MERS-CoV in sheep, goats, and cattle, United Arab Emirates, 2019: Virological and serological investigations reveal an accidental spillover from dromedaries

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Abstract

The recent COVID-19 pandemic has demonstrated again the global threat posed by emerging zoonotic coronaviruses. During the past two decades alone, humans have experienced the emergence of several coronaviruses, such as SARS-CoV in 2003, MERS-CoV in 2012, and SARS-CoV-2 in 2019. To date, MERS-CoV has been detected in 27 countries, with a case fatality ratio of approximately 34.5 %. Similar to other coronaviruses, MERS-CoV presumably originated from bats; however, the main reservoir and primary source of human infections are dromedary camels. Other species within the *Camelidae* family, such as Bactrian camels, alpacas, and llamas, seem to be susceptible to the infection as well, although to a lesser extent. In contrast, susceptibility studies on sheep, goats, cattle, pigs, chickens, and horses obtained divergent results. In the present study, we tested nasal swabs and/or sera from 55 sheep, 45 goats, and 52 cattle, collected at the largest livestock market in the United Arab Emirates, where dromedaries are also traded, for the presence of MERS-CoV nucleic acid by RT-qPCR, and for specific antibodies by immunofluorescence assay (IFA). All sera were negative for MERS-CoV-reactive antibodies, but the nasal swab of one sheep (1.8 %) was positive for MERS-CoV nucleic acid. Next generation sequencing (NGS) of the complete *N* gene of the sheep-derived MERS-CoV revealed >99 % nucleotide identity to MERS-CoV sequences of five dromedaries in nearby pens and to three reference sequences. The NGS sequence of the sheep-derived MERS-CoV was confirmed by conventional RT-PCR of a part of the *N* gene and subsequent Sanger sequencing. All MERS-CoV sequences clustered within clade B, lineage 5. In conclusion, our study shows that non-camelid livestock, such as sheep, goats, and cattle do not play a major role in MERS-CoV epidemiology. The one sheep that tested positive most likely reflects an accidental viral spillover event from infected dromedaries in nearby pens.

Short running title (less than 70 characters):

Study on MERS-CoV in sheep, goats, and cattle, UAE, 2019

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Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required for sampling of the involved animals.

Summary

The recent COVID-19 pandemic has demonstrated again the global threat posed by emerging zoonotic coronaviruses. During the past two decades alone, humans have experienced the emergence of several coronaviruses, such as SARS-CoV in 2003, MERS-CoV in 2012, and SARS-CoV-2 in 2019. To date, MERS-CoV has been detected in 27 countries, with a case fatality ratio of approximately 34.5 %. Similar to other coronaviruses, MERS-CoV presumably originated from bats; however, the main reservoir and primary source of human infections are dromedary camels. Other species within the *Camelidae* family, such as Bactrian camels, alpacas, and llamas, seem to be susceptible to the infection as well, although to a lesser extent. In contrast, susceptibility studies on sheep, goats, cattle, pigs, chickens, and horses obtained divergent results. In the present study, we tested nasal swabs and/or sera from 55 sheep, 45 goats, and 52 cattle, collected at the largest livestock market in the United Arab Emirates, where dromedaries are also traded, for the presence of MERS-CoV nucleic acid by RT-qPCR, and for specific antibodies by immunofluorescence assay (IFA). All sera were negative for MERS-CoV-reactive antibodies, but the nasal swab of one sheep (1.8 %) was positive for MERS-CoV nucleic acid. Next generation sequencing (NGS) of the complete *N* gene of the sheep-derived MERS-CoV revealed >99 % nucleotide identity to MERS-CoV sequences of five dromedaries in nearby pens and to three reference sequences. The NGS sequence of the sheep-derived MERS-CoV was confirmed by

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conventional RT-PCR of a part of the *N* gene and subsequent Sanger sequencing. All MERS-CoV sequences clustered within clade B, lineage 5. In conclusion, our study shows that non-camelid livestock, such as sheep, goats, and cattle do not play a major role in MERS-CoV epidemiology. The one sheep that tested positive most likely reflects an accidental viral spillover event from infected dromedaries in nearby pens.

