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Asymptomatic Middle East Respiratory Syndrome Coronavirus Infection in Rabbits

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The ability of Middle East respiratory syndrome coronavirus (MERS-CoV) to infect small animal species may be restricted given the fact that mice, ferrets, and hamsters were shown to resist MERS-CoV infection. We inoculated rabbits with MERS-CoV. Although virus was detected in the lungs, neither significant histopathological changes nor clinical symptoms were observed. Infectious virus, however, was excreted from the upper respiratory tract, indicating a potential route of MERS-CoV transmission in some animal species.

Middle East respiratory syndrome coronavirus (MERS-CoV) represents a novel betacoronavirus species closely related to clade 2c bat CoVs (1). MERS-CoV has been identified in patients who presented with acute pneumonia (2–4), and more recently also in dromedary camels (5, 6). The potential animal origin of MERS-CoV is consistent with *in vitro* studies showing that cells from different animal species, including bats, camels, goats, and nonhuman primates, allow MERS-CoV infection (7, 8). However, the ability of MERS-CoV to infect some other animal species may be restricted given the fact that mice, ferrets, and hamsters were shown to resist MERS-CoV infection (9–11). Detailed analysis of the MERS-CoV spike protein binding region in dipeptidyl peptidase 4 (DPP4)—the functional receptor for MERS-CoV (12)—in different animal species revealed two divergent loops in the DPP4 beta propeller region (11). Because the virus binding region in rabbit DPP4 closely resembles that in human DPP4 (11), we tested whether rabbits can be infected with MERS-CoV.

In a first set of experiments, we observed that MERS-CoV is able to infect rabbit primary kidney cells *in vitro*, which was blocked by antibodies against DPP4 (Fig. 1A and B). In addition, tissue slices of rabbit lungs and kidney infected with MERS-CoV for 24 h were found to stain for MERS-CoV nucleocapsid when tested by *in situ* hybridization (ISH) (Fig. 1C). The 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 and visualized using the substrate Fast Red. Subsequently, 16 female 6-month-old New Zealand White rabbits (*Oryctolagus cuniculus* [Harlan]), specific pathogen free, seronegative for MERS-CoV, and intraperitoneally transplanted with temperature loggers were inoculated with MERS-CoV ($n = 12$) or sham inoculated ($n = 4$). The virus-inoculated animals were euthanized at 3, 4, or 21 days postinfection (dpi), while sham-inoculated animals were euthanized at 4 dpi (all $n = 4$ per group). To infect all parts of the respiratory tract, the rabbits were inoculated both intranasally with 1×10^6 50% tissue culture infective doses ($TCID_{50}$) and intratracheally with 4×10^6 $TCID_{50}$ of MERS-CoV (EMC [Erasmus Medical Center] isolate) or cell culture medium as a control under ketamine-medetomidine anesthesia. Approval for animal experiments was obtained from the Institutional Animal Welfare Committee (no. 201300121), and the studies were performed under biosafety level 3 (BSL3) conditions. All animals remained free of clinical signs and maintained a relatively constant body temperature (Fig. 1D). The body weight loss did not show significant differences between virus- and sham-inoculated animals (data not shown). However, neutralizing antibodies were detected at 21 dpi in all four virus-inoculated rabbits (titers of 80 to 160).

Just before inoculation and at various dpi, animals were anesthetized with ketamine and nasal, pharyngeal, and rectal swabs were taken, which were placed in virus transport medium. Swabs were frozen at −70°C until analysis with reverse transcription-quantitative PCR targeting regions upstream of the E gene (UpE RT-qPCR) (13), confirmed by a nucleocapsid-specific RT-qPCR and virus titration on Vero cells (12). Infectious virus was detected in nasal swabs at 1 to 7 dpi (Fig. 1E), while pharyngeal swabs mostly were found negative (Fig. 1F), and no virus could be detected in rectal swabs (not shown). Samples of nasal conchae, trachea, bronchus, lung, tracheobronchial lymph node, olfactory bulb, cerebrum, cerebellum, kidney, liver, spleen, and intestine were collected and placed into transport medium or 10% neutral buffered formalin. Samples were collected in a standard manner from the cranial and caudal parts of the lung, embedded in paraffin, sectioned at 4 μm, and used for immunohistochemistry (IHC) with sera from human MERS patients, a monoclonal antibody to the MERS-CoV nucleocapsid protein (Sino Biological, Beijing), for *in situ* hybridization (ISH), or for histopathology after staining with hematoxylin and eosin (HE).

