

Middle East respiratory syndrome coronavirus (MERS-CoV) RNA and neutralising antibodies in milk collected according to local customs from dromedary camels, Qatar, April 2014

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Antibodies to Middle East respiratory syndrome coronavirus (MERS-CoV) were detected in serum and milk collected according to local customs from 33 camels in Qatar, April 2014. At one location, evidence for active virus shedding in nasal secretions and/or faeces was observed for 7/12 camels; viral RNA was detected in milk of five of these seven camels. The presence of MERS-CoV RNA in milk of camels actively shedding the virus warrants measures to prevent putative food-borne transmission of MERS-CoV.

In April 2014, serum, nasal swabs and rectal swabs were taken from 33 milking dromedary camels at two locations in Qatar (Al Shahaniya and Dukhan), areas with known Middle East respiratory syndrome coronavirus (MERS-CoV) circulation in camels [1] and data not shown. In addition, milk was collected from these animals according to local customs. Serum samples and milk were tested for the presence of MERS-CoV-specific antibodies by protein microarray, with confirmation by virus neutralisation. Swabs and milk were tested for the presence of MERS-CoV RNA by real-time reverse transcription (RT)-PCR testing for multiple genomic targets. Antibodies to MERS-CoV were detected in serum and milk from all camels at both locations. At the Dukhan location, none of the 21 animals tested was actively shedding viral RNA from the nose and/or in faeces and no evidence for the presence of MERS-CoV RNA in milk was observed. At the Al Shahaniya location, evidence for active virus shedding was observed for seven of the

12 camels tested. Viral RNA was detected in milk of five of the seven camels with active virus shedding.

Background

In 2012, MERS-CoV was identified in patients with severe respiratory illness in the Middle East [2]. As of 11 June 2014, a total of 683 cases including 204 deaths have been reported to the World Health Organization (WHO) [3]. All cases have had an epidemiological link to the Middle East, with confirmed cases in Iran, Jordan, Kuwait, Lebanon, Oman, Saudi Arabia, Qatar, United Arab Emirates and Yemen. Human-to-human transmission seems limited to family and healthcare settings and is assumed to have contributed to the recent upsurge of cases [4]. Overall, however, a large proportion of cases of MERS-CoV infection is community acquired, with suspected zoonotic transmission, although the extent thereof remains to be determined [5]. Dromedary camels (*Camelus dromedarius*) are the prime suspects to serve as an animal reservoir for MERS-CoV, although alternative sources remain possible [6-11].

In August 2013, dromedary camels were implicated for the first time as a possible source of the virus leading to human infection on the basis of the presence of MERS-CoV neutralising antibodies in dromedaries from Oman and the Canary Islands of Spain [6]. Since then, MERS-CoV-specific antibodies have been detected in camels across the Middle East and in several African countries [7-9]. Analysis of an outbreak of MERS-CoV infection

TABLE

Middle East respiratory syndrome coronavirus (MERS-CoV) analysis of serum, nasal and rectal swabs and milk of dairy dromedary camels, Al Shahaniya, Qatar, April 2014 (n=12)

Barn number	Camel dam ^a number	Age camel dam (years)	Age calf (months)	Real-time reverse transcription-PCR ^b							Serology			
				Nasal swab	Rectal swab	Whole milk	Milk fat	Skimmed milk	Cell pellet	Milk total ^c	Serum ^d	Milk ^d	Serum ^e	Milk ^e
1	1	8	3	-	-	-	-	-	-	-	+	+	≥1,280	80
	2	7	4	+	-	+	-	-	-	+	+	+	640	Eq.
	3	10	4	-	+	-	-	-	-	-	+	+	≥1,280	80
	4	8	5	+	-	-	-	-	-	-	+	+	≥1,280	Eq.
	5	7	6	+	+	+	-	+	+	+	+	+	≥1,280	40
	6	9	7	+	-	+	-	+	+	+	+	+	≥1,280	40
	7	9	7	+	-	+	-	+	+	+	+	+	≥1,280	40
2	8	10	5	-	+	+	-	+	+	+	+	+	≥1,280	40
	9	8	3	-	-	-	-	-	-	-	+	+	≥1,280	40
3	10	15	8	-	-	-	-	-	-	-	+	+	≥1,280	NT
	11	12	5	-	-	-	-	-	-	-	+	+	≥1,280	20
	12	10	7	-	-	-	-	-	-	-	+	+	≥1,280	80
Total number positive	NA	NA	NA	5	3	5	0	4	4	5	12	12	12	9

Eq.: equivocal (titre between ≥5 and <20); NA: not applicable; NT: not tested due to lack of sample.

A dash represents that the test was negative.

^a A dam is the femal parent of a livestock animal.

^b A sample is considered PCR positive for MERS-CoV when ≥2 targets (*UpE*, *Orf1a* and/or *N*) are reactive.

^c Summary results of whole milk, milk fat, skimmed milk and cell pellet.

^d Serology based on MERS-CoV S1 protein-microarray. Cut-off value 4,000 relative mean fluorescent intensity.

