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Cross-sectional study of MERS-CoV-specific RNA and antibodies in animals that have had contact with MERS patients in Saudi Arabia

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ABSTRACT

Background: Middle East respiratory syndrome coronavirus (MERS-CoV) is a newly emerged coronavirus that is associated with a severe respiratory disease in humans in the Middle East. The epidemiological profiles of the MERS-CoV infections suggest zoonotic transmission from an animal reservoir to humans.
Methods: This study was designed to investigate animal herds associated with Middle East respiratory syndrome (MERS)-infected patients in Saudi Arabia, during the last three years (2014–2016). Nasal swabs and serum samples from 584 dromedary camels, 39 sheep, 51 goats, and 2 cattle were collected. Nasal samples from camels, sheep, goats, and cattle were examined by real-time reverse-transcription PCR (RT-PCR) to detect MERS-CoV RNA, and the Anti-MERS ELISA assay was performed to detect camel humeral immune response (IgG) to MERS-CoV S1 antigen infection. The complete genome sequencing of ten MERS-CoV camel isolates and phylogenetic analysis was performed.
Results: The data indicated that seventy-five dromedary camels were positive for MERS-CoV RNA; the virus was not detected in sheep, goats, and cattle. MERS-CoV RNA from infected camels was not detected beyond 2 weeks after the first positive result was detected in nasal swabs obtained from infected camels. Anti-MERS ELISA assays showed that 70.9% of camels related to human cases had antibodies to MERS-CoV. The full genome sequences of the ten MERS-CoV camel isolates were identical to their corresponding patients and were grouped together within the larger MERS-CoV sequences cluster for human and camel isolates reported form the Arabian Peninsula.
Conclusions: These findings indicate that camels are a significant reservoir for the maintenance of MERS-CoVs, and they are an important source of human infection with MERS.

Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel betacoronavirus that recently emerged. MERS is considered a life-threatening disease with an extensive health impact. The clinical manifestations vary from subclinical manifestations to rapid progressive acute pneumonia. MERS-CoV-infected patients often present fever, sore throat, myalgia, cough, and shortness of breath, and occasionally hemoptysis [1–3]. MERS-CoV was first identified in 2012 in Jeddah, Saudi Arabia [4]. Since its identification, MERS has been responsible for 2040 cases and 712 deaths in 27 countries

Abbreviations: MERS, Middle East respiratory syndrome; MERS-CoV, Middle East respiratory syndrome coronavirus; TUU, Tabuk; AJF, Jouf; HAS, Hail; RAH, Northern Boundaries; ELQ, El-Qassim; MED, Al-Madina; RUH, Riyadh; DWD, El-Dowadmi; SHG, Shagraa; AKH, Alkharj; WAE, Wadi El-Dwasir; ZUL, Zulfi; MJH, Majmaa; TIF, Taif; MAK, Makkah; ABT, Bahaa; AHB, Asir; GIZ, Gizan; EAM, Najran; DMM, Shargia; HOF, Ihsaa.

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worldwide by July 21, 2017 [5]. The majority of cases and deaths occurred in Saudi Arabia, most of which were sporadic infections, sometimes leading to family or hospital clusters [6].

The exact source of MERS-CoV and how it is transmitted to humans is unknown. Initial investigations have indicated that MERS-CoV originated from bats; sequences related to MERS-CoV have been found in several bat species [6,7]. Several other animal species in the Arabian Peninsula have been serologically assessed for MERS-CoV infection [8]. Viral and serological surveillance revealed a high rate of seropositivity to MERS-CoV in dromedary camels, including detection of antibodies to MERS-CoV in sera of camels in different countries (i.e., Saudi Arabia, UAE, Oman, Egypt, Jordan, Nigeria, Tunisia, Ethiopia, Kenya, Canary islands, Pakistan, and Mali) [10–18]. Recovery of MERS-CoV genome sequences from camels with a high degree of homology to their counterparts in humans and isolation of MERS-like CoV from camels have been reported [9,10,14,15,19,20,21]. Together, these results suggest that the MERS-CoV detected in dromedary camels is the most likely source of the human infection [8,9,15,20,21]. The questions that need to be addressed are why the majority of human infections occur in Saudi Arabia, and whether interspecies transmission of the MERS-CoV to humans has only recently occurred or has only been recently recognized. The epidemiological factors related to these questions need to be investigated.