Keywords

Sheep, goat, cattle, Middle East respiratory syndrome, Middle East respiratory syndrome coronavirus, MERS-CoV

Introduction

Since the 1930s, members of the family *Coronaviridae* have been identified in humans and many different animal species, such as cats, dogs, horses, pigs, cattle, mice, and fowl . In the last 20 years, three zoonotic coronaviruses have emerged into the human population:*Severe acute respiratory syndrome-related coronavirus* (SARS-CoV) in 2003, *Middle East respiratory syndrome-related coronavirus*(MERS-CoV) in 2012, and most recently, SARS-CoV-2 in 2019, which led to the so far most devastating pandemic of the 21[st]century . Since 2012, MERS-CoV has been detected in 27 countries, with 84.0 % of cases found in Saudi Arabia, and a case fatality ratio of approximately 34.5 % (WHO, 2019). Similar to other coronaviruses, MERS-CoV presumably originated from bats; however, the main reservoir and primary source of human infections are dromedaries (*Camelus dromedarius*) . Although dromedaries usually remain asymptomatic, or develop only mild symptoms due to MERS-CoV infection, it has been shown that they can shed considerable amount of virus . Studies on Bactrian camels (*Camelus bactrianus*), hybrid camels, alpacas (*Vicugna pacos*), llamas (*Lama glama*), and even pigs (*Sus scrofa*), have revealed that these animals are also susceptible to MERS-CoV infection (Adney et al., 2019; Lau et al., 2020; Reusken et al., 2016; Vergara-Alert et al., 2017). In contrast, studies on sheep, goats, cattle, horses, and chickens have indicated that, although sheep and goats may produce antibodies to MERS-CoV, none of these species effectively shed the virus (Adney et al., 2016; Hemida et al., 2013; Reusken, Ababneh et al., 2013; Vergara-Alert et al., 2017). A study about domestic mammals in contact with infected dromedaries found MERS-CoV nucleic acid in nasal swabs not only from sheep and goats, but also from a cow and donkeys (Kandeil et al., 2019). To provide more information about the potential exposure and infection of other livestock, we performed a survey for MERS-CoV at a livestock market in the United Arab Emirates (UAE), as live animal markets are known to be common sources of virus spillover.

Materials and Methods

Sampling

Sampling was performed at the largest national livestock market in the UAE, the Al Ain central market in the emirate of Abu Dhabi, in October 2019. The market is divided into three main areas, with mostly mixed sheep and goat pens located between camel and cattle stalls (Fig. 1). The distance between camel pens and the closest pens holding sheep and goats is approximately 20 m.

In total, we sampled 152 animals, which appeared healthy at the time of sampling. Nasal swabs and blood samples were taken from 55 sheep (41 female and 14 male) with an age range from three months to four years (mean of 19 months), and 45 goats (31 female and 14 male) from three to 40 months old (mean of 12 months). In addition, we collected blood samples from 52 cattle (43 female and nine male) from one to six years (mean of 3.8 years). Nasal swabs were collected in tubes containing a virus inactivation solution (DNA/RNA Shield, ZymoResearch, Irvine, CA, USA). Serum was separated from each blood sample by centrifugation. All samples were then stored at -80degC at the laboratory of the College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, UAE, before shipment on dry ice to the University of Veterinary Medicine, Vienna, Austria, for virological and serological investigations.