^e Serology based on MERS-CoV neutralisation assay. Starting dilution 1:5. Neutralising antibody titres are shown.

in humans associated with a barn in Qatar in October 2013 found dromedaries and humans to be infected with nearly identical strains of MERS-CoV [1] and the virus was isolated from dromedaries shortly after [10]. Further support for camels as a reservoir came from a study in Saudi Arabia that found widespread circulation of different genetic variants of MERS-CoV in camels, and antibodies in samples taken since the early 90s [11].

Although camels are suspected to be the primary source of MERS-CoV leading to human infection, the routes of direct or indirect zoonotic transmission remain unknown. A possible route might be food-borne transmission through consumption of raw camel milk or undercooked meat. Here we report on our investigations into virus shedding of milking camels, in relation to the presence of MERS-CoV RNA in milk, as a first assessment of a potential role of consumption of raw camel milk in MERS-CoV transmission.

Analysis of dromedary serum, milk, nasal and rectal swabs

Sample collection

In April 2014, serum, nasal swabs, rectal swabs and milk were collected from 12 dromedary camels in three barns at the Al Shahaniya barn complex and 21 dromedary camels from a milking herd in the Dukhan area, Qatar. The milking camels at the barns at Al Shahaniya were kept together with racing camels that have regular contact with camels outside the barn at practice and racing events. Barn 1 held 22 racing and nine milking camels. Barn 2 held 18 racing and four milking camels, while Barn 3 held 15 racing and three milking camels. Each milking camel (dam) had their calf present. The age range of the calves was three to eight months (Table).

The herd in the Dukhan area was in a secluded area far from other animals. The age range of the calves was three to seven months. Both locations had known circulation of MERS-CoV in dromedaries at the end of 2013/beginning of 2014 [1] and data not shown.

FIGURE

Milking camels according to local customs, Al Shahaniya barn complex, Qatar, April 2014



Milk production is triggered by the calf: the calf is then set aside and the milk is collected.
Photographs by E. Farag.

No samples were collected from the calves. Serum and swabs from the dams were collected wearing a disposable gown, gloves, goggles and FFP2 mask, as described [1]. Milk was collected according to local customs as follows: dromedary calves were not weaned after delivery but kept at the farm in paddocks adjacent to their dams throughout lactation. Dams were reunited with their calf to trigger milk production. Once milk production was initiated, the milk samples were collected by the camel owner or handler according to regional customs. No specific hygienic precautions were taken (Figure). All samples were stored at -80°C until shipment to the Netherlands on dry ice. All sera and swabs were shipped in agreement with Dutch import regulations for animal samples from foot-and-mouth disease-endemic regions and stored and handled in a biosafety level 3 laboratory until inactivation by incubation for 4 hours at 56°C or addition of lysis buffer, respectively.

Sample testing

Total nucleic acids from swabs were isolated using an automated MagNAPure 96 extraction with the total nucleic acid isolation kit (Roche, Mannheim, Germany). Swabs were tested for MERS-CoV RNA by internally controlled real-time RT-PCR targeting *UpE* and *N* genes, as described [1,12]. Initial observations of reduced nucleic acid recovery when whole milk was extracted using routine protocols for clinical samples triggered us to test milk fractions, besides whole milk, for putative increase of sensitivity [13,14]. Total RNA was manually extracted from whole milk, skimmed milk, cellular pellet and cream components of milk samples using the High Pure RNA isolation kit (Roche, Mannheim, Germany). Extracts of whole milk and milk fractions were tested for MERS-CoV RNA by internally controlled

real-time RT-PCR targeting *Orf1A* and *UpE* genes, as described [1, 12]. According to international consensus, samples were considered positive for MERS-CoV RNA when at least two different targets were reactive [15].

At Al Shahaniya, seven of the 12 camels tested were actively shedding viral RNA from the nose ($n=5$) and/or faeces ($n=3$) with threshold cycle (Ct) values ranging between 23.0 and 29.7. Overall, milk obtained from five of the seven virus-shedding animals demonstrated presence of MERS-CoV RNA (Table) with Ct values ranging from 29.2 to 37.9. Sequence analysis of the PCR products from the milk fraction with the highest viral load confirmed the presence of MERS-CoV (data not shown).

At the Dukhan location, none of the 21 animals tested was actively shedding viral RNA and no evidence for the presence of MERS-CoV RNA in milk was obtained (data not shown). Milk fractions of bulk milk collected from dairy dromedaries in the Netherlands tested negative for MERS-CoV RNA (data not shown).

Serum and milk samples were tested for the presence of IgG antibodies reacting with MERS-CoV (residues 1–747), severe acute respiratory syndrome (SARS)-CoV (residues 1–676) and human coronavirus (HCoV)-OC43 (residues 1–760) spike domain S1 antigens using extensively validated protein-microarray technology, as described [6,16–18]. HCoV-OC43 S1 was used as proxy for bovine CoV (BCoV), which is known to circulate commonly in dromedaries [19,20]. All serum and milk samples from Al Shahaniya and the Dukhan location had MERS-CoV S1 binding antibodies (Table and data not shown).

