Supplementary Materials for

An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels


*Corresponding author. E-mail: b.haagmans@erasmusmc.nl (B.L.H.); gerd.sutter@lmu.de (G.S.); albert.osterhaus@tiho-hannover.de (A.D.M.E.O.)

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Materials and Methods

MERS-CoV.
Stocks of MERS-CoV were produced by preparing a seventh passage of the MERS-CoV EMC isolate on Vero cells. Cells were inoculated with virus in Dulbecco’s modified Eagle medium (BioWhittaker) supplemented with 1% serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. After inoculation, the cultures were incubated at 37°C in a CO₂ incubator and three days after inoculation supernatant from Vero cells was collected.

MVA-S vaccine.
The vaccine MVA-S used here is based on a recombinant MVA expressing the full-length MERS-CoV spike glycoprotein (20). Vaccine preparations were obtained and characterized as described previously (34). MVA-S was propagated on chicken embryo fibroblasts (CEF) prepared from 10 day-old chicken embryos (SPF eggs, VALO, Cuxhaven, Germany), purified by ultracentrifugation through 36% sucrose, and reconstituted to vaccine stocks in Tris-buffered saline pH 7.4. Vaccine preparations were tested by Western blot analysis and re-assessed for genetic identity and genetic stability by PCR analysis of genomic viral DNA. Viral titers were determined by plaque assay using MVA-specific immunostaining and titrated in plaque forming units (PFU).

Animal studies.
Eight 6-8 month-old healthy dromedary camels, negative for antibodies to MERS-CoV and MVA, were obtained from the Canary Islands and housed in biosecurity level 3 facilities (CReSA, Barcelona, Spain) in groups of four animals. Animal ethics approval for the experiment was obtained. All animals were vaccinated twice at a four week interval intranasally using a laryngo-tracheal mucosal atomization device (Teleflex, Research Triangle Park, US) and intramuscularly in the neck. We inoculated 4 dromedary camels twice with a 4 week interval with $2 \times 10^8$ plaque forming units (PFU) MVA expressing the full spike protein (MVA-S) via both nostrils and $1 \times 10^8$ PFU intramuscularly in the neck of the animal. Four control animals received the wild type MVA ($n = 2$) or PBS ($n = 2$).

On the day of MERS-CoV inoculation the animals were anaesthetized with Midazolam (5 mg/ml), with a dose depending on the size of the animals, between 18 and 30 mg per animal, and inoculated with $10^7$ half-maximal tissue-culture infectious dose (TCID₅₀) in 3 ml PBS intranasally in both nostrils using a laryngo-tracheal mucosal atomization device. Blood, nasal and rectal swabs were taken at 1, 2, 3, 4, 6, 8, 10 and 13 days pi.

Pathology
On 4 days pi, 2 vaccinated and 2 non-vaccinated animals were anaesthetized with Midazolam (5 mg/ml), with a dose depending on the size of the animals, between 18 and 30 mg per animal, and euthanized with an overdose of Pentobarbitone followed by exsanguination. The following samples were taken for virology and pathology: nose (frontal, medial, caudal), trachea (proximal, medial distal), large bronchus, small
bronchus, lung (left and right cranial tip, mediodorsal, caudal tip), palatum molle, tonsil, tracheal lymph node, mediastinal lymph node, cervical lymph node, parotid salivary gland, heart, liver, spleen, kidneys, adrenal, pancreas, duodenum, jejunum, rectum, brainstem and thyroid. Tissue for virology was placed in culture medium and stored at -70 °C. Tissue for pathology was fixed by immersion in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin for examination by light microscope. For detection of MERS-CoV antigen by immunohistochemistry (IHC), sequential slides were stained with a monoclonal antibody to the MERS-CoV nucleocapsid protein (Sino Biological, Beijing). In each staining procedure, an isotype control was included as a negative control and a lung section from a rabbit experimentally infected with MERS-CoV was used as a positive control. Virus antigen was demonstrated as a bright red staining in the cytoplasm. The in situ hybridization (ISH) probes targeting the nucleocapsid gene of MERS-CoV were designed by Advanced Cell Diagnostics (Hayward, CA) and ISH was performed according to the manufacturer’s instructions; ISH staining was visualized using substrate Fast Red (pink). Controls included probes against SARS-CoV nucleocapsid protein and tissues from non-infected animals (26).

**MERS-CoV neutralization assay.** Sera were heat-inactivated by incubation for 30 minutes at 56°C and then briefly centrifuged to spin down the serum from the lid. We tested the MERS-CoV neutralization activity of sera for their ability to neutralize MERS-CoV (EMC isolate). Briefly, virus (400 PFU) was premixed 1:1 with serial dilutions of sera from animal groups prior to inoculation onto Huh7 cells, and viral infection was monitored by the presence of viral antigen at 8 h pi. Formaldehyde fixed cells were stained using rabbit-anti-MERS-CoV antibodies, according to standard protocols using a FITC-conjugated swine-anti-rabbit antibody as a second step (17). PRNT<sub>90</sub> values were calculated for the MERS-CoV neutralization assay.

