Appendix 24

Dromedaries (Camelus dromedarius) are of very low susceptibility to experimental, high-dose inoculation with FMDV Serotype O and do not transmit the infection to direct contact camels or sheep

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Abstract:

Introduction, Materials and Methods: To further evaluate the susceptibility of camels to infection with FMDV, two fully grown female sheep were inoculated intradermally in the coronary band and five dromedaries (Camelus dromedarius) around 400-450 kg (three females and two castrated males of 7-10 years of age) were inoculated subepidermo-lingually each with $10^{7.8}$ TCID$_{50}$ of a first cattle passage FMDV type O UAE 7/99 inoculum in a volume of 0.25 ml. The two female sheep and three female dromedaries were naïve in respect to FMDV before inoculation while the two castrated male dromedaries had been inoculated 9 months earlier with a cell-culture passaged preparation of the same FMDV isolate (before cattle passage), but had shown no signs of infection and stayed antibody negative. An additional five female dromedaries of approximately the same weight and age were kept in the same pen as the inoculated camels as continuous direct contacts and another 4 sheep were kept in a corner of the camel pen as contacts from the day after inoculation.

Results: The two inoculated sheep developed elevated body temperatures, had, albeit minor, local swelling and inflammation at the site of inoculation and were lame on one to four feet for several days typical for FMD in sheep. Infectious FMDV and FMDV RNA, respectively, were detected at relatively high levels in sera on days 1-4 after inoculation of the sheep. Infectious virus was isolated from a probang sample from one of the inoculated sheep on day 3 after inoculation and RNA was detected in probang samples from the two inoculated sheep on days 3, 6, 10 and 14 after inoculation and in mouth swabs from day 1 to 4. Moreover, the inoculated sheep developed an antibody titre against FMDV at 4 days after inoculation and increasing high titres from day 10. None of the camels or the contact sheep developed any clinical signs of disease nor had the inoculated camels any signs of vesicular lesions, swelling or inflammation. However, one of the inoculated female dromedaries had a significantly raised body temperature of 37.9°C at 3 days pi and this camel also had a viraemia on days 2-4 after inoculation as determined by virus isolation and by detectable FMDV RNA in the serum samples taken at 2-6 and 10 days after inoculation. No infectious virus was isolated from probang samples from this animal, but very low levels of FMDV RNA was detected by real time RT-PCR in a probang sample taken at 6 days after inoculation while probang samples taken at 3, 10, 14, 21 and 28 days were clearly negative. Real time RT-PCR analysis of mouth swabs indicated a low amount of FMDV RNA to be present from day 1 to 3 (and up to day 5 in the camel with viraemia) after inoculation in 4 of 5 of the inoculated camels while no infectious FMDV or FMDV RNA was detected in sera, probang or mouth swab samples from the 5 contact camels or the 4 contact sheep. The 5 contact camels, the 4 contact sheep and the 2 inoculated female camels, that did not develop a viraemia, did not develop any antibodies to FMDV when tested up to 28 days after inoculation. In contrast, the female camel with viraemia started to produce detectable antibodies to FMDV at 14 days after inoculation while the two previously exposed male camels, despite having no detectable viraemia, had detectable antibodies to FMDV from 10 days after inoculation. However, neither of these dromedaries developed any antibodies against FMDV non-structural proteins (NSPs).

Discussion: Based on these results in addition to our previous preliminary findings we conclude that dromedaries are of very low susceptibility to infection with FMDV serotype O, and that they are unlikely to play any significant role in the natural epidemiology of FMD.

Introduction:

Opinions vary widely whether animals of the camelidae family are susceptible to FMD or not, or if they may serve as viral reservoirs. Several investigations appear to indicate that dromedaries can contract the disease after experimental infection and through close contact with FMD diseased livestock, but that they do not present a risk in transmitting FMD to susceptible animals (reviewed in 17). With the experiment described here, we wanted to follow up on a recent pilot study 18.
indicating that dromedaries may be of low susceptibility to FMD virus type O. Serotype O FMDV is the most widespread serotype worldwide and in particular the PanAsia lineage used here appears to be very capable of replicating and causing disease in several species. Moreover, serotype O are present in areas of the world where camels play an important role and consequently, may come in contact with infected fully susceptible animals such as cattle, buffalo, small ruminants and perhaps pigs.