Current epidemiological data on MERS infections refers to zoonotic transmission from an animal reservoir to humans. In this study, animals (camels, sheep, goats, and cattle) related to human cases were examined for MERS-CoV RNA and antibodies. Furthermore, complete genome sequences of MERS-CoV isolated from camels and patients were compared to assess the potential zoonosis of MERS infection.

Materials and methods

Samples

This study was carried out during 2014, 2015, and 2016 at the Ministry of Environment, Water and Agriculture (MEWA), Riyadh, Saudi Arabia. This was part of a cooperative epidemiological response to the confirmed human cases of MERS with a history of animal contact. Notifications were issued by the Ministry of Health (MOH), Riyadh, Saudi Arabia. We received 167 notifications from the MOH, distributed throughout all regions of Saudi Arabia. Sixty-eight of these notifications were associated with camels, while 14 were related to other animals. Seventy-two notifications had no relationship with animals, and in the remaining 13 notifications, the owners did not allow us to investigate their animals (Fig. 1).

A total of 780 nasal swabs were collected from all animals with a history of contact with MERS-patients (595 dromedary camels, 93 sheep, 90 goats, and 2 cattle) of different ages and sex in different regions of Saudi Arabia. Nasal swabs from animals were transferred to the Riyadh veterinary laboratory in transport medium (COPAN Italia, Italy) within 24–72 h after collection. All animals positive for MERS-CoV RNA were isolated, quarantined, and examined weekly until two consecutive negative samples were obtained to determine the maximum period of viral shedding.

Blood samples were collected from dromedary camels, centrifuged to separate the sera, and then frozen at −20 °C. Serum samples were tested for the presence of IgG antibodies reacting with MERS-CoV.

Nucleic acid extraction, real-time reverse transcription-PCR, and sequencing

RNA was extracted from nasal swabs samples, using a Qiagen viral RNA extraction kit in accordance with the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Real-time reverse transcription-PCR (rtRT-PCR) targeting the envelope protein gene upstream (UpE) of MERS-CoV was conducted for screening [22,23]. Open reading frame (ORF) 1a was used to confirm the MERS-CoV diagnosis, based on the recommendation of the World Health Organization (WHO) [24]. Briefly, 5 μL of extracted RNA was subjected to rtRT-PCR using UpE primers, as described elsewhere [22]. The rtRT-PCR was performed using LightMix Molecular Dx MERS-CoV upE kits (Roche) according to the manufacturer's protocol. All positive samples from the UpE screening assay were confirmed by ORF1a as previously described [22,23].

Ten positive RT-PCR samples {Riyadh (3), Jeddah (3), Unayzah (1), Quwaiyah (1), Artawiyah (1), and Taif (1)} were subjected to full genome sequencing. Phylogenetic analyses based on full genome sequencing of MERS-CoV were carried out with MEGA7 [25]. The evolutionary distances were estimated by means of the neighbor-joining method [26] based on the Tajima-Nei method [27]. Bootstrap analyses were performed with 1000 repeat samples of the data sets [28].