**Camelpox virus neutralizing antibodies** For analysis of camelpox virus (CMLV) neutralizing antibodies we used the CMLV reference strain CP-1 (35). CMLV was grown and titrated in the African green monkey kidney cell line MA-104 (General Cell Collection, Cat. No. 85102918, Public Health England, UK) and purified by ultracentrifugation through sucrose cushions. Serum samples from immunized animals or mock-vaccinated animals were tested in triplicate in plaque reduction assays on MA-104 cell monolayers grown in 24-well tissue culture plates. Eight two-fold serial dilutions of serum were mixed with 200 PFU of CMLV at 37°C for 2 hours. Following incubation, 100 µl of the reaction mix was loaded onto the MA-104 cell monolayers. Cells were over-laid with medium and incubated at 37°C with 5% CO<sub>2</sub> for 48h. In the following media were removed and the cells were fixed with ice-cold methanol-aceton for 5 min. Fixed cell monolayers were immunostained using primary rabbit anti-vaccinia virus antibody (diluted 1:2000) and secondary antibody (peroxidase labeled goat anti-rabbit-Ig, Dianova, Hamburg) followed by incubation with True Blue Peroxidase Reagent (KPL, Medac GmbH, Wedel Germany). Blue plaques were counted and CMLV neutralization titers were determined to be the last dilution of serum that reduced the number of plaques by 60% compared with control wells.
Analysis of MVA neutralizing antibodies.
For analysis of MVA neutralizing antibodies we used MVA (clonal isolate F6) grown and titrated in CEF and purified by ultracentrifugation through sucrose cushion (35). Serum samples from immunized animals or mock-vaccinated animals were tested in triplicate in plaque reduction assays on DF-1 cell (ATCC CRL-12203) monolayers grown in 24-well tissue culture plates. Eight two-fold serial dilutions of serum were mixed with 200 PFU of MVA at 37°C for 2 hours. Following incubation, 100 μl of the reaction mix was loaded onto the DF-1 cell monolayers. Cells were overlaid with medium and incubated at 37°C with 5% CO₂ for 48h. In the following media were removed and the cells were fixed with ice-cold methanol-aceton for 5 min. Fixed cell monolayers were immunostained using primary rabbit anti-VACV antibody (diluted 1:2000) and secondary antibody (peroxidase labeled goat anti-rabbit-Ig, Dianova, Hamburg) followed by incubation with True Blue Peroxidase Reagent (KPL, Medac GmbH, Wedel Germany). Blue plaques were counted and MVA neutralization titers were determined to be the last dilution of serum that reduced the number of plaques by 60% compared with control wells.

MERS-CoV S1 ELISA.
Microtiter plates were coated with MERS-CoV-S1 protein (17) in PBS at 1.0 μg/ml and incubated o/n at 4 °C. Next day plates were washed 3 times with PBS/0,05% Tween-20 and blocked with 200 μl PBS/0.5% tween20 supplemented with 1% BSA, for 1h at 37 °C. After washing, serial diluted samples were incubated for 1 h at 37 °C. After washing, wells were incubated with a goat anti lama biotin conjugate at 1:1000 diluted in blocking buffer, followed by incubation with streptavidin peroxidase for 1 h at 37 °C. After washing with PBS, TMB substrate was added to each well. The reaction was stopped with 2M H₂SO₄ after 10 min of incubation and the OD was measured at 450 nm with a reader.

RNA-extraction and quantitative RT-PCR.
Samples were analysed with the UpE PCR as described (17) and confirmed by a nucleocapsid specific PCR. RNA from 200 μl of tissue homogenate was isolated with the Magnapure LC total nucleic acid isolation kit (Roche) and eluted in 100 μl. MERS-CoV RNA was quantified on the ABI prism 7700, with the TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems) using 20 μl isolated RNA, 1× Taqman mix, 0.5U uracil-N-glycosylase, 45 pmol forward primer (5’-GGGTGTACCTCTTTAATGCCAATTC-3’), 45 pmol reverse primer (5’-TCTGTCCTTGCTCCGCAAAT-3’) and 5 pmol probe (5’-FAM-ACCCCTGCGCAAATGCTGGG-BHQ1-3’). Amplification parameters were 5 min at 50°C, 20 sec at 95°C, and 45 cycles of 3 s at 95°C, and 30 sec at 60°C. RNA dilutions isolated from an MERS-CoV stock were used as a standard.