Materials and methods:

Virus and inoculation: The virus used was FMDV O UAE 542-99 (World Reference Laboratory designation: FMDV O UAE 7/99) isolated in early 1999 at CVRL in Dubai from epithelial lesions from Arabian gazelles in which it produced only minor lesions. The virus was isolated in Baby Hamster Kidney (BHK 21, clone 13) cells and the virus amplified by a further 5 passages in these cells. The initial inoculum was the supernatant of this 5th passage and contained $10^{7.9}$ TCID$_{50}$/ml when titrated in Bovine Thyroid (BTY) cells. Sequencing of the VP1 region of this virus showed that the virus belonged to the FMDV type O PanAsia lineage of viruses and was quite closely related to e.g. the FMDV O UKG 2001 virus (Nick Knowles, personal communication). In a previous study $^{18}$, two heifers (heifer 144 and 146) were inoculated subepidermo-lingually with 0.5 ml of this FMD O inoculum containing $10^{7.6}$ TCID$_{50}$/ml as shown by titration on BTY cells. Consensus sequencing of the entire L fragment (7802 nucleotides) of the FMDV in this inoculum showed 98.7% identity to the FMDV O UKG 35/2001 isolate $^{18}$ at the nucleotide level, and at the amino acid level only 11 coding differences (3 in L, 1 in VP2, 1 in VP3, 3 in VP1, 1 in 2B, 1 in 2C and 1 in 3A) were found in the entire open reading frame of 2332 residues, i.e. more than 99.5% identity. Furthermore, the capsid coding region did not have positively charged residues at key positions (data not shown) characteristic of cell culture adapted, in vivo attenuated FMDV $^{6,14}$ and therefore consistent with a virus derived directly from cattle vesicular epithelia and being fully virulent.

Interestingly, when also consensus sequencing the entire L fragment of the parental 5th BHK passage inoculum used in the initial study $^{18}$ we found 8 nucleotide differences between this virus and the virus in the inoculum after a single passage in heifer 144. Of the 8 differences the 3 were non-coding and located with 2 differences in 2C and 1 difference in 3D. Of the 5 coding differences, 1 is in VP-3 (changing the BHK inoculum virus at aa # 158 from proline to serine); 2 differences are in VP-1 (changing aa #13 alanine to threonine and aa # 144 alanine to valine); and 2 differences in 3A at amino acid # 104 (changing a to an asparagine) and #129 (alanine in the BHK inoculum to a threonine in the heifer 144 inoculum virus). Thus, it appears that the 5 passages in BHK cells potentially had selected for a cell culture adapted virus population which quickly changed character after a single cattle passage. Consequently, based on these results the heifer 144 inoculum could potentially provide a more severe challenge than the BHK passaged inoculum used in the initial trial $^{18}$.

Animals: Two local female sheep of around 30 kg (sheep No. 532 and 537 both fully grown) and serologically negative for antibodies to FMDV were housed in a closed building at the CVRL in Dubai and inoculated intradermally in the coronary band of a left forefoot. Also at the CVRL, but in an outdoors pen, five dromedaries (Camelus dromedarius) around 400-450 kg and of 7-10 years of age (three females (camel No. 32, 34 and 35) and two castrated males (camel No. 30 and 33)) were inoculated subepidermo-lingually each with $10^{7.6}$ TCID$_{50}$ of the cattle passaged foot-and-mouth disease virus (FMDV) type O UAE 7/99 inoculum in a volume of 0.25 ml. The three female dromedaries (camel No. 32, 34 and 35) were naïve in respect to FMDV before inoculation while the two castrated male dromedaries (camel No. 30 and 33) 9 months earlier had been inoculated with a cell-culture passaged preparation of the same FMDV isolate (before cattle passage), and at that occasion no signs of infection or development of antibodies were detectable $^{18}$. Another five naïve female dromedaries (camel No. 26-29 and 31) of approximately the same weight and age were kept in the same pen (approximately 10 by 10 metres) as continuous direct contacts. An additional 4 local sheep (2 females of around 30 kg (sheep No. 412 and 732) and 2 rams of around 35-50 kg (sheep No. "Big Black" and "Black with horn"), all fully grown, were kept in a pen (approximately 3.5 by 3.5 metres) made in a corner within the camel pen as contacts from the day after inoculation. Although these sheep were separated from the camels by an approximately 1.20 metres high fencing there was good possibility of contact as the camels easily could reach over the fencing. The animals were examined clinically each day for signs of FMD. Rectal temperatures were recorded daily from before inoculation until 14 days pi for the inoculated sheep (killed on day 14 pi) and from before inoculation until 6 days pi as well as on days 10, 14, 21 and 28 pi for the other.
animals except for one of the contact sheep which died between day 21 and 28 due to unrelated causes (chronic enteritis). Rectal temperatures were also taken daily from the camels for a week before inoculation in order to establish their normal temperature levels when handled.