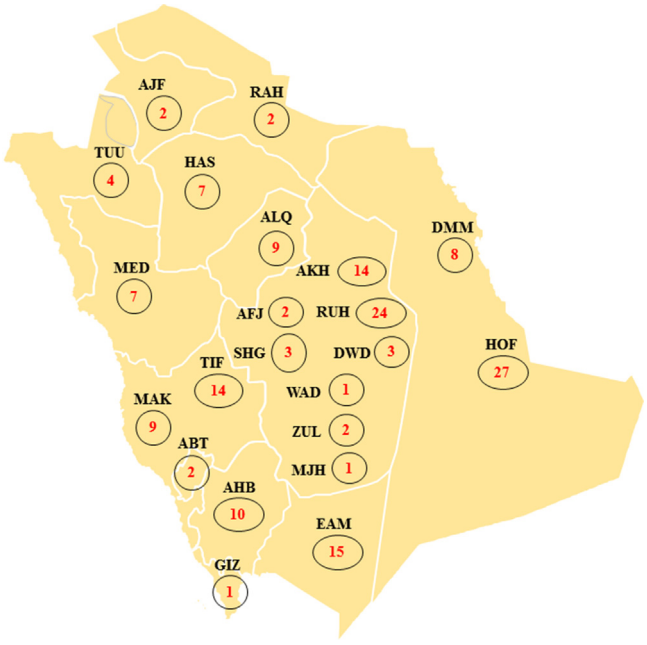

Fig. 1. Distribution of confirmed human cases of MERS with a history of contact with animals in different regions of Saudi Arabia, notifications issued by the Ministry of Health (MOH).

Anti-MERS-CoV ELISA

Camel serum samples were collected and assayed for MERS-CoV specific antibodies, using Anti-MERS-CoV ELISA Camel (IgG) (EUROIMMUN, Lübeck, Germany). The ELISA test kit allows a semi-quantitative assay for IgG antibodies against MERS coronavirus in plasma or serum from camels. The ELISA assay was carried out according to the manufacturer's protocol. In summary, the serum samples were diluted 1:101 in the sample buffer; the diluted samples were incubated in wells coated with the purified S1 antigen of MERS coronavirus (MERS-CoV S1). In the case of positive samples, the specific antibodies will bind to MERS antigens. A second incubation is carried out using an enzyme-labeled anti-camel IgG (enzyme conjugate) stimulating the reaction to detect the bound antibodies [12,29].

Evaluation of Anti-MERS-CoV ELISA kit with MERS-CoV pseudoparticle neutralization assay (ppNT)

To evaluate commercial Anti-MERS-CoV ELISA kits, 100 serum samples were re-tested with ppNT. For the ppNT assay, HIV/MERS pseudoparticles containing 5 ng of p24 were incubated with diluted serum for 30 min at 4 °C to infect the Vero E6 cells in triplicate. The remaining virus was assayed at 2 days post infection. The highest serum dilution resulting in a 90% reduction of luciferase activity was regarded as the ppNT antibody titer [13].

Data management and statistical analysis

The data collected from the questionnaire was transferred into a Microsoft Excel spreadsheet database and then imported into the Statistical Package for Social Sciences (SPSS) for Windows® Version 22.0 (SPSS Inc., Chicago, Illinois) for statistical analyses appropriate for each variable. Univariate and multivariate analyses were also performed by the 2-tailed chi-square test and using the logistic regression model. The association in the chi-square test and logistic regression model were considered significant when $P \leq 0.05$.

Results

Detection of MERS-CoV RNA by real-time PCR in animal samples

Epidemiological investigations were conducted for all 167 notifications of MERS patients throughout regions of Saudi Arabia that were issued by the MOH, 84 of which were linked to animals. There were 68 cases connected to camels, and 14 were associated with other animals. There were 20 positive notifications for MERS-CoV RNA after animals were screened (Fig. 1).

In total, 780 nasal swabs were collected from animals associated with MERS patients (595 dromedary camels, 93 sheep, 90 goats, and 2 cattle) in different parts of Saudi Arabia. The MERS-CoV was detected in 75 camels (12.6%) by rtRT-PCR assays; the virus was not detected in any of the nasal swabs collected from sheep, goats, and cattle (Table 1).

The MERS-CoV RNA has been detected in dromedary camels related to human cases in different regions of Saudi Arabia. There were 16 camels positive for MERS-CoV RNA in Shargia, 14 in Al-Madina, 11 in Makkah, 10 in Taif, 5 in Ihsaa, 6 in Riyadh, 4 in Alkharj, 3 in Asir, 2 in El-Dowadmi and Aflaj, 1 in both Najran and El-Qassim. However, no animals were detected shedding MERS-CoV RNA in Shagraa, Northern Boundaries, Hail, Bahaa, Gazan, Zulfi, Majmaa, Tabuk, and Wadi El-Dawasir (Fig. 2).

