Extended Viral Shedding of MERS-CoV Clade B Virus in Llamas Compared with African Clade C Strain

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Middle East respiratory syndrome coronavirus (MERS-CoV) clade B viruses are found in camelids and humans in the Middle East, but clade C viruses are not. We provide experimental evidence for extended shedding of MERS-CoV clade B viruses in llamas, which might explain why they outcompete clade C strains in the Arabian Peninsula.

Middle East respiratory syndrome coronavirus (MERS-CoV) infections cause severe pneumonia, acute respiratory distress syndrome, and even lethal disease in humans. High case-fatality rates are reported in the Middle East (*1*), where the virus is endemic and represents a major human health threat. Although major travel-associated outbreaks have occurred and nosocomial transmissions have been documented, MERS-CoV is primarily carried and transmitted to humans by dromedary camels, which are the natural reservoirs and main source of zoonotic events (*2*). All primary cases of MERS-CoV in humans reported during July–December 2021 occurred in persons who had been exposed to dromedary camels (*3*). Susceptible camelid species, such as dromedaries, llamas, and alpacas (*4*), as opposed to humans, do not experience severe disease upon MERS-CoV infection. Infection in camelids is characterized by upper respiratory tract replication, abundant infectious viral shedding, and high transmission potential (*2*). Furthermore, robust and transient innate immune responses in alpacas correlate with virus clearance in the respiratory epithelia (*5*,*6*).

High seroprevalences and active circulation of MERS-CoV have been determined in dromedary camels from the Arabian Peninsula and Africa (*7*). Although >80% of the global camel population is found in Africa (https://www.fao.org/faostat) and MERS-CoV infection is widespread in dromedaries in Africa, zoonotic disease has only been reported in the Arabian Peninsula. Serologic and molecular evidence of MERS-CoV infection in camel handlers exists (*8*–*11*), but no zoonotic transmission has been reported in Africa. Despite continuous trade of dromedaries into the Arabian Peninsula, African clade C MERS-CoV strains have not been detected in the region. One explanation for the dominance of clade B strains in the Middle East could be their increased fitness compared with African clade C viruses. A recent study demonstrated increased replication competence of MERS-CoV clade B Arabian viruses compared with different clade C African strains in human lung ex vivo cultures and in a transgenic mouse model expressing the human cell receptor for MERS-CoV (human dipeptidyl peptidase-4 [hDPP4]) (*12*). However, the replication and

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transmission competence of Arabian and African viruses in camelid reservoir species remains unknown.

The kinetics by which llamas shed infectious MERS-CoV are similar to those of dromedary camels, so they are considered a reliable surrogate model for transmission experiments (*2*,*4*). Therefore, we experimentally investigated transmission of MERS-CoV viruses in llamas.

The Study

We used a previously developed direct-contact model in which the transmission of a MERS-CoV clade B isolate (Qatar15/2015) was assessed in llamas (*13*,*14*). In brief, we kept a group of 5 llamas inside an experimental enclosure (Appendix Figure 1, panel A, https://wwwnc.cdc.gov/EID/article/29/3/22-0986-App1.pdf) to study the transmission capabilities of a MERS-CoV clade C isolate (MERS-CoV/Egypt2013) that was obtained from an infected dromedary (*15*). We inoculated 2 llamas with MERS-CoV and placed them in direct contact with 3 sentinels at 2 days postinoculation (dpi) (Appendix). We monitored clinical signs and body temperature and collected nasal swab specimens for virologic studies. In addition, we retrieved experimental data from MERS-CoV Qatar15/2015-inoculated and in-contact llamas (*13*,*14*) and performed comparative analyses.

We specifically selected the animals used in the transmission studies to be 6-to-10-month-old juveniles of similar geographic origin, sex, and health status background. All animal experimentation and MERS-CoV handling were conducted at the Biosafety Level 3 facilities of the Biocontainment Unit of IRTA CReSA (Barcelona, Spain). Animal handling and experimental procedures were approved by the Ethical and Animal Welfare Committee of IRTA and by the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia (approval nos. FUE-2017–00561265 and CEA-OH/10942/1).