MERS-CoV nucleic acid screening

Nasal swabs of sheep (n=55) and goats (n= 45), as well as serum samples of all animals (n=152) were screened

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for MERS-CoV nucleic acid by reverse transcription real-time (RT-q)PCR. For this, 200 µl of each sample were subjected to automated nucleic acid extraction employing QIAamp Viral RNA Mini Kit on QIAcube HT (both Qiagen, Hilden, Germany). MERS-CoV-specific RT-qPCR was performed using qScript XLT One Step RT-qPCR ToughMix (QuantaBio, MA, USA) and previously described primers and probe in the open reading frame (ORF) $1a$ gene . RT-qPCR-positive samples were additionally tested by conventional RT-PCRs (OneStep RT-PCR Kit, Qiagen, Hilden, Germany) employing two previously published primer pairs within the nucleocapsid (N) and $ORF1b$ genomic regions of MERS-CoV . Amplicons from the RT-PCRs were subjected to Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). In addition, next generation sequencing (NGS) was performed on putative positive samples and five selected MERS-CoV-positive samples from dromedaries in nearby pens. For this, adapter sequences were trimmed using bcl2fastq2 conversion software v2.20.0, and high-quality sequencing reads were aligned to three reference MERS-CoV genome sequences (GenBank acc. numbers KF192507, MG757604 and MK462253) using BWA v0.7.17. Consensus sequences were generated for the N gene using GATK v3.8–1–0 and BCFtools v1.3.1. Multiple sequence alignment of the N gene of MERS-CoV (1.2 kb) derived from one sheep and five dromedary camels was performed using MAFFT v7.455.

MERS-CoV antibody testing

Altogether 148 serum samples from sheep (n=55), goats (n=45), and cattle (n=48) were available in sufficient volumes to perform MERS-CoV antibody testing. MERS-CoV spike protein-based immunofluorescence assay (IFA) was done as described previously . Initial screening was performed with sample dilutions of 1:40, retesting of putative positive (questionable) samples was done with a dilution series of 1:10 – 1:320. Detection was facilitated using 1:200 dilutions of donkey-anti sheep, donkey-anti goat, or goat-anti bovine secondary antibodies, all labelled with Alexa Fluor 488 (Dianova, Hamburg, Germany).

Results

MERS-CoV nucleic acid and antibody prevalence

The nasal swab of one sheep (1.8 %) repeatedly tested positive for MERS-CoV nucleic acid by RT-qPCR (Ct values 32.5-34.8; Table 1), and the nasal swab of one goat tested borderline positive (Ct values 38.1-39.4; Table 1). The positive sheep was a two-year-old female, presumably locally raised Nuaimi breed. It was kept in a pen at the market used for sheep only, which was the second closest pen to the camel area (approximately 25 m away; Fig. 1, Supplementary Table 1). The borderline positive goat, a one-year-old locally raised male Omani goat, was kept in one of the pens directly adjacent to the camel pens, with only approximately 20 m between them (Fig. 1, Supplementary Table 1).

The RT-qPCR-positive sheep sample was confirmed by two different RT-PCRs. The obtained sequences [lengths without primer sequences: N – 293 bp (GenBank acc. number: MZ558082), $ORF1b$ – 341 bp (GenBank acc. number: MZ558083)] were >99 % identical to many MERS-CoV sequences belonging to clade B, lineage 5 [according to the classification by , including human and camel strains from the UAE, Saudi Arabia, South Korea, Jordan, Qatar, and the United Kingdom. Unfortunately, we were not able to uncover the complete MERS-CoV genomes using NGS; however we succeeded to establish the complete N gene sequences of the virus-positive sheep and five selected MERS-CoV-positive dromedaries (Figure 2). The sheep-derived N gene sequences obtained by NGS and Sanger sequencing were identical. By NGS, the average sequencing coverage of the N gene from the sheep (S90_N) and five camel samples (C53_N, C5_N, C6_N, C28_-N, and C50_N) was >6X across all samples (Figure 2A). The percentage of sequence identity of the N gene of the same samples along with three reference MERS-CoV sequences (GenBank acc. numbers KF192507, MG757604 and MK462253) revealed that the sheep sample showed high nucleotide identity (>99 %) with all other MERS-CoV sequences (Figure 2B). In detail, multiple sequence alignment revealed five positions of nucleotide variations between the sheep-derived MERS-CoV sequence and the camel-derived sequences and/or reference sequences (representative positions 533 and 637 of the N gene are shown in Figure 2C). The GenBank accession numbers of the complete MERS-CoV N gene sequences are: sheep-derived (S90_-N): MZ558076; dromedary-derived: C53_N - MZ558080; C5_N - MZ558077; C6_N - MZ558078; C28_N -

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MZ558079; and C50_N - MZ558081. Unfortunately, the borderline-positive result of the goat sample could not be confirmed by either RT-PCR or NGS.