Sample Collection: Blood samples and mouth swabs were taken before inoculation (control samples), then daily for the first 6 days and then on days 10 and 14 and for most animals also days 21 and 28 pi. The blood samples were transported to the laboratory and serum was separated after storage at 4°C for up to 24 h and then stored at ~80°C. Swabs were placed in 0.5 ml of RLT lysis buffer (Qiagen) and stored at ~80°C. Probang samples were taken at 3, 6, 10 and 14 days pi and for most animals also on days 21 and 28. For RNA extraction, an aliquot of serum, swab or probang samples were thawed and a volume of 0.14 ml of sample added to 0.56 ml of Qiagen AVL buffer using the QIAamp Viral RNA mini spin column (Qiagen GmbH, Hilden, Germany) for nucleic acid extraction.

Assay for Infectious Virus: The infectivity in the inoculum and in serum and probang samples were assayed by inoculation of monolayer cultures of BTY cells using either roller tubes (0.2 ml sample per tube, 3-5 tubes per sample) or using 96-well plates (0.05 ml per well, 11 wells per sample). The specificity of the cytopathogenic effect observed in cell cultures was confirmed by antigen ELISA.

Quantitative RT-PCR: Quantitative "real-time" RT-PCR was used to determine the amount of FMDV RNA in extracts of total nucleic acid from blood, probang and swab samples. The assay method was described in detail elsewhere. Each extraction required 0.14 ml of sample and the nucleic acids were finally eluted in a volume of 0.06 ml. All estimations included a dilution series of standard reactions based on in-vitro transcribed, molecularly cloned FMDV RNA with a known content of FMDV genome-equivalents and the method is influenced minimally by sample type.

Assay for Antibodies: Serum samples were tested for antibodies by an enzyme-linked immunosorbent assay (ELISA), and samples in the inconclusive range were verified by virus neutralization assay, as described in the Manual of Standards for Diagnostic Techniques and Vaccines, l’Office International des Epizooties, OIE, 2000. Testing for antibodies against FMDV non-structural proteins (NSPs) were also by ELISA. The tests mentioned are all blocking type tests and are therefore supposed to work well for most/all species including camels.

Results:

Clinical signs, FMDV Load in Serum and Swabs and Seroconversion: The two inoculated sheep developed elevated body temperatures (both up to 41.9-42.0°C), stopped eating and were depressed, had, albeit minor, local swelling and inflammation at the site of inoculation, had markedly warm feet and were lame on one to four feet on days 1-6 pi typical for FMD in sheep. Infectious FMDV and FMDV RNA were detected at relatively high levels in the serum samples from the inoculated sheep on days 1 and 2 after inoculation in regard to virus isolation and days 1-4 in regard to detection of FMDV RNA (up to $10^{4.2}$ to $10^{5.6}$ TCID$_{50}$/ml corresponding to $10^{7.4}$ and $10^{8.1}$ copies of FMDV RNA/ml on day 2 after inoculation). Infectious virus was isolated from a probang sample from one of the inoculated sheep on day 3 after inoculation and RNA was detected in probang samples from the two inoculated sheep on days 3, 6, 10 (only one of the sheep positive on this day) and 14 after inoculation at levels from $10^{3.7}$ and $10^{5.8}$ copies of FMDV RNA/ml of diluted probang sample. RNA at levels up to $10^{15}$ copies of FMDV RNA/ml was found in swab samples from these sheep from pi day 1-4.

These sheep also developed an antibody titre against FMDV at 4 days after inoculation increasing to high titres (higher than 200 in both ELISA and VNT) from day 6-10 after inoculation and, additionally, had significant levels of antibodies against FMDV NSPs from 10 days after inoculation indicating active viral replication. None of the camels or the contact sheep developed any obvious clinical signs of disease nor had the inoculated camels any signs of vesicular lesions, swelling or inflammation even at the inoculation sites. Camel 33 had visible injection sites at 3 and 4 days pi but these healed immediately and there were no signs of vesicular lesions or inflammation apart from the physical trauma caused by the needle. Camel 30 had a 3 x 0.5 cm mucosal lesion above the canine tooth on the left dorsal labia on 3 and 4 days pi, however, this was almost certainly inflicted by physical trauma during the physical handling of the camels (this is supported by us finding no FMDV in swab samples from the lesion or in blood and probang samples from this camel).

