VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 17, Number 2, 2017
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DOI: 10.1089/vbz.2016.2062

High Prevalence of Middle East Respiratory Coronavirus in Young Dromedary Camels in Jordan

Neeltje van Doremalen,[1] Zaidoun S.K. Hijazeen,[2] Peter Holloway,[3] Bilal Al Omari,[4] Chester McDowell,[5] Danielle Adney,[6] Hani A. Talafha,[4] Javier Guitian,[3] John Steel,[7] Nadim Amarin,[8] Markos Tibbo,[9] Ehab Abu-Basha,[4] Ahmad M. Al-Majali,[4] Vincent J. Munster,[1,*] and Juergen A. Richt[5,*]

Abstract

Prevalence of Middle East respiratory syndrome coronavirus (MERS-CoV) was determined in 45 dromedary camels from two geographically separated herds in Jordan. Virus shedding was only detected in swabs obtained from the respiratory tract and primarily observed in camels younger than 3 years. MERS-CoV seroprevalence increased with age of camels. Bovine and sheep sera were seronegative. Phylogenetic analysis of partial S2 clustered the Jordanian MERS-CoV strains with contemporary MERS-CoV strains associated with nosocomial outbreaks.

Keywords: dromedary camel, Jordan, MERS-CoV, phylogeny, serology

Introduction

Since the identification of Middle East respiratory syndrome coronavirus (MERS-CoV) as the causative agent of a fatal case of respiratory tract disease in the Kingdom of Saudi Arabia (KSA) in 2012, the virus has caused >1700 laboratory-confirmed cases of disease, including >600 fatal cases (WHO 2016). The first known MERS-CoV outbreak in humans, diagnosed retrospectively, occurred in Jordan in 2012 (Hijawi et al. 2013). The dromedary camel has been identified as the primary reservoir of MERS-CoV, and direct evidence for zoonotic transmission from camels has been reported in KSA and Qatar (Haagmans et al. 2014, Memish et al. 2014). A limited number of seropositive camels in Jordan have been described previously (Reusken et al. 2013), but no MERS-CoV has been detected in camels in Jordan.

Materials and Methods

During May 2016, we collected swabs (from nasal, urogenital, and rectal areas) and blood samples from camels at two locations in Jordan. Two camel herds were identified to study (1) a traditional bedouin camel herd, in which camels are allowed to graze and browse freely (Azraq, Zarqa) and (2) a more conventional mixed farm setting, where camels were kept in pens on one farm (Ramtha) (Fig. 1A). In Ramtha, blood samples from adult sheep and cattle were additionally collected. Swab samples were collected in virus transport medium. RNA was extracted from samples using the QiaAmp Viral RNA kit (Qiagen). Five microliters of RNA was used in a one-step real-time RT-PCR UpE assay for MERS-CoV using the Rotor-Gene™ probe kit (Qiagen). Positive samples (cycle threshold [Ct] < 37) were tested using the ORF1A assay (Corman et al. 2012), and samples were excluded from further analysis when ORF1A testing was negative. cDNA was synthesized using random hexamer and used to PCR amplify the MERS-CoV spike S2 domain (nucleotides 23781–24395 of HCoV-EMC/2012) as described previously (Smits et al. 2015). Sequences were assembled on SeqMan Pro and analyzed on MegAlign (DNASTAR). Phylogenetic trees of the S2 domain were generated using Mega 6.0.6 with the maximum likelihood statistical method

[1]Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana.
[2]Food and Agriculture Organization of the United Nations (FAO), Amman, Jordan.
[3]Veterinary Epidemiology, Economics and Public Health Group, The Royal Veterinary College, Hatfield, United Kingdom.
[4]Department of Veterinary Medical Sciences, Faculty of Veterinary Medicine, Jordan University of Science and Technology, Irbid, Jordan.
[5]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas.
[6]Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado.
[7]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia.
[8]Boehringer Ingelheim, MENA, Dubai, United Arab Emirates.
[9]FAO Regional Office for the Near East and North Africa, Cairo, Egypt.
*These authors contributed equally to this work.