The serological survey revealed that, although in the initial screening with 1:40 dilutions 15 serum samples (eight sheep, four goats, three cattle) appeared questionable, the presence of antibodies could not be confirmed in any sample by retesting using a dilution series of 1:10 – 1:320 (Supplementary Tables 1 and 2).

Discussion

Previous studies have demonstrated that, besides dromedaries, several other members of the *Camelidae* family are susceptible to MERS-CoV infection, such as Bactrian camels, alpacas, and llamas , although human contact infections have only been reported from dromedary camels . However, the existing data on the susceptibility of non-camelids are conflicting. Our results are in line with a series of publications demonstrating that non-camelids do not play a major role in the transmission cycle . For instance, in a seroepidemiological study in Saudi Arabia (2010-2013), no antibodies were detected in sheep, goats, cattle, or chickens, but a high prevalence was found in camels . Similarly, after an experimental challenge of sheep, goats, and horses by intranasal MERS-CoV inoculation, none of the animals effectively shed the virus, and neutralizing antibodies were only found in goats . Further regarding the family *Equidae* , including horses, donkeys, and mules, although viral replication has been observed in primary horse kidney cells, no antibodies have been found in any of 1,053 equid sera collected in the UAE and Spain . A study on livestock cell lines from the Arabian Peninsula showed that MERS-CoV can replicate in goat, alpaca, and dromedary cells, in contrast to sheep, cattle, rodent, and insectivore cells . In a study experimentally challenging animals from France and Spain, MERS-CoV nucleic acid in the respiratory tract as well as seroconversion were detected only in llamas and pigs, but not in sheep and horses . A serosurvey in a MERS-CoV-affected area in Jordan identified MERS-CoV neutralising antibodies in the sera of all camels but none in goats and cattle, while 4.8 % of sheep sera reacted with MERS-CoV antigen . In a monitoring study of several mammalian species in direct contact with infected dromedaries in Egypt, Tunisia, and Senegal (2015-2017), antibodies were identified in an astonishing 55.6 % of sheep from Senegal, 1.8 % of sheep from Tunisia, and 0.9 % of goats from Egypt. MERS-CoV RNA was detected in nasal swabs from 1.2 % sheep and 4.1 % goats from Egypt and Senegal, as well as 1.9 % cattle and 7.1 % donkeys from Egypt . In conclusion, it seems that several non-camelid species may become infected with MERS-CoV during viral spillover events when they are in direct contact with virus-shedding dromedaries. Maybe this susceptibility is due to the close relatedness of the *Betacoronaviruses* (specifically their receptors) infecting camels and especially bovids, but also humans, porcines, and equids . However, whether these animals are implicated in the MERS-CoV transmission cycle remains unknown, but seems to be highly unlikely.

The fact that none of the sheep, goats, and cattle in our survey tested positive for antibodies indicates that none of them had previously experienced a MERS-CoV infection, although with an age range of three months to six years, all animals could have encountered the virus in the past. Moreover, as the average time of stay at the market for goats is usually 7-10 days, 10-14 days for sheep, and between 14 and 30 days for cattle, there is also a not negligible risk of infection at the market. However, the nasal swab of one sheep (1.8 %) contained MERS-CoV nucleic acid (Supplementary Table 1), suggesting either a current infection, or a spillover event from nearby dromedaries, which appears to be more likely since no antibodies were detected and the sheep was kept in a pen in close proximity to the camel pens (approximately 25 m away; Fig. 1). Of note, we additionally sampled 90 dromedaries at the same market and at the same time as the non-camelids, of which 35.6 % were positive for MERS-CoV nucleic acid, and another 24.4 % tested borderline positive (Ct values 38.6-41.2). Moreover, 91.1 % of the 90 camel sera were MERS-CoV antibody-positive by ELISA, and 96.7 % by IFA (data can be provided upon request), further supporting the high prevalence of MERS-CoV infections in dromedaries. This hypothesis is also supported by our latest study, in which we screened 76 dromedaries during spring and autumn 2019 at the same market, and found MERS-CoV nucleic acid in 57.9 % of their nasal swabs .

