC, respectively.

Complete genomic sequence was also obtained for virus cultured from two DC nasal swab samples. Vero E6 cells were inoculated with sterile filtered nasal swab/viral transport medium (VTM) samples (KSA-363-Taif-21 and KSA-378-Taif-36) or a rectal swab/VTM sample (KSA-371-Taif-29 [10]). Viral proliferation was monitored by real-time "upstream-of-E" (UpE) PCR after 48 h and 66 h in comparison to residual inoculum measured after removal of the inoculum and washing of the cells at 2.5 h postinfection ($t = 0$) (Fig. 1A). Virus growth was observed with the two nasal swab samples but not with the rectal swab sample. Total nucleic acid extracts obtained from the 48-h samples were subjected to random sequencing on the Ion Torrent platform, yielding full-length genomic sequence. No differences were observed in the consensus sequences obtained using template from extracts of nasal swabs or cultured virus.

The five consensus full-genome sequences (KSA-363, -378, -376, -503, and -505) were aligned to other genome-length human MERS-CoV sequences available in GenBank. Analysis of the five consensus sequences confirmed earlier work with short PCR products obtained from DC (10) that suggested that DC in Saudi Arabia harbor the same virus that causes MERS in humans (Fig. 1B).

However, detailed inspection of the multiple sequence alignment indicated that our DC sequence assemblies showed frequent IAUC codes for two-base wobbles in positions where high divergence between human MERS-CoV sequences is observed (Fig. 1C, i). To ensure that the appearance of multiple sequence variants in individual DC samples was genuine rather than a sequencing artifact, we amplified representative regions by specific PCR, cloned the products, and sequenced individual clones by the dideoxy chain termination method. Alignment of the clone-derived sequences confirmed the presence of multiple sequence species in several individual DC samples (Fig. 1C, ii and iii). Sequence diversity in sample KSA-363 decreased over a period of 48 h in culture (see Fig. S1 in the supplemental material).

The role of DC in human MERS-CoV infection is unclear. Studies throughout the Middle East conducted independently by several research teams have described antibodies to MERS-CoV in DC (3–12). In recent work in KSA, we found antibodies to MERS-CoV in 95% of adult DC and MERS-CoV sequences in 35% of juvenile DC (10). However, direct exposure to DC is only rarely reported in human cases. Furthermore, there are no published reports of MERS-CoV virus isolation from DC and only a single near-full-genome sequence from an African DC that indicates that viruses related to human MERS-CoV circulate in African DC (12). Here, we confirm that DC may harbor infectious virus and that whole-genome consensus sequences obtained from nasal isolates align with whole-genome sequences recovered from humans.

Our analysis of whole-MERS-CoV-genome sequences recovered from DC revealed the presence of sequence variants within single samples (also known as quasispecies [13]). One amino acid in the spike protein (A520S, corresponding to nucleotides [nt] 23013 to 23015 in GenBank accession no. JX869059) was changed within the receptor-binding domain; however, all other changes occurred outside the receptor-binding domain. It is unclear whether this has any functional implications. In other viral systems, genetic diversity has been linked to pathogenicity and shown to enable adaptation to new environments such as those associated with movement into new hosts (13, 14). No sequence variants have been described in individual human MERS-CoV samples. Whether this means that only consensus sequences are reported or that human sequences truly represent clonal virus populations within individual cases cannot be discerned from published data. If the latter, we must entertain a model for interspecies transmission, wherein only specific genotypes, which may