The body temperatures of the camels were taken for one week before inoculation and the temperature fluctuated between 35.5 and 37.5°C (average 36.3; st.dev. 0.53; N = 80). The temperature after inoculation and contact were in the same range (average 36.5; st.dev. 0.43; N =
Sequencing of virus from infected camel 34. Serum taken at pid 3 from camel 34 was cultured for a single passage in BHY cells for 48 hours and the supernatant prepared for direct consensus sequencing. The consensus sequence of the entire L fragment of this virus was identical to the given inoculum from heifer 144, suggesting that although BHK passage had introduced/selected variants that were significantly different from those selected after a single cattle passage, this cattle-passaged virus did not change at all even when passaged in a camel. We can not formally exclude that the single BHY passage changed a potential camel-selected virus population back to the original cattle virus, but we have attempted sequencing of virus RNA directly from the serum samples from the infected camel with viraemia to define this better. However, we could only sequence around 7000 nucleotides from this material due to the relatively low content of viral RNA in the in vivo sample and we have repeatable failed to sequence a segment of around 800 nucleotides including VP1. However, the sequence of the other 7000 nucleotides is identical to the sequence obtained from the first BHY cell culture passage and thus provide a strong indication that this sequence is valid for the virus in the infected camel.

Discussion:

Two sheep and five dromedaries were each inoculated intradermally in the coronary band or subepidermo-lingually with a foot-and-mouth disease virus (FMDV) type O from samples from Arabian gazelles originally isolated in BHK cells but passaged once in cattle before use. While the two inoculated sheep got infected, excreted virus and developed clinical signs typical of FMD in sheep, none of the camels or the contact sheep developed any clinical signs of disease nor had the inoculated camels any signs of vesicular lesions, swelling or inflammation. However, one of the three inoculated naive camels (camel 34) had a raised body temperature of 37.9°C for one day at 3 days pi and this camel also had a viraemia on days 2, 3, 4, 5, 6 and 10 days after inoculation as determined by virus isolation and by detectable FMDV RNA in the serum samples taken at 2, 3, 4, 5, 6 and 10 days after inoculation (up to $10^{5.6}$ copies of FMDV RNA/ml on day 4). No infectious virus was isolated from probang samples from this animal, but very low levels of FMDV RNA ($10^{3.1}$ copies of FMDV RNA/ml of diluted probang sample) were detected by real time RT-PCR in a probang sample taken at 6 days after inoculation while probang samples taken at 3, 10, 14, 21 and 28 days were clearly negative. Examination of mouth swabs from the inoculated camels by real time RT-PCR showed that FMDV RNA was present from pi day 1-3 (and up to day 5 in camel 34 with the viraemia) at levels up to $10^{5.8}$ copies of FMDV RNA/ml in 4 out of 5 inoculated camels (only camel 35 being below detection limit). No infectious FMDV or FMDV RNA (detection limit $<10^{2.2}$ TCID50/ml of infectious FMDV and $<10^{3.2}$ copies of FMDV RNA/ml, respectively) was isolated in sera, probang, or mouth swab samples from the other 5 contact camels or the 4 contact sheep. The 5 contact camels, the 4 contact sheep and the 2 inoculated female camels that did not develop a viraemia (camel 32 and 35), did not develop any antibodies to FMDV when tested up to 28 days after inoculation, despite camel 35 having been positive for FMDV RNA in mouth swabs from 1-3 days pi. Inoculated camel 34 (the one with viraemia) started to produce detectable antibodies to FMDV at 10-14 days after inoculation, however, the titre was very modest with a titre of 15 or less in ELISA and 56 or less in VNT. The two previously exposed male camels (inoculated camels No. 30 and 33), despite having no detectable viraemia, had detectable antibodies to FMDV from 6-10 days after inoculation at modest titres of less than 13-19 in ELISA and less than 48-68 in VNT. Interestingly, none of the camels had any reaction for antibodies against FMDV NSPs indicating that there either is a very low level or no viral replication in these animals, or, alternatively, that the NSP test is of limited value for use in Camels.
day 5 in camel 34 with viraemia) in 4 out of 5 inoculated camels. In clear contrast, no infectious FMDV or FMDV RNA was isolated in sera, probang, or mouth swab samples from the 5 contact camels or the 4 contact sheep. The 5 contact camels, the 4 contact sheep and the 2 inoculated naïve camels that did not develop a viraemia did not develop any detectable antibodies to FMDV when tested up to 28 days after inoculation. This is despite the fact that camel 35 was positive for FMDV RNA in mouth swabs from 1-3 days pi. The naïve camel that developed a one day fever and a viraemia (camel 34) on days 2-4 pi (RNA present from day 2 to 10) started to produce detectable, albeit very modest, antibody levels to FMDV at 10-14 days after inoculation while the two previously exposed male camels, despite having no detectable viraemia, developed a similarly modest, but detectable antibody response to FMDV from 6-10 days after inoculation. Interestingly, none of the camels had any reaction for antibodies against FMDV NSPs indicating that there either is a very low level or no viral replication in these animals, or, alternatively, that the NSP test is of limited value for use in Camels. Consequently, although 1 out of 3 inoculated naïve camels developed a relatively long lasting viraemia, this did not lead to much clinical affection, only fever for one day and no signs of vesicular disease. The infected camel excreted very little virus as indicated by negative findings in probang samples apart from a single probang sample at pid 6 that contained a minimal amount of viral RNA only detected by RT-PCR. Importantly, despite the relatively long viraemia in this infected camel, and despite the fact that this camel together with 3 of the other 4 inoculated camels had FMDV RNA present in mouth swab samples for 3-5 days shortly after tongue inoculation, infection was not transferred to direct contact camels or sheep.