Confirmation of array results from Al Shahaniya was done by MERS-CoV neutralisation assays, as described [6]. Neutralising antibody titres varied between 640 and $\geq 1,280$ for serum and between 10 and 80 for milk with 9 out of 11 having titres fourfold above the starting dilution of 1:5 (Table). Control serum ($n=3$) and bulk milk collected from dairy dromedaries in the Netherlands were negative (data not shown). All serum and milk samples from both locations in Qatar and the Netherlands reacted with HCoV-OC43 S1 confirming common circulation of BCoV in camelids. All samples tested negative for SARS-CoV (data not shown).

To gain insight into possible faecal contamination of the milk samples, the samples were analysed for the presence of *Escherichia coli* by a quantitative PCR based on the *E. coli uidA* gene, with a limit of quantification of $<10^3$ genome copies per ml [21]. The presence of *E. coli* was not consistently detected in repeated testing (data not shown).

Discussion

Raw milk from dromedaries has been consumed by humans for thousands of years and is thought to have healing properties when consumed 'hot', directly out of the udder [22]. Nowadays, dromedaries are still an important source of milk in rural areas of arid countries such as Qatar and other countries in the Middle East and parts of Africa [23]. Food-borne transmission is a putative route of zoonotic transmission of MERS-CoV that needs further investigation. Recent data demonstrated that MERS-CoV experimentally introduced into camel milk can survive for up to 72 hours at 4 °C and 22 °C and it has been suggested that consumption of MERS-CoV-containing milk might result in introduction of the virus into the oral cavity and subsequent infection of the lower respiratory tract [24].

Here, we detected the presence of MERS-CoV RNA in five milk samples collected from seven animals shedding MERS-CoV from the nose and/or feces at Al Shahaniya. Although shedding of infectious virus in ruminant milk and infection of humans due to the consumption of raw milk have been described for several viruses [25,26], it cannot be concluded from our data that this holds true for MERS-CoV as well. The milk samples were collected according to local customs in which camel udders are not normally cleaned before milking and hygienic conditions are such that udders and milk can be contaminated with nasal secretions or faeces from the camel, saliva of the calves, which are allowed to suckle prior to milking to initiate the milk flow, or dirt from the bowl or the hands of the milker. Additional studies under controlled hygienic conditions are ongoing to determine whether MERS-CoV replicates in the udder or could be introduced as contaminant during the milking process.

It remains to be seen if the results reflect the presence of infectious virus in the milk samples. The RNA loads in the milk samples were too low to attempt virus

isolation; we have observed that samples containing MERS-CoV RNA with Ct values >30 in general do not contain infectious virus particles. Experiments aiming at determining the amount of infectious virus present in milk samples such as those collected in our study should be conducted locally, avoiding detrimental effects of shipment and freeze-thaw cycles on virus viability. In addition, the presence of substantial levels of MERS-CoV neutralising antibodies in the milk samples might neutralise any infectious virus present during in vitro testing, which may differ from the in vivo situation, particularly if the virus is resistant to gastric juice and passage of infectious virus through the stomach occurs [27]. Nevertheless, it can be concluded that the presence of MERS-CoV RNA in raw milk as consumed locally might represent a source for zoonotic transmission of MERS-CoV and prudence is called for. Munster et al. showed that heat treatment (30 minutes at 63 °C) of MERS-CoV-containing camel milk reduced levels of infectious virus below detection level [24]. Boiling milk before consumption could be an easy, achievable local measure to prevent transmission and to preserve consumption of camel milk.

An interesting observation is the difference in virus shedding between the herds at Al Shahaniya and Dukhan (7/12 and 0/21, respectively) although virus circulation had been detected in the Dukhan location earlier (data not shown). While the current study provides only a snapshot, it suggests that herd management practices may influence virus circulation. In addition, the nasal and/or faecal shedding of MERS-CoV by animals with high levels of neutralising antibodies suggests that the presence of antibodies does not confer sterilising immunity.

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Conflict of interest

None declared.

Authors' contributions

CR: coordination of the study in the Netherlands, assisted in designing the study, analysed data, wrote manuscript.

EF: coordination of the study in Qatar, assisted in designing the study, read and revised manuscript. MJ: protocol development, performed laboratory testing, analysed data, read and revised manuscript. GJG: performed laboratory testing, analysed data, read and revised manuscript. AES: field work Qatar, read and revised manuscript. SP: performed laboratory testing, analysed data, read and revised manuscript. VSR: performed laboratory testing, analysed data, read and revised manuscript. KM: field work Qatar, read and revised manuscript. HAM: performed laboratory testing, analysed data, read and revised manuscript. HG: read and revised manuscript. FAH: read and revised manuscript. AI: field work Qatar, read and revised manuscript. BJB: design antigen production, provided antigens, read and revised the manuscript. HAR: read and revised manuscript. SKP: read and revised manuscript. MAT: read and revised manuscript. SAM: read and revised manuscript. MAH: overall coordination collaboration Qatar-the Netherlands, assisted in designing the study, read and revised manuscript. BH: data analysis, assisted in designing the study, read and revised manuscript. MK: overall coordination collaboration Qatar-the Netherlands; assisted in designing the study, data analysis, read and revised manuscript.

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