Rectal temperatures of all animals remained at basal levels (37°C–40°C), and no animals displayed clinical signs during the study. We detected no gross or microscopic lesions in the upper or lower respiratory tracts of any studied llama, independent of their experimental group. Animals inoculated with a high dose of either MERS-CoV Egypt/2013 (clade C) or Qatar15/2015 (clade B) had similar levels of genomic and subgenomic viral RNA in nasal swab specimens for 2 weeks (Figure 1, panels A, B). They also shed

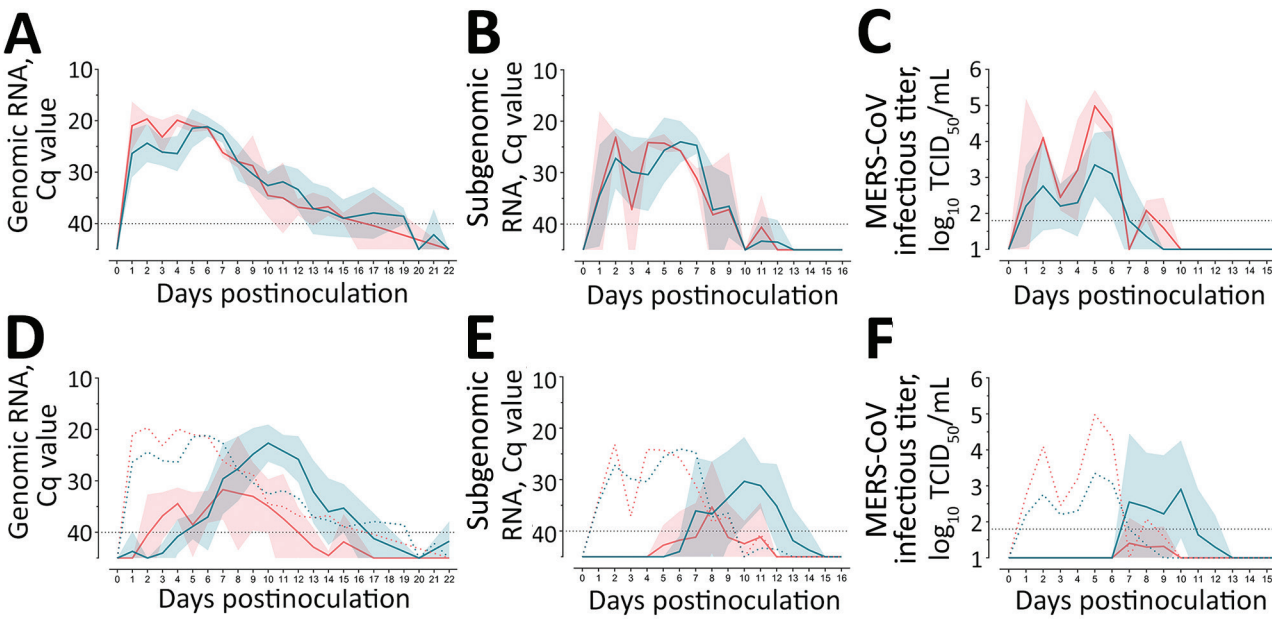

Figure 1. MERS-CoV RNA and infectious virus shedding in llamas experimentally infected with MERS-CoV Egypt/2013 (red) or Qatar15/2015 (blue) strains. A–C) Viral RNA and infectious MERS-CoV shedding of inoculated animals. Genomic (A) and subgenomic (B) viral RNA were quantified in nasal swab samples collected at different times after MERS-CoV inoculation. Infectious MERS-CoV titers (C) were demonstrated in nasal swab specimens collected on different days after MERS-CoV inoculation. Solid lines indicate mean values determined for different MERS-CoV–inoculated groups; shadings represents SD intervals. D–F) Infection profile of naive in-contact llamas). Genomic (D) and subgenomic (E) viral RNA quantified in nasal swab samples collected at different times after MERS-CoV inoculation. Infectious MERS-CoV titers (F) were demonstrated in nasal swab samples collected on different days after MERS-CoV inoculation. Solid lines indicate mean values of the groups of animals infected by contact; shaded areas represent SD intervals. Colored dashed lines indicate mean values calculated for MERS-CoV–inoculated animals. Horizontal dashed lines depict detection limits of assays. Cq, quantification cycle; MERS-CoV, Middle East respiratory syndrome coronavirus; $TCID_{50}$, 50% tissue culture infective dose.