The positive sheep from this study was a two-year-old female, presumably locally raised Nuaimi breed. However, due to the fact that sex was not equally distributed across the animals we sampled (115 females,

75.7 %, compared to 37 males, 24.3 %), no conclusions can be drawn regarding the susceptibility of the different sexes. The same is true for the different breeds, with only one positive animal, we cannot infer any breed-specific genetic predispositions to MERS-CoV infection.

In conclusion, our study shows that non-camelids such as sheep, goats, and cattle do not play a major role in MERS-CoV epidemiology. The one sheep that tested RNA-positive in our study most likely reflects a viral spillover event from infected dromedaries in nearby pens. Furthermore, serology revealed that the sheep did not develop an antibody response, suggesting that the infection was very recent, or the sheep only carried viral particles on its nasal mucosa without actually being infected.

Acknowledgements

Conflict of Interest Statement

The authors declare no conflict of interest.

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Table 1. Results of the MERS-CoV screening in sheep, goats, and cattle. Nasal swabs from sheep and goats, and sera from all animals were tested for MERS-CoV nucleic acid by *ORF1a* RT-qPCR. Serum samples from all but four animals (*i.e.* cattle sera with insufficient volumes) were investigated for MERS-CoV-specific antibodies by IFA.

Species	n (female/male)	Age range (average)	Nucleic acid-positive Nasal swabs	Nucleic acid-positive Serum samples	Antibody
Sheep	55 (41/14)	3-48 months (19 months)	1 (1.8 %)	0	0
Goat	45 (31/14)	3-40 months (12 months)	1 borderline (2.2 %)	0	0
Cattle	52 (43/9)	1-6 years (3.8 years)	0	0	0

Figure 1: Image of the Al Ain central market in Abu Dhabi. Camel pens are located on the left side, cattle stalls on the right side, and sheep and goats are kept in between. The distance between camel pens and the closest pens holding sheep and goats is approximately 20 m (separated by a road). The red asterisk indicates sheep pen S18, in which the MERS-CoV-positive sheep was sampled, the green asterisk indicates the mixed pen S17, in which the borderline positive goat was kept. Image retrieved from Google Maps.

Figure 2: Next generation sequencing analysis of the MERS-CoV N gene of sheep and camels. A) Sequencing coverage of the MERS-CoV N gene derived from one sheep (S90_N) and five camels (C53_N, C5_N, C6_N, C28_N, and C50_N). The average coverage of the N gene across all samples was >6X. X axis = N gene of MERS-CoV (1.2 kb) and Y axis = sequencing coverage; red line = sheep-derived MERS-CoV, blue lines = dromedary-derived MERS-CoV. B) Percentage of N gene sequence identity of the same samples along with three reference MERS-CoV sequences (GenBank acc. numbers KF192507, MG757604 and MK462253). The sample collected from the sheep showed high nucleotide identity (>99 %) with all camel-derived MERS-CoV sequences as well as the reference sequences. C) Multiple sequence alignment of the N gene of the same MERS-CoV samples (1.2 kb). Two short representative regions with nucleotide sequence variations are shown: positions 533 and 637 of the N gene (highlighted by a red vertical box); conserved positions are indicated by asterisks (*). Multiple sequence alignment was performed using MAFFT v7.455.

a)

Sequencing coverage

N gene of MERS-CoV (1.2 kb)