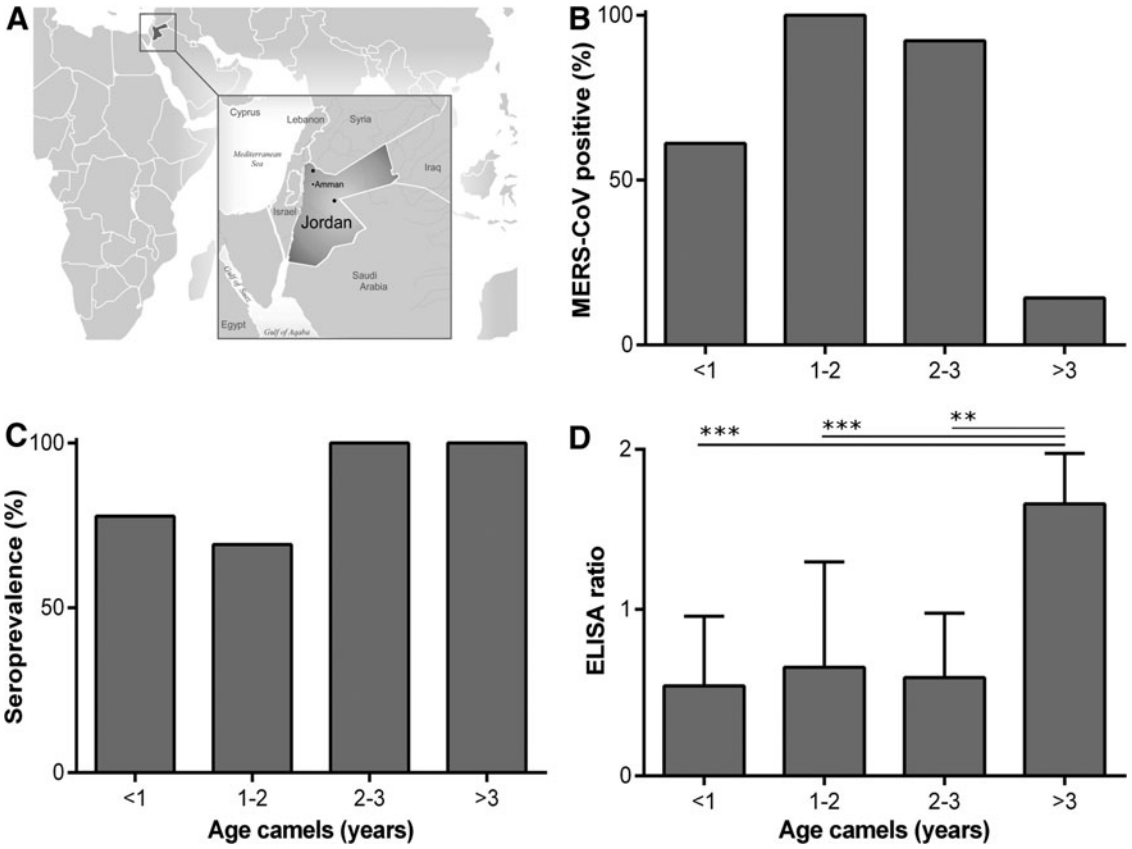

FIG. 1. MERS-CoV prevalence in dromedary camels in Jordan. **(A)** Locations of dromedary camels (north: Ramtha; east: Azraq). **(B)** Percentage of MERS-CoV RNA shedding dromedary camels as detected by UpE and ORF1A qRT-PCR assay in nasal swab, stratified by age. **(C)** Percentage of MERS-CoV S1-specific seropositive dromedary camels, stratified by age. **(D)** ELISA ratio of seropositivity of dromedary camels, stratified by age. The ELISA ratio was calculated by dividing the OD of each serum sample by a constant positive sample on the ELISA plate. ELISA, enzyme-linked immunosorbent assay; Middle East respiratory syndrome coronavirus (MERS-CoV), OD, optical density.

based on the GTR+G+I model with 1000 bootstraps replicates. Sera were analyzed by MERS-CoV spike protein (S) enzyme-linked immunosorbent assay (ELISA); Maxisorp (Nunc) plates were coated overnight with S1 protein (Sino Biological) and blocked with 1% milk. Sera (400× dilution) were added to the plate in duplicate. MERS-CoV S1-specific antibodies were detected using anti-llama (Agrisera), anti-bovine, or anti-sheep (Jackson) IgG (H&L) HRP-conjugated antibody on development with peroxidase-substrate reagent (KPL); optical density (OD) was measured at 405 nm. The threshold of positivity was mean OD + 10× standard deviation of negative sera obtained from camels raised in captivity in the United States.