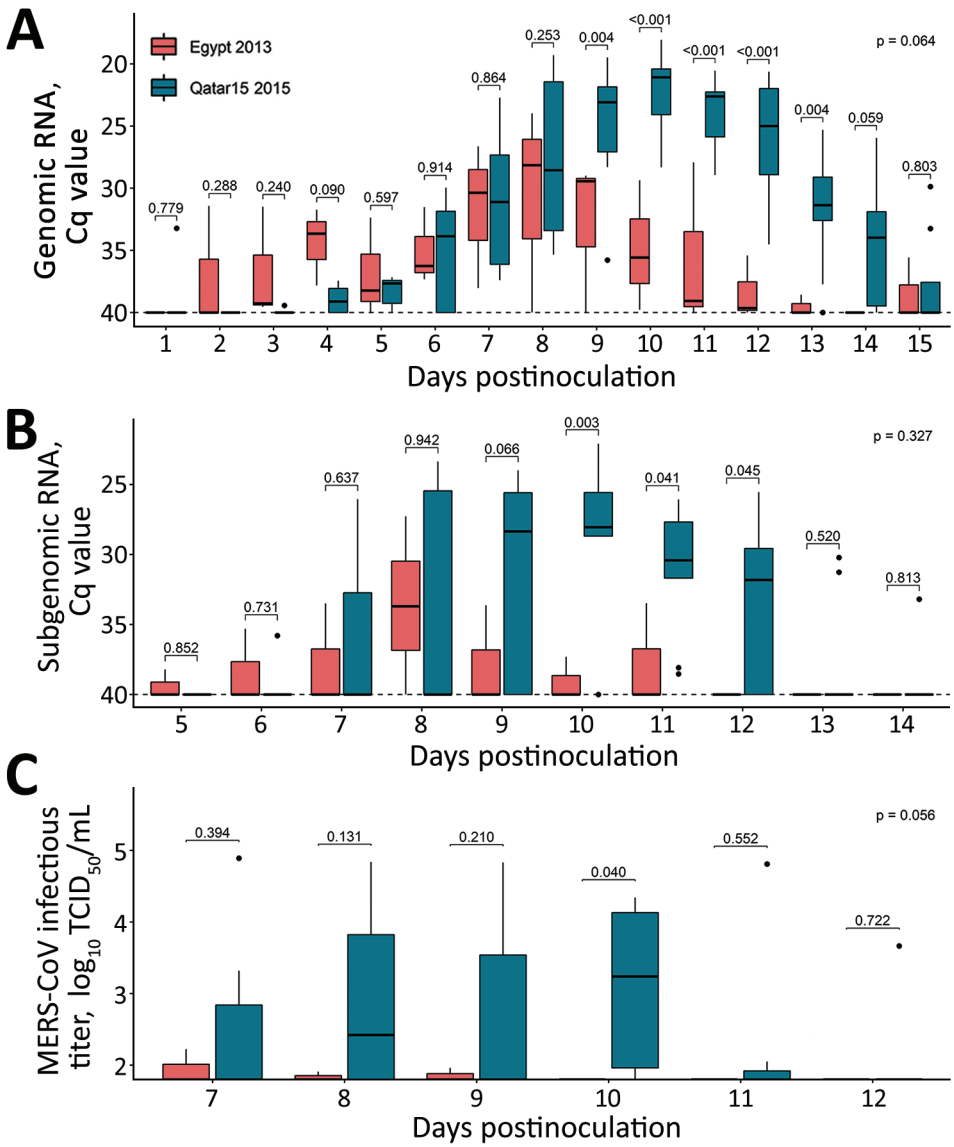

Figure 2. Mixed model analyzing the transmission competence of each MERS-CoV strain over time in investigation of llamas experimentally infected with MERS-CoV. Boxplots show daily virus shedding of sentinel llamas infected with MERS-CoV Egypt/2013 (red) or Qatar15/2015 strains (blue) after direct exposure to inoculated llamas. A, B) Genomic (A) and subgenomic (B) viral RNA quantification in nasal swabs collected throughout the study. C) Infectious MERS-CoV titers. Only the time points considered in the mixed models are represented. Horizontal lines within boxes indicate medians; box tops and bottoms indicate interquartile ranges; error bars indicate 95% CIs; black dots indicate outliers. p values are indicated above the boxes; p values indicate statistical differences between areas under the curve of the experimental groups, as calculated by Wilcoxon test. Cq, quantification cycle; MERS-CoV, Middle East respiratory syndrome coronavirus; $TCID_{50}$, 50% tissue culture infective dose.

high titers of infectious virus during the first week after inoculation in a similar biphasic pattern (Figure 1, panel C), indicating that the doses used to inoculate the animals caused productive infection with both strains. Although viral shedding was comparable between both experimental groups, higher infectious titers were detected in Egypt/2013-inoculated llamas. The infection was characterized by a first peak of shedding at 2 dpi and a subsequent reduction in MERS-CoV viral loads, followed by a secondary peak before viral clearance.

The African MERS-CoV isolate was transmitted to 2 of 3 in-contact animals in this study, as determined by quantitative reverse transcription PCR (Figure 1, panels D, E), but infectious virus shedding in contact animals largely remained below threshold levels (Figure 1, panel F). Infectious MERS-CoV Egypt/2013 could only be isolated sporadically and at titers close to the limit of detection. In contrast, the Arabian MERS-CoV Qatar15/2015 isolate was transmitted to all direct-contact llamas, leading to productive infection (Figure 1, panels D–F). Of note, genomic and subgenomic MERS-CoV Egypt/2013 RNA was detected at lower levels and cleared faster in direct-contact llamas than in sentinels infected with the MERS-CoV Qatar15/2015 strain (Figure 1, panels D, E). In the remaining sentinel, a productive infection did not develop, but the animal was naturally exposed to MERS-CoV Egypt/2013, as indicated by traces of genomic RNA in NS at 3–7, 10, and 12 dpi (cycle quantitation values >37) and development of serum neutralizing antibodies (nAbs) to MERS-CoV (Appen-