The percentage of positivity for MERS-CoV RNA in male camels was 20%, while it was 4.9% in females. Camels less than 2 years old showed a high virus load (15.4%) compared with other camels (2–4 years) (6.3%), (4–6 years) (4.2%), and camels more than 6 years old (7.4%) (Table 1).

MERS-CoV RNA was not detected from infected camels after more than 2 weeks from the first positive result as detected in nasal swabs.

Detection of MERS-CoV antibodies by Anti-MERS ELISA

In total, 595 serum samples were collected from camels that had been linked to the MERS human cases from different regions of Saudi Arabia (Fig. 3); MERS-CoV-specific IgG was identified by ELISA assays in 422 camel serum samples (70.9%). MERS-CoV specific IgG antibodies in camels from different regions of Saudi Arabia varied from 100% positive in Shagraa, Bahaa, Zulfi, and Wadi El-Dwasir to 37% in Alkharj (Table 2).

There were 127 of the 152 male camels positive for MERS-CoV antibodies (83.5%), while 295 female camels were positive (66.5%). The camels more than 6 years old showed a high prevalence of MERS-CoV-specific IgG (86.6%) compared to other camels (4–6 years) (80.6%), (2–4 years) (76.9%), and less than 2 years of age with 57.7% (Table 2).

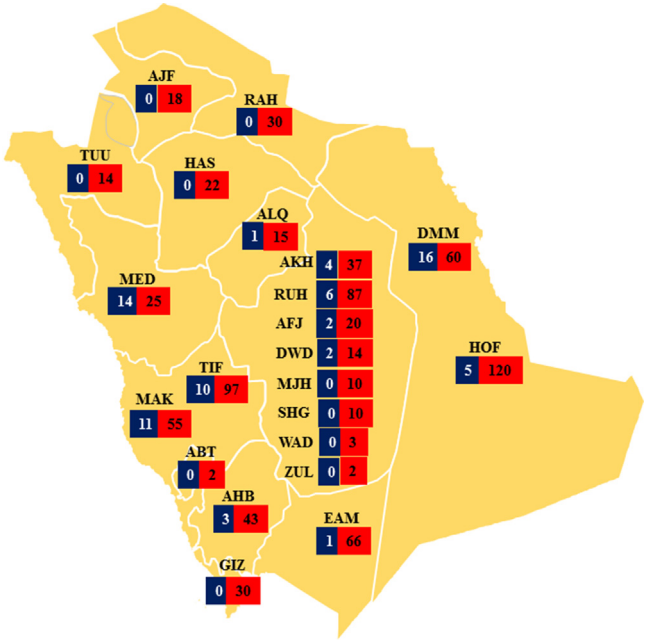

Fig. 2. Distribution of animals examined for MERS-CoV RNA, linked to MERS-human cases in different regions of Saudi Arabia. Total number of animals examined by real-time PCR for MERS-CoV RNA in each region (red square), number of positive animals in each region (blue square).

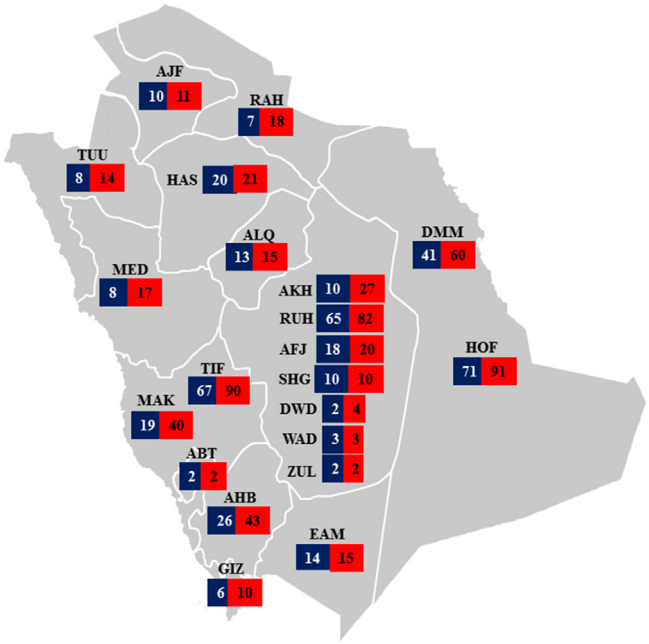

Fig. 3. Distribution of animals examined for MERS-CoV specific IgG antibodies, linked to MERS-human cases in different regions of Saudi Arabia. Total number of animals examined by Anti-MERS ELISA for MERS-CoV IgG in each region (red square) and the number of positive animals in each region (blue square).