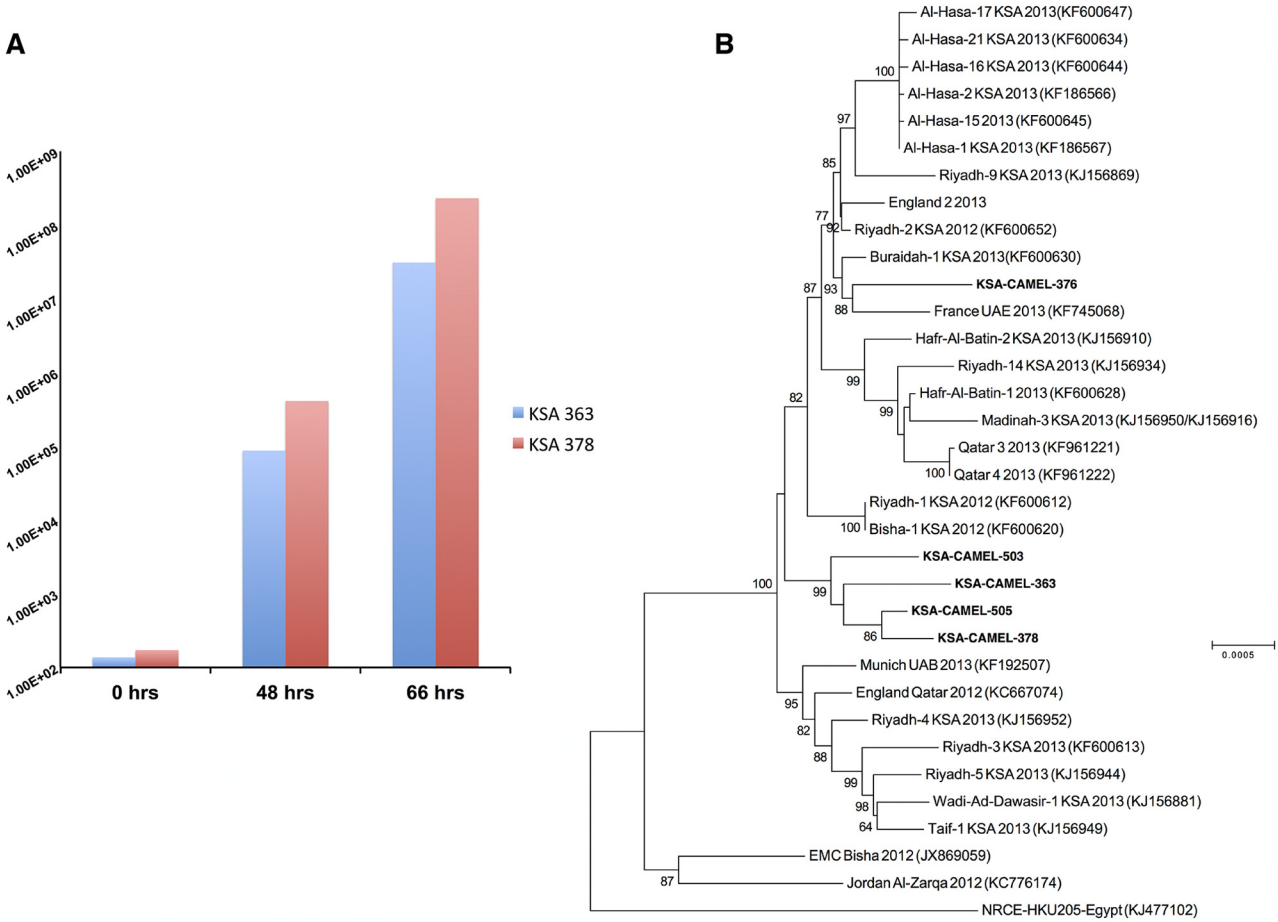

FIG 1 (A) Real-time PCR analysis of cell culture supernatant after inoculation of Vero cells with nasal swab samples KSA-363 and KSA-378. (B) Phylogenetic analysis of MERS-CoV sequences from dromedary camels in Saudi Arabia and other genome-length MERS-CoV sequences available on 7 April 2014. GenBank accession numbers are given in parentheses for each sequence (England2 sequence is available at http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/MERSCoV/respPartialgeneticsequenceofnovelcoronavirus/); bootstrap values of >60% indicate statistical support for the respective nodes; the scale bar indicates the number of substitutions/site. (C) (i) Clippings from the multiple MERS-CoV sequence alignment indicating sequence variation among human MERS-CoV sequences and potential variation of sequences within individual DC samples (indicated by x). (ii) Sequences obtained by direct sequencing of PCR products from the same region. (iii) Sequence analysis of individual clones generated from the PCR amplification products. Two PCRs were performed, including nt 24190 to 24300 and 24510 to 2530.
(Continued on following page)

not be present in every infected DC, are capable of passing bottleneck selection. Such a model would not abrogate a role for host susceptibility in infection and disease but might provide insights into the rarity of human cases of MERS.

Nucleic acid extraction, high-throughput sequencing, and PCR. Total nucleic acids from nasal swab, rectal swab or cell culture supernatant samples were extracted on a QiaCube with Cador reagent kits (Qiagen, Hilden, Germany). Superscript III and random hexamer primers were used to generate cDNA preparations (Life Technologies, Carlsbad, CA, USA). Second-strand cDNA synthesis for high-throughput sequencing was carried out by random primer extension with Klenow enzyme (New England Biolabs, Ipswich, MA, USA).