Results

Twenty-three camels sampled from the Bedouin herd in Azraq ranged in age from 4 months to 8 years, whereas 22 camels sampled at the farm in Ramtha ranged in age from 4 months to 3 years (Table 1). Ten sheep and five cows were sampled. Nasal discharge was observed at the time of sampling in some of the MERS-CoV-positive camels <1 year old but not in older camels.

RNA from 42/45 camels was tested positive for the presence of MERS-CoV nucleic acid. MERS-CoV RNA was solely detected in nasal swabs. Importantly, urogenital and rectal swab samples were negative. One nasal swab originating from camel 40 was excluded from analysis; while the UpE assay resulted in a Ct value of <37, the ORF1A assay was negative. An unpaired two-tailed Student's *t*-test comparing the age of viral RNA-positive versus RNA-negative camels was significant ($p = 0.0311$). Only 1/7 camels older than 3 years was positive for viral RNA, whereas 11/18 camels <1 year old, 4/4 camels 1–2 years old, and 12/13 camels 2–3 years old were positive for viral RNA in nasal swabs (Fig. 1B). MERS-CoV-specific antibodies were observed in the majority of animals: 78% of camels <1 year old, 69% of camels 1–2 years old, and 100% of camels >2 years old were seropositive (Fig. 1C). No MERS-CoV S1-specific antibodies were found in sheep or bovine serum samples.

To better assess the potential significance of ELISA values as a correlate of MERS-CoV susceptibility, we calculated the ratio of the ELISA value of each sample to that obtained from an included camel reference serum on each plate in the assay. By this approach, we show that camels <3 years old exhibited an average ELISA ratio of 0.5–0.65, whereas older animals exhibited an average ELISA ratio of 1.63. This difference was significant as tested via a two-tailed Mann–Whitney test (Fig. 1D). Spike S2 partial domain sequences were obtained from 16/28 samples (accession

Table 1. Age, Location, and Assay Results Per Dromedary Camel

Camel	Age	Herd	Virus positive	TCID50eq/swab		Seropositive	ELISA ratio
				UpE	ORF1A		
40	2M	Ramtha	0			1	1.15
41	2M	Ramtha	0			1	0.50
42	2M	Ramtha	1	135	156	0	0.11
07	4M	Azraq	1	12	37	0	0.18
13	4M	Azraq	1	15	93	0	0.19
33	4M	Ramtha	1	252	196	1	0.17
36	4M	Ramtha	0			1	0.79
09	5M	Azraq	1	200	728	1	0.43
12	5M	Azraq	1	21	35	0	0.21
16	5M	Azraq	0			1	0.35
35	5M	Ramtha	1	92	109	1	0.29
06	6M	Azraq	1	81	264	1	0.30
17	6M	Azraq	1	188	174	1	1.27
24	6M	Ramtha	0			1	1.43
34	6M	Ramtha	0			1	0.43
37	6M	Ramtha	1	2729	1256	1	0.30
10	7M	Azraq	0			1	0.58
11	7M	Azraq	1	70	209	1	1.07
25	12M	Ramtha	1	9575	4420	0	0.13
26	13M	Ramtha	1	1569	2643	0	0.09
30	14M	Ramtha	1	5139	6054	0	0.13
32	14M	Ramtha	1	5907	2760	0	0.09
18	15M	Azraq	1	56	802	1	1.46
08	18M	Azraq	1	76	86	1	1.25
14	18M	Azraq	1	128	371	1	1.05
15	18M	Azraq	1	23	119	1	1.35
27	18M	Ramtha	1	1067	1074	1	0.16
28	18M	Ramtha	1	507	876	1	0.15
29	18M	Ramtha	1	168	368	1	0.63
31	18M	Ramtha	1	491	230	1	0.21
21	19M	Azraq	0			1	1.79
38	30M	Ramtha	1	280	452	1	1.10
39	30M	Ramtha	1	152	155	1	0.54
44	2Y	Ramtha	1	112	157	1	0.57
45	2Y	Ramtha	1	76	219	1	0.15
01	3Y	Azraq	0			1	1.76
20	3Y	Azraq	0			1	1.74
43	3Y	Ramtha	1	87	179	1	0.82
02	4Y	Azraq	0			1	1.83
04	5Y	Azraq	0			1	1.85
22	5Y	Azraq	0			1	1.89
03	6Y	Azraq	ND			1	1.76
19	6Y	Azraq	0			1	1.23
05	8Y	Azraq	ND			1	1.81
23	8Y	Azraq	ND			1	1.67