dix Figure 2). Subgenomic RNA analyses indicated no evidence for either viral replication or shedding in this llama throughout the study. Statistical analyses in sentinel animals showed a significant reduction in MERS-CoV Egypt/2013 replication and shedding period compared with those observed in llamas exposed to MERS-CoV Qatar15/2015 strain (Figure 2). Regardless of the MERS-CoV strain investigated, nAbs were detected starting at 2 weeks after infection in all inoculated animals and in-contact sentinels (Appendix Figure 2). We did not find statistical differences in serum nAb levels among experimental groups.

Altogether, our data demonstrated transmission of both MERS-CoV strains, which resulted in decreased viral replication and shedding capabilities of the Egypt/2013 strain compared with the Qatar15/2015 strain in sentinel llamas infected by contact. Therefore, the Egypt/2013 strain might have a lower potential of transmission than the Qatar15/2015 strain.

Conclusions

The results of our experimental investigation might explain why MERS-CoV clade C strains have not been established in the Arabian Peninsula after being introduced through imported camels and competing with enzootic clade B viruses. However, further studies are needed to determine whether this potentially reduced transmissibility is a common feature of the diverse MERS-CoV lineages found in dromedaries in Africa. Specific amino acid substitutions in the spike protein or in other genomic regions of African clade C viruses might be determinant of the low replication phenotype observed in the in-contact animals in our study, as has been previously observed in human cells (*12*). However, viral or host factors that play a key role in conferring replication and transmission competence remain to be explored in camelid reservoirs. High MERS-CoV genome stability was previously described in llamas infected with the Qatar15/2015 strain (*13*). Thus, eventual mutations arising from animals infected with the Egypt/2013 strain were not expected, and no sequencing was performed in those infected animals. Nonetheless, our study provides in vivo experimental data demonstrating reduced MERS-CoV fitness of 1 African clade C isolate to in-contact camelids compared with an Arabian clade B isolate. In addition, reduced MERS-CoV shedding from infected camelids might limit spillover to humans. Introducing MERS-CoV clade B strains to Africa through infected camelids must be avoided, because these strains might outcompete African MERS-CoV clade C strains and pose a greater zoonotic and pandemic threat in Africa.