High-throughput sequencing was performed in parallel on random-primed cDNA preparations and MERS-CoV-enriched PCR product pools. To enrich for MERS-CoV sequences from total nucleic acid extracts, PCR amplifications employing a set of overlapping PCR primers spanning the whole genome in approximately 2.0- to 2.5-kb fragments were performed as described (15). PCR products were pooled and sequenced on both the Ion Torrent and Illumina platforms.

Sequencing on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) resulted in an average of approximately 20 to 50 million reads per sample. cDNA preparations were sheared (E210 sonicator; Covaris, Woburn, MA, USA) for an average fragment size of 200 bp and added to Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) for purification, and libraries were prepared with Kapa high-throughput library preparation kits (Kapa Biosystems, Wilmington, MA, USA). Sequencing was performed using a read length of 100 nt, followed by an independent read of the 6-nt bar code. Samples were demultiplexed using Illumina-supplied CASAVA software and exported as FastQ files. More than 90% of Illumina reads passed the Q30 filter. Demultiplexed FastQ files were mapped against GenBank scaffolds (KF600620 and KF186567) with Bowtie 2 mapper 2.0.6 (http://bowtie-bio.sourceforge.net [16]). Sequencing on the Ion Torrent PGM platform was performed with Ion PGM Sequencing 200 kits on Ion 318 chips (Life Technologies), yielding on average 1.5

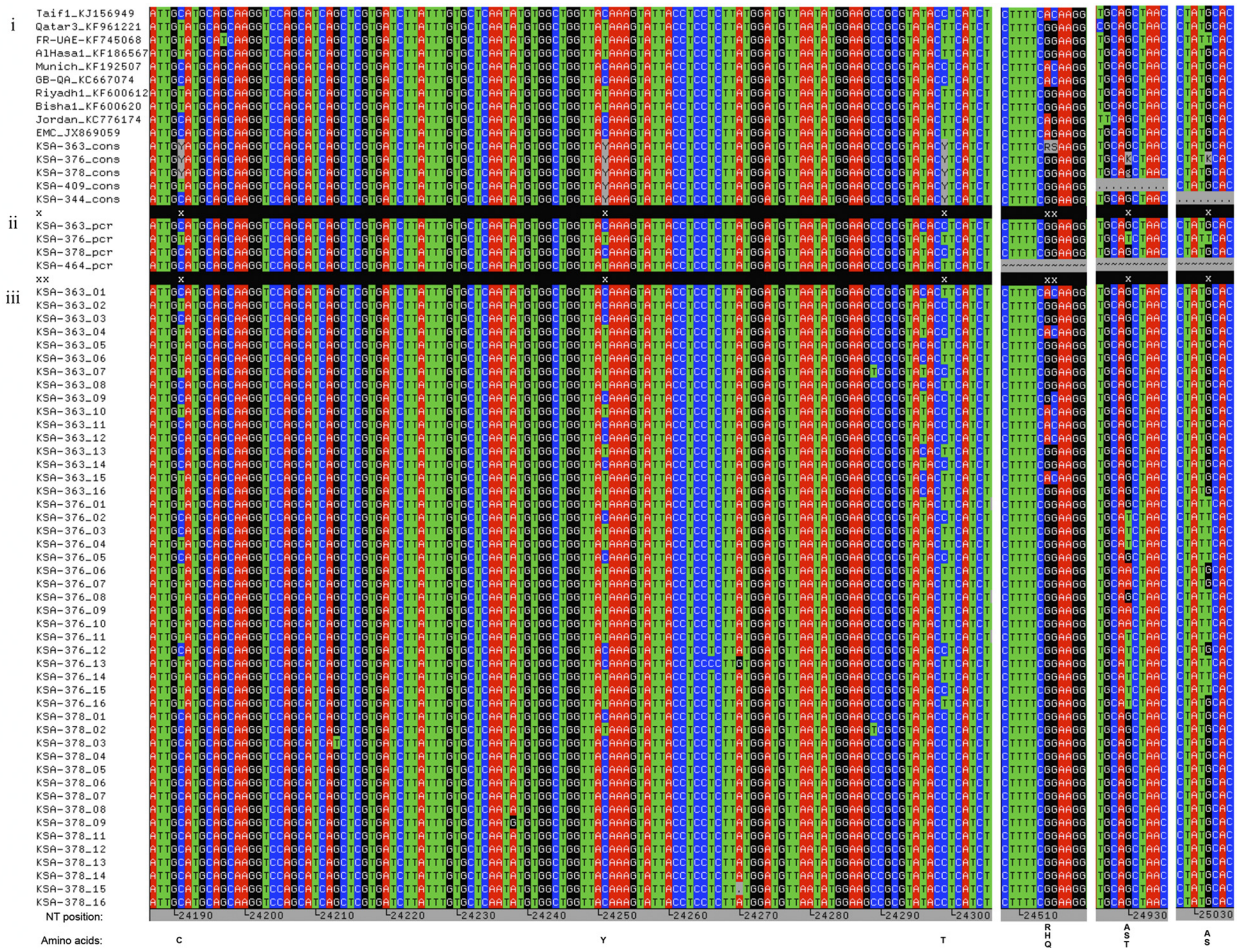

FIG 1 (Continued)