Most data from the field indicate either a very low or no susceptibility to infection with FMDV. Kumar et al. (8) described isolation of FMDV serotype O from one of two randomly selected dromedaries in India and Moussa et al. (19) in Egypt have suggested that dromedaries are susceptible to natural FMD. The authors described ruptured vesicles and ulcers on the upper lips of four dromedaries. Additionally, ulcerations were seen between the teeth and on the teats. Two viral strains were isolated from these lesions and both were identified as FMDV, serotype O. However, to the knowledge of the authors these results have not been confirmed by others, and moreover, were performed under less than optimal conditions. Our present experiment supports our earlier findings and clearly shows that dromedaries are of very low susceptibility to infection of the FMDV isolate O from Arabian gazelles even derived directly from bovine vesicular epithelium. In our previous trial only 2 camels were inoculated, but in the current study we used a total of 10 camels of which 5 were inoculated and the other 5 kept in direct contact from the outset and consequently must have been exposed to live virus from the inoculum; all 10 without any signs of vesicular disease and with laboratory results indicating a limited infection (viraemia and viral RNA in mouth swabs up to day 5) in a single inoculated camel while another 4 inoculated camels rapidly cleared the virus inoculated directly under the tongue epithelia and thus virus (RNA) was only detectable in mouth swabs up to day 3 or less. Moreover, the 4 contact sheep being exposed from 1 day pi did not become infected either, further substantiating the lack of transmission from the single infected camel or from the additional 3 inoculated camels that did for a short period contain FMDV RNA in mouth swab samples. The virus used came directly from a secondary vesicle from an infected heifer, were given at a very high titre (10^7 TCID50; this titer is likely to correspond to enough virus to infect and cause disease in 1 thousand to 1 million fully susceptible animals such as e.g. cattle). The virus used by us came from clinically infected cattle and produced typical FMD in the inoculated sheep. Thus, the lack of acute disease and lack of contact transmission indicate a true low susceptibility of dromedaries and, importantly, a complete lack of transmission from dromedary camels even when infection is achieved by experimental, high-dose inoculation. Our results suggest that dromedaries, which are being listed in the OIE Code Chapter as being susceptible to FMD similar to cattle, sheep, goats and pigs, are much less susceptible or non-susceptible to FMD and as they do not transmit infection they play no significant role in the epidemiology of FMD. Therefore, the importance of FMD in dromedary camels should be re-addressed by the relevant authorities. To aid such an analysis, we are planning on continuing these experiments to provide better statistical background for our observations and to expand the findings to FMDV serotype A, another serotype of potential importance in relation to possible susceptibility of dromedary camels.

Conclusions:

- We conclude that dromedaries (Camelus dromedarius) are of very low susceptibility to infection with FMDV serotype O.
- Although they may become infected at low level after direct inoculation, they appear not to transmit infection even by close direct contact.
- Consequently, dromedary camels are unlikely to play any significant role in the natural epidemiology of FMD.
- Dromedary camels appear to only mount a limited initial antibody response to FMDV and no antibodies to NSP were detected!
Recommendations:

- Continued systematic and well-planned experiments with other serotypes and isolates should be encouraged and funded by relevant authorities and should in due time lead to a needed re-evaluation of the significance, if any, of FMD in dromedary camels and perhaps also in other members of the camelidae family.

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