Legend: S90_N, C53_N, C5_N, C6_N, C28_N, C50_N
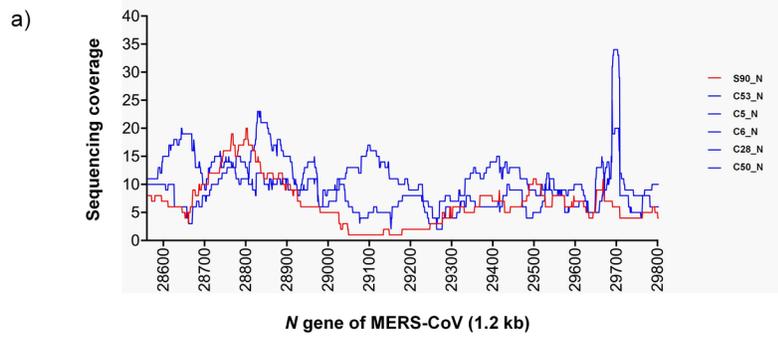
b)

	KF192507_N (UAE 2013)	MG757604_N (KSA 2016)	MK462253_N (KSA 2018)	S90_N	C53_N	C5_N	C6_N	C28_N	C50_N
KF192507_N (UAE 2013)	100	99.84	99.60	99.84	99.60	99.60	99.60	99.76	99.76
MG757604_N (KSA 2016)	99.84	100	99.43	99.67	99.43	99.43	99.43	99.59	99.59
MK462253_N (KSA 2018)	99.60	99.43	100	99.60	99.84	99.84	99.84	99.84	99.68
Sheep S90_N	99.84	99.67	99.60	100	99.60	99.76	99.76	99.76	99.92
Camel C53_N	99.60	99.43	99.84	99.60	100	99.84	99.84	99.84	99.68
Camel C5_N	99.60	99.43	99.84	99.76	99.84	100	100	99.84	99.84
Camel C6_N	99.60	99.43	99.84	99.76	99.84	100	100	99.84	99.84
Camel C28_N	99.76	99.59	99.84	99.76	99.84	99.84	99.84	100	99.84
Camel C50_N	99.76	99.59	99.68	99.92	99.68	99.84	99.84	99.84	100

c)

Virus designation	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
KF192507_N (UAE 2013)	a	g	c	c	t	c	t	a	g	c	g	t	a	a	g	c
MG757604_N (KSA 2016)	a	g	c	c	t	c	t	a	g	c	g	t	a	a	g	c
MK462253_N (KSA 2018)	a	g	c	c	t	c	t	a	g	c	g	c	a	a	g	c
Sheep S90_N	a	g	c	c	t	c	t	a	g	c	g	t	a	a	g	c
Camel C53_N	a	g	c	c	t	c	t	a	g	c	g	c	a	a	g	c
Camel C5_N	a	g	c	c	t	c	t	a	g	c	g	c	a	a	g	c
Camel C6_N	a	g	c	c	t	c	t	a	g	c	g	c	a	a	g	c
Camel C28_N	a	g	c	c	t	c	t	a	g	c	g	c	a	a	g	c
Camel C50_N	a	g	c	c	t	c	t	a	g	c	g	c	a	a	g	c

Virus designation	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
KF192507_N (UAE 2013)	t	a	g	g	a	g	g	t	g	a	t	c	t	a	c	t
MG757604_N (KSA 2016)	t	a	g	g	a	g	g	t	g	a	t	c	t	a	c	t
MK462253_N (KSA 2018)	t	a	g	g	a	g	g	t	g	a	t	t	t	a	c	t
Sheep S90_N	t	a	g	g	a	g	g	t	g	a	t	c	t	a	c	t
Camel C53_N	t	a	g	g	a	g	g	t	g	a	t	t	t	a	c	t
Camel C5_N	t	a	g	g	a	g	g	t	g	a	t	t	t	a	c	t
Camel C6_N	t	a	g	g	a	g	g	t	g	a	t	t	t	a	c	t
Camel C28_N	t	a	g	g	a	g	g	t	g	a	t	t	t	a	c	t
Camel C50_N	t	a	g	g	a	g	g	t	g	a	t	c	t	a	c	t

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