Table 1
The prevalence of MERS-CoV RNA in animals linked to human cases in different regions, sex and ages in Saudi Arabia.

	No of tested animals	No of positive animals	Percentage of positivity	χ^2 test	P-value
Regions					
El-Dowadmi	14	2	14.2%	132.826	0.000**
Shagraa	10	0	0%		
Riyadh	87	6	6.89%		
Alkharj	37	4	10.8%		
EL-Qassim	15	1	6.6%		
Shargia	60	16	26.6%		
Northern Boundaries	30	0	0%		
Najran	66	1	1.5%		
Ihsaa	120	5	4.16%		
Jouf	18	0	0%		
Al-Madina	25	14	56%		
Hail	22	0	0%		
Bahaa	2	0	0%		
Gazan	30	0	0%		
Makkah	55	11	20%		
Asir	43	3	6.9%		
Taif	97	10	10.3%		
Zulfi	2	0	0%		
Aflaj	20	2	10%		
Majmaa	10	0	0%		
Tabuk	14	0	0%		
Wadi El-Dwasir	3	0	0%		
Sex					
Male	245	49	20%	44.32	0.000**
Female	535	26	4.9%		
Age					
0–2 years	298	46	15.4%	19.688	0.000**
2.1–4 years	202	13	6.4%		
4.1–6 years	144	6	4.2%		
More than 6 years	136	10	7.4%		
Tested animals					
Dromedary Camel	595	75	12.6%	25.801	0.000**
Sheep	93	0	0%		
Goat	90	0	0%		
Cattle	2	0	0%		

NS Non significant difference at ($P > 0.05$).
* Significant difference at ($P \leq 0.05$).
** Highly significant difference at ($P \leq 0.01$).

Evaluation of Anti-MERS ELISA with pseudoparticle virus neutralization test (ppNT)

The MERS ppNT assay was conducted to examine 100 randomly selected camel serum samples from Saudi Arabia and was compared to the commercial Anti-MERS-CoV-specific IgG ELISA kit. Seventy dromedary camel serum samples that were positive in the ELISA screening assay were found to have a high neutralization activity in the ppNT assay showing ELISA specificity of 100%. In addition, three serum samples that were negative in the ELISA assay had weak activity (low MERS ppNT titers ranging from 40 to 160), but could be detected in the ppNT test showing ELISA sensitivity of 95.98% (Table 3).

Sequencing and phylogenetic analysis

The total nucleic acid extracts obtained from the nasal samples were subjected to random sequencing on the Ion Torrent platform, yielding full-length genomic sequences. The complete genomic sequences were obtained from ten camel's nasal swab samples, and then aligned to the corresponding sequences of human cases as well as other MERS-CoV genomic sequences retrieved from the GenBank. Alignment analysis showed complete identity between four MERS-CoV camel isolates (Unayzah, Quwaiyah, Jeddah-1/2015, and Jeddah 2016) and their corresponding isolates from MERS patients (KT806000.1 Hu/Unayzah-KSA-3249/2015, KT805966.1 Hu/Quwaiyah-KSA-3405/2015, KT805995.1 Hu/Jeddah-KSA-C22351/2015, and KX154690 Hu/Jeddah-KSA-161RS1146/2016). The other six MERS-CoV camel isolates (Camel/Riyadh-1/2015, Camel/Riyadh-2/2015, Camel/Artawiyah-1/2016, Camel/Taif-1/2015, Camel/Jeddah-2/2015, and Camel/Riyadh-1/2016) showed 99% identity with MERS-CoV sequences from related patients (KU851860.1 Hu/Riyadh-KSA-16121/2015, KU851860.1 Hu/Riyadh-KSA-16121/2015, KX154694.1 Hu/Artawiyah-KSA-13328/2016, KT806017.1 Hu/Taif-KSA-15167/2015, KT806045.1 Hu/Jeddah-KSA-C21271/2015, and KX154684.1 Hu/Riyadh-KSA-11739/2016) (Fig. 4). Such data confirmed that the dromedary camels in Saudi Arabia harbor the same virus that causes MERS infection in humans.