Virus titration in Vero cells
After euthanasia 0.2-0.5 grams of lung tissue was collected from each animal and the samples were transferred to 5 ml tubes containing 2 ml transport medium (Hanks balanced salt solution supplemented with 10% glycerol, 200 U of penicillin per ml, 200
ug of streptomycin per ml, 100 U of polymyxin B sulphate per ml, 250 μg of gentamycin per ml and 50 U of nystatin per ml). The exact weight of each sample was determined and the samples were homogenized on ice and centrifuged for 10 minutes at 2000 rpm. Homogenates were diluted in IMDM containing 5% FBS in ten-fold serial dilutions, starting with a dilution of 1:10. The diluted lung homogenates were transferred to Vero cells, the plates were sealed with adhesive tape and incubated for five days at 37°C. Plates were monitored daily under the light microscope and wells were scored after five days for presence of MERS-CoV induced CPE and the virus titer was calculated according to the Kärber method. The amount of infectious virus in lung homogenates was calculated by determining the dilution that caused CPE in 50% of the inoculated cell cultures (50% tissue culture infectious dose endpoint, TCID₅₀).

**Analysis of vaccine escape mutation.**

Viral RNA was extracted from 140 µl of cell culture supernatant using the QIAamp viral RNA mini kit (Qiagen) and the RNA was eluted with 40 µl buffer according to the manufacturer's instructions. Ten microliter RNA was reverse transcribed with the Superscript III first strand synthesis system (Invitrogen Corp) using random hexamers. The cDNA was used to amplify complete MERS-CoV spike gene (nucleotides positions 21304 to 25660, Genbank accession no JX869059) with a overlapping PCR approach using the PfuUltra II Fusion HS DNA polymerase polymerase (Agilent Technologies). The PCR was carried out as follows: one initial denaturation step of 95°C for 3 min, followed by 39 cycles of 95°C for 20 s, 48°C for 20 s, 72°C for 45 s and a final extension of 72°C for 5 min. Then the amplicons were sequenced directly on both strands using the BigDye Terminator version 3.1 Cycle sequencing kit on an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

**Statistics.**

A Student’s t test was used to analyze differences in mean values between groups. All results are expressed as means ± SEs of the means. P values < 0.05 were considered significant. Statistical analysis was performed with Prism 5.0 (Graphpad).
Fig S1. MERS-CoV S1 reactive antibody responses in MVA vaccinated dromedary camels. Serum antibody responses were analyzed in control vaccinated (open bars) and MVA-S vaccinated (closed bars) dromedary camels at different time points after immunization. Shown are O.D. values of the reactivity’s as measured in an ELISA assay using recombinant MERS-CoV S1 protein.
Fig S2. Change in body temperature after MERS-CoV inoculation in dromedary camels. Rectal body temperatures were measured at different time points after MERS-CoV challenge in control vaccinated dromedary camels (closed circles) and MVA-S vaccinated dromedary camels (closed squares).
**Fig S3. Sequence analysis of the receptor binding domain of MERS-CoV.**

The amino acid sequence of the receptor binding domain of MERS-CoV (amino acids 358-588) was determined from virus isolated at day 6 pi from a dromedary camel vaccinated with MVA-S (camel_5_6dpi) and compared to the virus that was used to inoculate the animals at day 0 (EMC_isolate_P7).
Fig S4. MERS-CoV neutralizing antibody responses in MVA vaccinated dromedary camels after MERS-CoV challenge. Serum antibody responses were analyzed in control vaccinated (closed bars) and MVA-S vaccinated (open bars) dromedary camels at different time points after challenge. Shown are MERS-CoV PRNT90 neutralization titers.
Fig. S5. MERS-CoV RNA detection in rectal swabs of vaccinated dromedary camels. MERS-CoV RNA expressed as GE (TCID$_{50}$/ml) was determined in rectal swabs at different time points after MERS-CoV challenge in dromedary camels vaccinated with MVA-S (open bars) or MVA-wt/PBS (closed bars).
Nose frontal: respiratory epithelium
Nose medial: respiratory epithelium
Nose caudal: olfactory epithelium
Nose medial: olfactory epithelium
Palatum molle (oropharyngeal arch)
Trachea proximal

Fig. S6. Detection of MERS-CoV in tissues of vaccinated dromedary camels at 14 days pi. MERS-CoV viral RNA was determined in tissue homogenates from dromedary camels vaccinated with MVA-S (green and black bars) or control vaccinated (red and blue bars) at 14 days after challenge.
Fig. S7. Viral antigen present in control vaccinated dromedaries challenged with MERS-CoV. (A to D) At 4 days post challenge, no virus antigen was present in the lungs (A), but few epithelial cells had virus with viral antigen in the trachea (B), occasional large mononuclear cells were positive in the tracheal lymph node (C) and a few cells were positive in overlying epithelial cells of the tonsil (D), as determined by immunohistochemistry.
Table S1. Detection of MERS-CoV antigen positive cells in the tissues of dromedary camels.

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</tbody>
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+++: many cells with MERS-CoV presence of virus antigen, ++: moderate number of cells, +: few cells, +/-: occasional cell(s), np: not present
REFERENCES AND NOTES


18. H. Mou, V. S. Raj, F. J. van Kuppeveld, P. J. Rottier, B. L. Haagmans, B. J. Bosch, The receptor binding domain of the new Middle East respiratory syndrome coronavirus maps


23. Materials and methods are available as supplementary materials on *Science* Online.