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Dr. Rodon conducted his doctoral research at the Animal Health Department of IRTA-CReSA, Barcelona, Spain. His primary research interest is reemerging viral zoonotic diseases with pandemic potential, with particular focus on the One Health approach.

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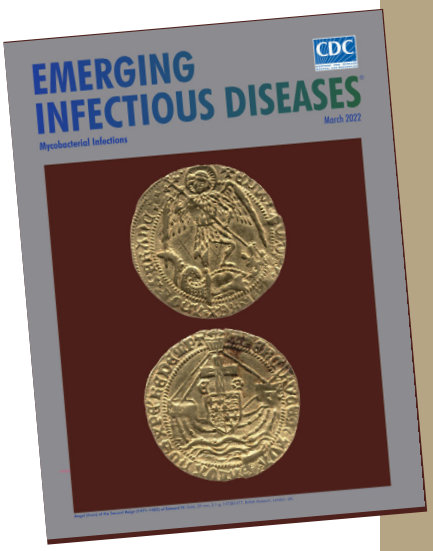

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Schizophyllum commune

[skiz-of'-ĭ-ləm kom'-yoon]

Schizophyllum commune, or split-gill mushroom, is an environmental, wood-rotting basidiomycetous fungus. *Schizophyllum* is derived from "*Schíza*" meaning split because of the appearance of radial, centrally split, gill like folds; "*commune*" means common or shared ownership or ubiquitous. Swedish mycologist, Elias Magnus Fries (1794–1878), the Linnaeus of Mycology, assigned the scientific name in 1815. German mycologist Hans Kniep in 1930 discovered its sexual reproduction by consorting and recombining genomes with any one of numerous compatible mates (currently >2,800).

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Appendix

Materials and Methods

Cell Culture and Viruses

Vero cells (CRL-1586, ATCC, USA) were cultured in Dulbecco's modified Eagle medium (DMEM; Lonza, Switzerland) supplemented with 5% fetal calf serum (EuroClone, Italy), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine (all ThermoFisher Scientific, USA). Calu-3 cells were cultured in Opti-MEM I (1X) supplemented with GlutaMAX (GIBCO, USA), 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. MERS-CoV Qatar15/2015 (clade B strain; GenBank accession no. MK280984.2) passage-3 stocks were prepared as previously described (*1*,*2*), and a passage 6 of the MERS-CoV Egypt/2013 strain (*3*) (clade C; GenBank accession no. KJ477103) was propagated for 3 days at 37°C and 5% CO_2 in Vero cells. Viral stocks were sequenced (GenBank accession no. OP906306) to ensure that critical mutations for virion infectivity (in the RBD or the S1/S2 cleavage site) were not acquired in cell culture. Three aa mutations were detected in the Egypt/2013 inoculum: D1418G (ORF1ab), S1251F (Spike, S2 subunit), T8I (M). Infectious virus titers were determined in Vero cells and calculated by determining the dilution that caused 50% cytopathic effect (CPE) in cell cultures (50% tissue culture infectious dose endpoint, $TCID_{50}$).