Discussion

Since 2012, MERS-CoV infection has been occurring among countries in the Middle East. The majority of cases have been reported in Saudi Arabia, and most of them were reported in hospitals and healthcare units (nosocomial infection). The incidence of primary cases draws attention to a possible animal source, indicating a potential zoonotic infection. Several studies have reported a high prevalence of MERS-CoV antibodies in dromedary camels [9,11,14]. Interestingly, antibodies to MERS-CoV have been detected in camel sera collected more than 10 years ago from the United Arab Emirates (UAE) [11]. In addition, MERS-CoV has been reported in camels in many cross-sectional and longitudinal studies [10,19,20,21]. However, other farm animals (cattle, sheep,

Table 2
The prevalence of MERS-CoV antibodies in dromedary camel linked to human cases in different regions, sex and ages in Saudi Arabia.

	No of tested animals	No of positive animals	Percentage of positivity	χ^2 test	P-value
Regions					
El-Dowadmi	4	2	50%	1.725	0.164[NS]
Shagraa	10	10	100%		
Riyadh	82	65	79.2%		
Alkharj	27	10	37%		
EL-Qassim	15	13	86.6%		
Shargia	60	41	68.6%		
Northern Boundaries	18	7	38.8%		
Najran	15	14	93.3%		
Ihsaa	91	71	78%		
Jouf	11	10	90.9%		
Al-Madina	17	8	47.5%		
Hail	21	20	95.2%		
Bahaa	2	2	100%		
Gazan	10	6	60%		
Makkah	40	19	47.5%		
Asir	43	26	60.4%		
Taif	90	67	74.4%		
Zulfi	2	2	100%		
Aflaj	20	18	72%		
Tabuk	14	8	57.1%		
Wadi El-Dwasir	3	3	100%		
Sex					
Male	152	127	83.5%	10.021	0.007**
Female	443	295	66.5%		
Age					
0–2 years	251	145	57.7%	14.244	0.003**
2.1–4 years	156	120	76.9%		
4.1–6 years	98	79	80.6%		
More than 6 years	90	78	86.6%		
Tested animals					
Dromedary Camel	595	422	70.9%		

[NS]Non significant difference at ($P > 0.05$).
*Significant difference at ($P \leq 0.05$).
** Highly significant difference at ($P \leq 0.01$).

and goats) were negative for MERS-CoV antigen and antibodies [14,15]. These results strongly suggest that the dromedary camels are the most likely source of the MERS-CoV infection; furthermore, these findings necessitate the investigation of MERS-CoV in animals related to MERS patients.

This study showed that MERS-CoV RNA was detected in 12.6% of dromedary camels associated with MERS-patients, while the MERS-CoV genome was not detected in sheep, goats, and cattle; this is in accordance with previous studies [14,15,30]. The prevalence of MERS-CoV RNA in different regions of Saudi Arabia varied from 60% in Al-Madina to 0% in Shagraa, Northern Boundaries, Hail, Baha, Gizan, Zulfi, Majmaa, Tabuk, and Wadi El-Dwasir. Available data confirmed that dromedary camels were the primary reservoirs of MERS-CoV and direct contact with dromedaries in Saudi Arabia was found to be independently associated with MERS-CoV illness, while contact with goats, sheep, or cattle was not associated with human disease [31,32]. The analysis of nasal swabs revealed that the maximum period for viral shedding from infected camels was 2 weeks after the initial detection; this is in accordance with previous studies [33].