ELISA ratio was defined by dividing the OD of each serum sample by a constant positive sample on the ELISA plate. All viral RNA was detected in respiratory tract swab samples, none in urogenital or fecal swab samples. Positivity is marked as ''1,'' negativity as ''0.''

ELISA, enzyme-linked immunosorbent assay; M, month; OD, optical density; Y, year.

numbers KX443663–KX443678). We were unable to obtain sequences of 12 positive samples with a lower viral load (Ct >33). We performed phylogenetic analysis with a selection of MERS-CoV S2 sequences using representatives of known clades, as performed previously (Smits et al. 2015). The phylogenetic analysis of the partial S2 sequences placed the novel Jordanian viruses within the B1 cluster representing contemporary camel and human MERS-CoVs. Three sequences were identical to spike S2 sequences of human isolates of MERS-CoV obtained in Jordan in 2015 (Lamers et al. 2016). Twelve samples differed by two synonymous mutations (C23837T, T24074G), whereas the remaining samples contained a mixture of 24074T and 24074G combined with 23837T. Importantly, all novel sequences were found in cluster B1, containing the most recent MERS-CoV sequences originating from camel and human viruses (Fig. 2). Thus, the circulating MERS-CoV strains in the Jordan dromedary camel population are closely related to virus strains known to be capable of zoonotic transmission and to cause disease in the human population.