Study Design

To study the transmission of a MERS-CoV clade C strain, five healthy llamas were purchased from a private animal facility and housed at the animal BSL-3 facilities of the IRTA-CReSA Biocontainment Unit (Barcelona, Spain). The experimental box was set up as in previous MERS-CoV transmission studies (*1*,*2*,*4*) (Appendix Figure 1, panel A). Two llamas were

intranasally inoculated with $10^{6.4}$ $TCID_{50}$ of MERS-CoV Egypt/2013 strain in 3 mL saline solution, using a nebulization device (LMA MADgic, Teleflex Inc., USA) and administrating 1.5 mL into each nostril. At 2 days postinoculation (dpi), inoculated llamas were placed in direct contact with the remaining three sentinel llamas (Appendix Figure 1, panel B). Clinical signs of all animals were monitored for 3 weeks, and rectal temperatures were recorded until 15 dpi with a fast display digital thermometer (AccuVet, Infratec, Italy). Nasal swabs were obtained daily until 15 dpi, plus at 17 and 22 dpi. Whole blood samples of all animals were collected from the jugular vein using Vacutainer tubes (Beckton Dickinson, USA) and serum samples were obtained before MERS-CoV challenge and at 7, 14 and 22 dpi. Animals were euthanized at 22 dpi with an overdose of pentobarbital, followed by a complete necropsy with special focus on upper and lower respiratory tract lesions.

Additionally, aiming to understand differential transmission patterns between MERS-CoV clades, experimental data was retrieved from previous MERS-CoV Qatar15/2015 strain (clade B) transmission studies in llamas and new analyses were performed (*1*,*2*). Briefly, the MERS-CoV Qatar15/2015 strain was previously used to demonstrate transmission from experimentally infected llamas to naïve animals, and to evaluate the efficacy of two distinct vaccine prototypes in blocking virus transmission (*1*,*2*). Animals used in these studies were also allocated in the biocontainment facilities of IRTA-CReSA and experimental procedures followed the same study design, sample collection and the experimental process than the present study with MERS-CoV Egypt/2013 strain. Therefore, data obtained from these studies were considered appropriate for further comparative analyses. Viral RNA and infectious MERS-CoV Qatar15/2015 (clade B) shedding data from eight inoculated and nine contact animals was used to compare the transmissibility versus the Egypt/2013 (clade C) strain in camelids. All non-protected animals met the inclusion criteria and were used for subsequent comparative analyses.

MERS-CoV RNA Detection

Viral RNA was extracted from nasal swab samples with the IndiMag pathogen kit (Indical Biosciences, Germany) using a Biosprint 96 workstation (Qiagen, Germany), following the manufacturer's instructions. Genomic and subgenomic RNA extracts were detected by the UpE and M mRNA RT-qPCR assays, respectively (*5*,*6*). Viral RNA was detected from nasal swabs by RT-qPCR as previously described (*1*,*2*,*4*,*7–9*). RT-qPCR was carried out with the AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems, Life Technologies, USA) and

amplification was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, USA). Thermal profile followed 10 min at 50°C, 10 sec at 95°C, and 45 cycles of 15 s at 95°C and 30 sec at 58°C. Samples with a quantification cycle (Cq) value ≤40 were considered positive for MERS-CoV genomic or subgenomic RNA.

Virus Titration

The presence of infectious MERS-CoV in nasal swab specimens was evaluated in Vero E6 cells, as previously reported (*1*,*2*,*4*,*7–10*). Briefly, an initial 1/10 dilution and subsequent ten-fold dilutions of the samples were transferred to Vero E6 monolayers. Cells were monitored daily under a light microscope and they were evaluated for the presence of virus-induced cytopathic effect at 6 days after inoculation. The infectious virus concentration in nasal swabs was calculated by determining the dilution that caused 50% CPE in cell cultures ($TCID_{50}$/mL). The limit of detection of the technique was established at 1.8 TCID50/mL.