The study showed high seroprevalence rates of dromedary camels associated with MERS-patients to MERS-CoV-specific IgG antibodies (70.9%). The prevalence of MERS-CoV antibodies in different regions of Saudi Arabia ranged from 100% in Shagraa, Bahaa, Zulfi, and Wadi El-Dwasir to 37% in Alkharj. This result is in agreement with several reports from other studies, which showed high seroprevalence rates in camels, with 84.5% of camels in Egypt carrying antibodies to MERS-CoV [34] and more than 90% in northern Mali [18]. In addition, 46.9% of camels in Laikipia County, Kenya showed seropositivity to MERS-CoV antibodies [35].

The analysis of age-based results showed that adult camels had a higher seroprevalence of MERS-CoV antibodies (86.6%) compared to young camels less than 2 years (57.7%). While young animals less than 2 years showed a high positivity (15.4%) to MERS-CoV RNA compared to adult animals, previous studies have shown a high seropositivity in adults compared to juvenile camels that exhibited a high infection rate [36,37]. In addition, male camels showed a higher positivity (83.5%) to MERS-CoV antibodies than the female camels (66.5%). Moreover, for MERS-CoV RNA the male camels showed 20% positivity while female camels exhibited 4.9%. A previous study stated that there was no difference in the seroprevalence rates between female camels (82.7%) and males (85.1%), while MERS-CoV RNA level was higher in females (7.1%) than in males (2.6%) [34]. This variation is likely related to the difference in sample numbers and the age of animals included in both studies.

The commercial Anti-MERS-CoV-specific IgG ELISA kit was compared with the MERS ppNT assay for examining MERS-CoV antibodies in camel serum. ELISA specificity was 100% while the sensitivity was 95.98%. This difference may be account for the three negative sera samples that had ELISA titers near the positive results. A previous report indicated that the MERS-CoV ppNT assay is a safe and specific assay for serological studies in a variety of animal species and humans [13]. Furthermore, many previous studies tested serum samples for MERS-CoV antibodies by a recombinant MERS-CoV spike protein-based ELISA [11,12,18,35].

The relative phylogenetic data showed that there were identical sequences in four MERS-CoV isolates recovered from camels (Unayzah 2015, Quwaiyah 2015, Jeddah-1/2015, and Jeddah 2016) and from their corresponding patients (KT806000.1 Hu/Unayzah-KSA-3249/2015, KT805966.1 Hu/Quwaiyah-KSA-