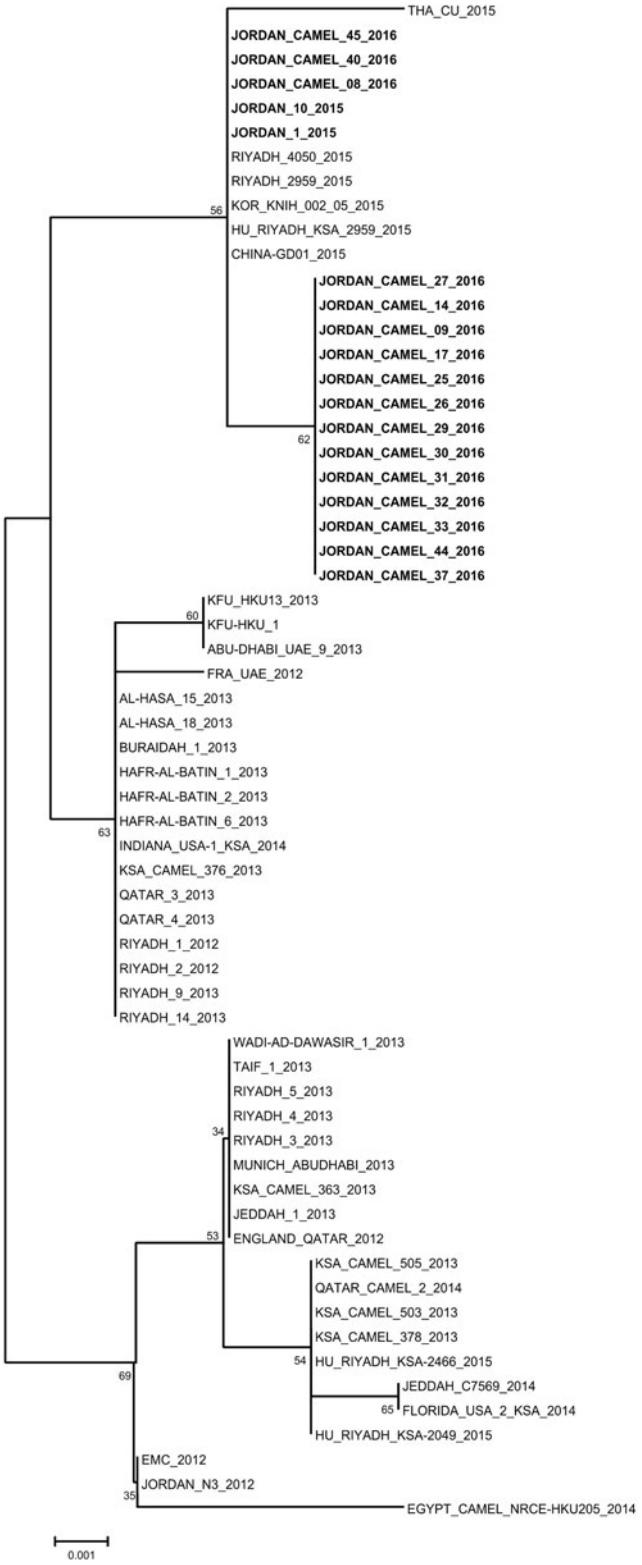

FIG. 2. Phylogenetic analysis of a partial spike S2 domain. A maximum likelihood tree based on the GTR+G+I model using 1000 bootstraps was generated from a spike S2 domain genome fragment corresponding to nucleotides 23781–24395 of HCoV-EMC/2012. The newly identified MERS-CoV sequences are depicted in *bold*, recent MERS-CoV sequences associated with an outbreak in Jordan in 2015 are depicted in *bold*. Bootstrap values of <50 were not shown.

Discussion

This study confirms the circulation of MERS-CoV within the dromedary camel population of Jordan in line with MERS-CoV detection in camels throughout the Middle East (Reusken et al. 2016). Importantly, MERS-CoV RNA could be detected in nasal swabs of seropositive dromedary camels. Antibodies against MERS-CoV were found at a young age in dromedary camels. The presence of maternal antibodies via the intake of colostrum during the first 24 h postparturition could play a role in the detection of MERS-CoV-specific antibodies in animals <6 months old and might not reflect actively acquired antibodies (Kamber et al. 2001, Meyer et al. 2016).

We observed shedding of MERS-CoV in the presence of antibodies, which suggests either reinfection of seropositive animals or shedding of virus/viral RNA during early stages of seroconversion. Previously, a lack of correlation was observed between virus/viral RNA shedding and the presence of neutralizing antibodies; these data highlight the potential for reinfection of seropositive animals (Farag et al. 2015). The ELISA ratio might be a better predictor of MERS-CoV susceptibility than seropositivity; 24/29 animals with an ELISA ratio of <1.1 were MERS-CoV positive, whereas only 4/16 animals with an ELISA ratio of ≥1.1 were positive for viral RNA in nasal swabs. In contrast, 20/34 seropositive dromedary camels were MERS-CoV viral RNA positive versus all (8/8) seronegative dromedary camels. This might indicate that sterile immunity is only reached at high levels of antibody titers, in line with a previous study conducted in the United Arab Emirates (Meyer et al. 2016).

The phylogenetic analysis of the partial S2 sequences placed the circulating viruses identified in the camel population within the B1 cluster representing contemporary dromedary and human MERS-CoVs. The clustering with human MERS-CoVs known to have caused nosocomial outbreaks in the KSA, South Korea, and Jordan underlines the zoonotic potential of these camel-derived MERS-CoVs.

Conclusions

While the most recent nosocomial outbreaks in Jordan were linked to travel-related cases from KSA as reported by the WHO, the detection of B1-cluster-like MERS-CoV in dromedary camels indicates that local introductions of MERS-CoV into the human population is a real possibility in Jordan, in addition to introductions via travel-associated cases.

Acknowledgments

This research was supported in part by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, the Jordan University of Science and Technology project 272/2015, internal support from Emory University, the Food and Agriculture Organization of the United Nations' component of the USAID-funded Emerging Pandemic Threats phase 2 (EPT-2) Program and the Kansas Bioscience Authority. We want to sincerely thank Boehringer Ingelheim for logistic assistance provided before and during research, and Austin Athman for assistance with figures.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Juergen A. Richt
Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, KS 66506-0100

E-mail: jricht@ksu.edu

Vincent J. Munster
Laboratory of Virology
Division of Intramural Research
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Rocky Mountain Laboratories
903 South 4th Street
Hamilton, MT 59840

E-mail: vincent.munster@nih.gov