Plaque Reduction Neutralization Assay

Sera samples collected weekly were tested for the presence of neutralizing antibodies against MERS-CoV (EMC/2012 isolate; GenBank accession no. NC_019843.3) using a plaque reduction neutralization (PRNT) assay according to a previously published protocol (*1*,*2*,*10*), with minor modifications. Briefly, serum samples were inactivated at 56°C for 30 min. Then, 50 µl of 2-fold serially diluted sera were mixed 1:1 with 400 PFU of MERS-CoV, transferred to Calu-3 cells monolayers and incubated at 37°C and 5% CO_2. After 8 h of infection, cells were fixed, permeabilized with 70% ethanol, and stained using mouse anti-MERS-CoV nucleocapsid protein (SinoBiological, China; diluted 1:1000 in 0.1% bovine serum albumin (BSA) -PBS) followed by goat anti-mouse Alexa Fluor 488 antibody (Invitrogen, 1:2000 in 0.1% BSA in PBS). Plates were scanned on the Amersham Typhoon Biomolecular Imager (GE Healthcare, USA). Data was analyzed using ImageQuantTL 8.2 image analysis software (GE Healthcare). The PRNT90 titer was defined as the reciprocal value of the sample dilution that showed 90% reduction of virus growth. Dose-response curves of serum samples were adjusted to a nonlinear fit regression model in Graphpad Prism 9 software, with bottom constraints of 0% and top constraints of 100%. Sera samples from previous studies (*1*,*2*) were re-analyzed to fulfil the above-described criteria.

Data Analyses

To statistically evaluate the transmission potential of each strain, we used two different approaches. First, genomic and subgenomic RNA, and infectious MERS-CoV shedding data were used to calculate areas under the curve of each animal. A Wilcoxon Test was applied to compare the shedding of MERS-CoV/Egypt2013 and MERS-CoV Qatar15/2015 strains within inoculated or in-contact llamas. Second, to compare the results at each time point, a mixed model was adjusted using the shedding data of each individual as a fixed factor and the corresponding MERS-CoV strain and days post-inoculation as random factors, along with a contrast of the estimated marginal means. Only the days whose values differed from the limit of detection were used for the mixed models, which encompassed values from 1 to 15 dpi for genomic RNA, from 5 to 14 dpi for subgenomic RNA and from 7 to 12 dpi for infectious virus. The model validation showed that the residuals of infectious virus shedding data were not normally distributed. Therefore, a log(x+1) transformation was applied only for this dataset. All analyses were performed using *DescTools* (https://cran.r-project.org/web/packages/DescTools/citation.html), *emmeans* (https://github.com/rvlenth/emmeans), *lme4* (https://cran.r-project.org/web/packages/lme4/citation.html), *stats*, and *tidyverse* (https://joss.theoj.org/papers/10.21105/joss.01686) statistic packages for the R software (https://www.r-project.org/). Results of the statistical analyses in sentinel animals are shown in Figure 2.