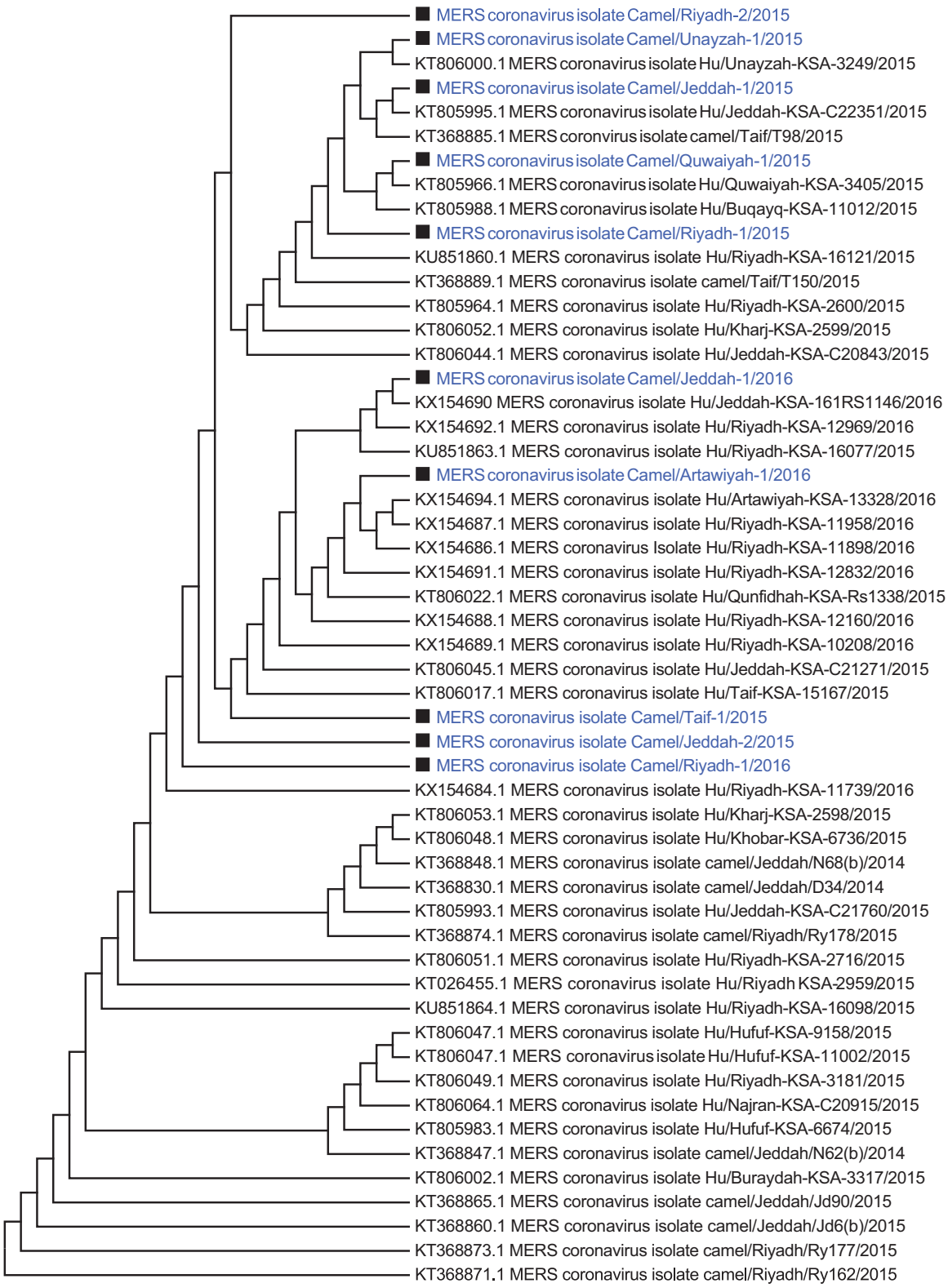

Fig. 4. Phylogenetic analysis of MERS-CoV found in ten RT-PCR-positive camels, using MEGA7. Full genome sequences of the ten MERS-CoV camel samples and the sequences from corresponding patients were aligned with sequences of MERS-CoV reference strains available from GenBank. Phylogenetic analysis was inferred using the neighbor-joining method and distance calculations were computed using the Tamura-Nei model. Sequences from the current study are indicated by a solid square.

3405/2015, KT805995.1 Hu/Jeddah-KSA-C22351/2015, and KX154690 Hu/Jeddah-KSA-161RS1146/2016) and 99% sequence similarity of the six remaining MERS-CoV camel isolates and corresponding patients. Evidence suggests that camels were the source of MERS-CoV that infected patients who had had close contact with the camel's nasal secretions. The presence of identical sequences of MERS-CoV isolates recovered from patients and related camels indicates that direct cross-species transmission probably occurred, and dromedary camels were the source of MERS-CoV that infected the patients who had close contact with them. These data show

Table 3
Evaluation of Anti-MERS ELISA kit with peudoparticle virus neutralization test.

Pesudoparticle virus neutralization test			
	+ve samples	−ve samples	Total samples
Anti-MERS ELISA			
+ve samples	70	0	70
−ve samples	3	27	30
Total samples	73	27	100
Sensitivity%	95.98%		
Specificity%	100%		

evidence of potential zoonosis and have a common origin. This is consistent with previous studies [31,33,38,39].

These results indicate the need for further studies to clarify the mechanisms by which the MERS-CoV is transmitted from camels to humans and the heterogeneity of human susceptibility to this virus.

Conclusion

Available data on the detection of MERS-CoV RNA and antibodies in camels related to MERS-patients support that human infection might be acquired by direct contact with infected camels. However, in order to elucidate the epidemiology of MERS-CoV in Saudi Arabia, further studies are required.

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Competing interests

None declared.

Ethical approval

MEWA ethical committees approved the study.

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