to 2.5 million reads per sample with a mean length of approximately 166 nt. cDNA preparations were sheared (Ion Shear Plus kit; Life Technologies) for an average fragment size of 200 bp and added to Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) for purification, libraries were prepared with Kapa library preparation/Ion Torrent series kits (Kapa), and emulsion PCR was performed with Ion PGM Template OT2 200 kits (Life Technologies). Ion Torrent reads were demultiplexed and exported as FastQ files by the Ion Torrent PGM software. After bar code and adaptor trimming, length filtering, masking of low-complexity regions, and subtraction of ribosomal and host sequences, reads were mapped as described for Illumina data. Consensus sequences from mapping assemblies were generated by using SAMtools/BCFtools 0.1.19 software (http://samtools.sourceforge.net [17]).

Based on available sequence information, a set of 25 nested consensus primer sets were designed to generate overlapping PCR products of approximately 1.3 kb that comprise the full genome (see Table S1 in the supplemental material). Random-primed cDNA was PCR amplified with individual primer pairs and AmpliTaq Gold (Life Technologies). The PCR products were purified by agarose gel electrophoresis and QIAquick gel extraction kits (Qiagen) and subsequently sequenced on both strands by the dideoxynucleotide chain termination method (GeneWiz, South Plainfield, NJ, USA). Products from selected PCRs were also cloned into pGEM-T Easy plasmid vector (Life Technologies), and 16 individual clones were dideoxy sequenced in order to assess clonal sequence diversity.

Quantitative real-time PCR used OneStep Real-Time qPCR buffer (Life Technologies) and UpE primer/probes (18).

Virus isolation. One hundred fifty microliters of nasal swab in universal virus transport medium (Becton, Dickinson, Franklin Lakes, NJ, USA) was filtered (0.45 μm; Millipore, Billerica, MA, USA), and the filtrate was inoculated on Vero E6 cells grown to semiconfluence in T25 culture flasks with Dulbecco modified Eagle medium (DMEM)-10% fetal calf serum. The inoculum was removed after 2.5 h, cells were gently rinsed, and fresh medium was added (T-0). Supernatant was tested for MERS-CoV by quantitative real-time PCR after 48 h (T-48), and supernatant as well as cell homogenate harvested at 66 h postinfection (T-66).

Bioinformatics and phylogenetic analysis. Sequence data were analyzed using software packages Geneious (Biomatters, Auckland, New Zealand), MEGA (http://www.megasoftware.net [19]), and Wisconsin GCG (Accelrys Inc., San Diego, CA). Phylogenetic analysis was performed by the neighbor-joining method implemented in MEGA 5.2, running 1,000 pseudoreplicate analyses to assess statistical support.

Nucleotide sequence accession numbers. Full genomic sequences of MERS-CoV from DC were deposited in GenBank under the indicated accession numbers: samples KSA-363-Taif-21, KJ713298; KSA-378-Taif-36, KJ713296; KSA-376-Taif-34, KJ713299; KSA-503-Taif-45, KJ713297; and KSA-505-Taif-47, KJ713295.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01146-14/-/DCSupplemental.

Figure S1, PDF file, 0.5 MB.

Table S1, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Ahmed A. Al-Qahtani, Research Center, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia, for the gift of Vero cells and Yasser A. Elnakady, Zoology Department, King Saud University, for generous technical support. We also thank Joel Garcia, Vishal Kapoor, James Ng, Stephen Sameroff, and Maria Sanchez-Leon for technical assistance; Kawthar Muhammad and Devon Welsh for project management; and Ellie Kahn for editorial contributions.

The KSU Mammals Research Chair is supported by the Deanship of Scientific Research, King Saud University. Work in the Center for Infection and Immunity is supported by awards from the National Institutes of Health (AI057158) and the United States Agency for International Development Emerging Pandemic Threats program, PREDICT project, under the terms of cooperative agreement GHN-A-OO-09-00010-00.

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