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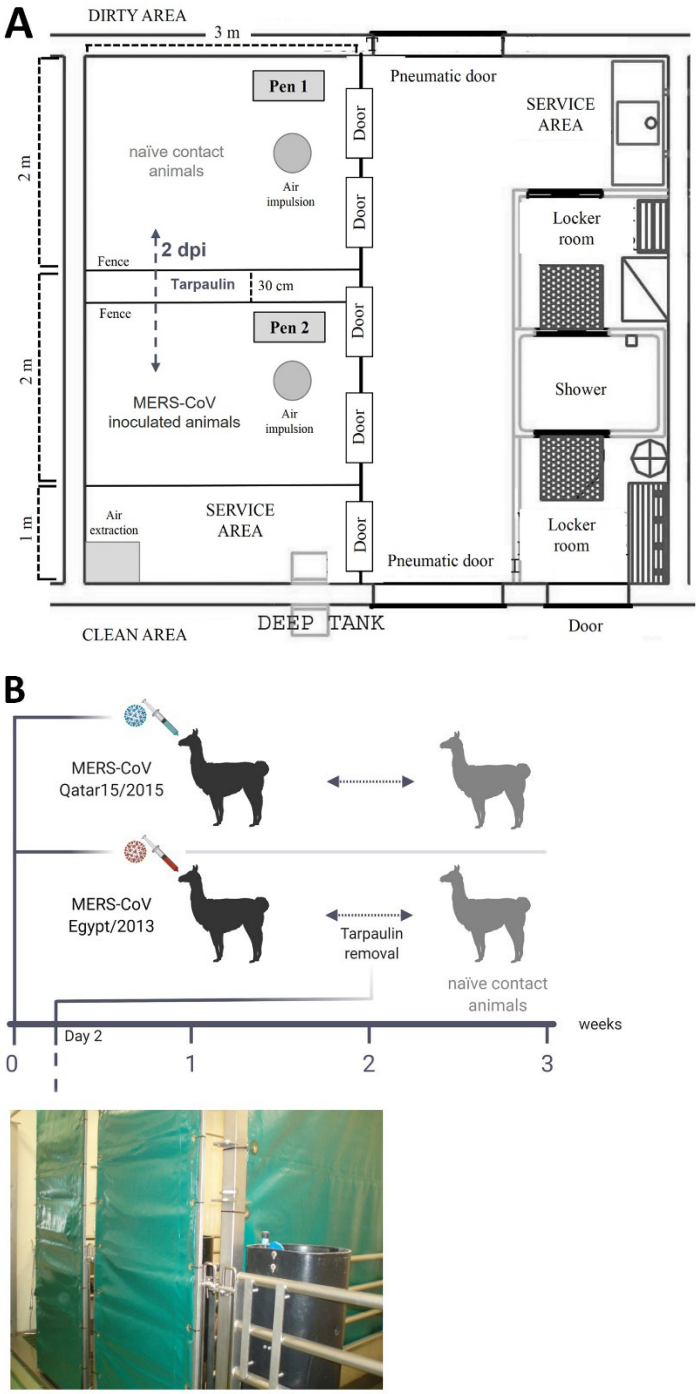
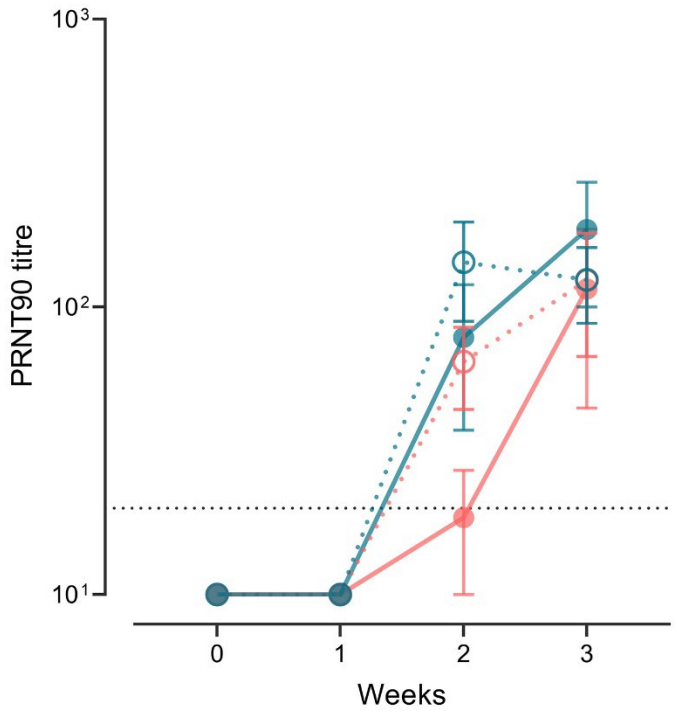


Appendix Figure 1. Schematic representation of the experimental set up. A) A group of llamas (black) was intranasally inoculated with MERS-CoV Qatar15/2015 (blue) or Egypt/2013 (red) and placed in separated experimental boxes. B) Inoculated llamas were kept separated from naïve llamas (gray) using a tarpaulin to avoid direct contact. Two days post MERS-CoV inoculation, tarpaulin was removed, and animals got in direct contact. Afterwards, the viral shedding and humoral responses were monitored for 3 weeks as indicated in the timeline.



Appendix Figure 2. Development of neutralizing humoral responses by llamas infected with MERS-CoV Egypt/2013 or Qatar15/2015. The plot displays levels of serum neutralizing antibodies elicited in llamas upon MERS-CoV Egypt/2013 (red) or Qatar15/2015 (blue) inoculation (dashed lines) or direct exposition to inoculated ones (solid lines). Each line represents mean group values and error bars represent standard error mean intervals. Grey dashed lines depict the detection limits of the assays. PRNT90, 90% plaque reduction neutralization titer.