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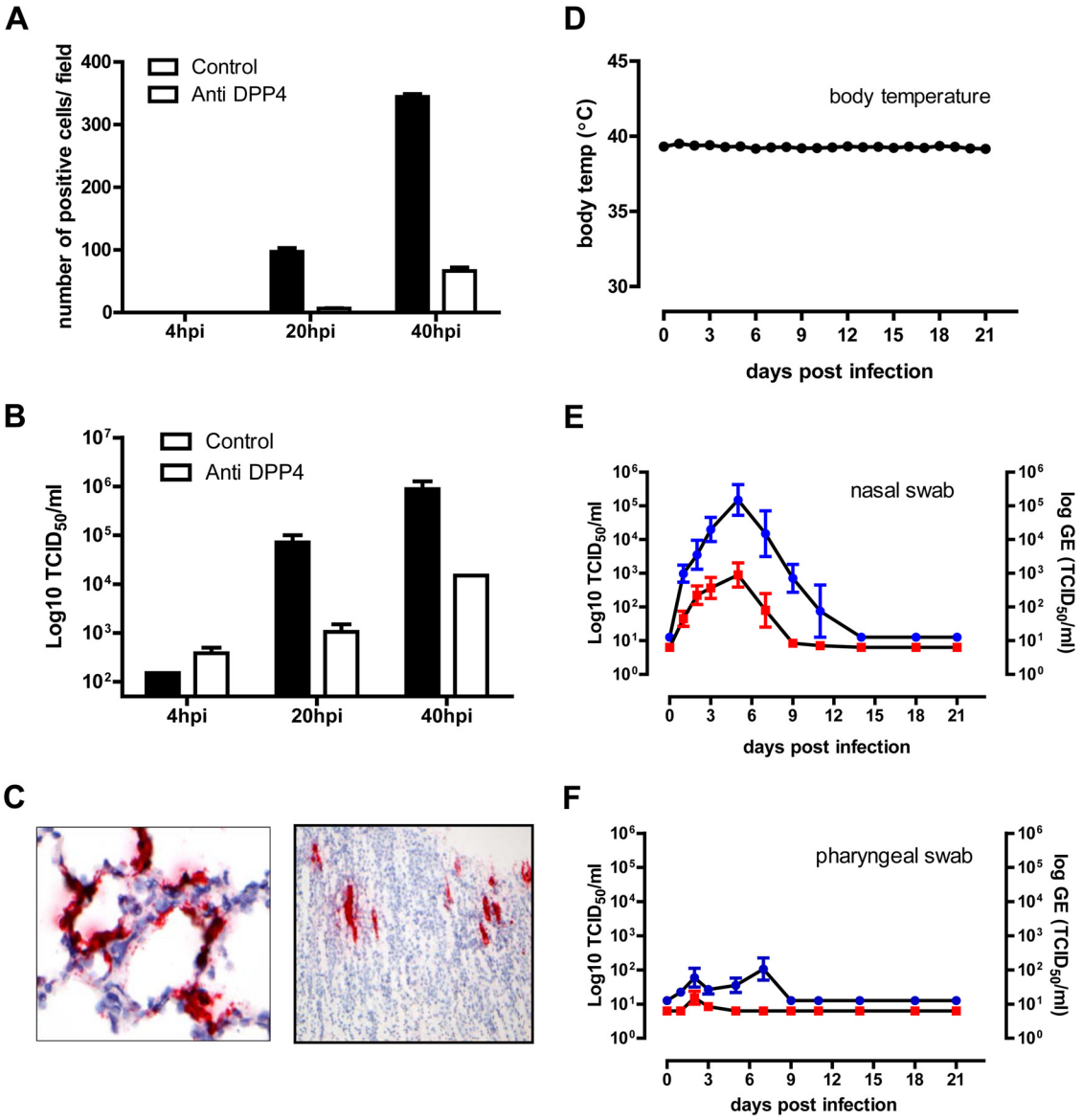

FIG 1 Infection of rabbits with MERS-CoV. Shown is *in vitro* infection of rabbit primary kidney cells with MERS-CoV in the presence of antibodies to DPP4 (open bars) or control serum (closed bars) revealing the number of MERS-CoV-infected cells (A) and infectious virus titers in the supernatant (B). (C) *In situ* hybridization of rabbit lung tissue (left panel) and kidney tissue slices cultured for 24 h *in vitro* after MERS-CoV infection using a MERS-CoV-specific probe. (D) Small fluctuations in mean body temperatures measured by intraperitoneal temperature loggers in four MERS-CoV-inoculated rabbits. Temperatures are shown until 21 dpi. (E) Virus excretion in nasal swabs determined by RT-qPCR and shown as genome equivalents (GE) per milliliter (blue circles) or virus titration (red squares). (F) Mean MERS-CoV excretion detected in pharyngeal swabs determined by RT-qPCR (blue circles) or virus titration (red squares).

The macroscopic appearances of the respiratory tracts of the virus- and sham-inoculated rabbits were similar. Microscopically, however, focal mild to moderate rhinitis with heterophils in the epithelium and lamina propria and focal mild to moderate necrosis and epithelial hyperplasia and hypertrophy related to regeneration were observed in the noses of MERS-CoV-inoculated animals at 3 and 4 dpi. Also, predominantly centered around the terminal bronchioles, the alveolar septa were mildly thickened, with increased numbers of heterophils in the septa and lumina and mild hypertrophy of type II pneumocytes. In addition, there was a moderate proliferation of the bronchus-associated lymphoid tissue. In sham-inoculated control rabbits no such lesions were observed. In the alveoli of most animals, including controls, moderate numbers of alveolar macrophages were observed in some alveoli (see Fig. 3). No significant lesions were seen in the nonrespiratory tissues. Overall, these results indicate that there are relatively limited microscopic changes in the upper and lower respiratory tracts of MERS-CoV-infected rabbits. A more severe course of the infection after 4 dpi is not likely as reflected by unchanged body temperatures during the 21-day period after inoculation (Fig. 1D).

MERS-CoV was detected in several respiratory tissues, including the lungs and nasal conchae at 3 dpi (Table 1). In contrast to the results obtained with the nasal swabs, infectious virus was detected at very low levels in the lungs compared to the MERS-CoV RNA levels. Similar results were obtained when rabbits were

TABLE 1 Detection of MERS-CoV by UpE RT-qPCR in different organs 3 days postinoculation with MERS-CoV

Animal	Expression (log_{10} GE/ml or log_{10} GE/g) in[a]:										
	Turbinate	Pharynx	Trachea	Lung	TBLN	Olf. bulb	Brain	Kidney	Liver	Spleen	Intestine
6634	5.8	NA	7.2	7.5	5.0	3.5	3.1	<2.8	<2.8	3.9	<2.8
7097	3.9	<2.7	4.6	7.0	4.9	<3.2	<2.6	<3.2	<2.8	3.6	<2.8
8805	6.6	4.0	7.2	4.7	5.9	3.5	<2.5	<2.6	<2.9	<3.0	<2.7
7960	5.3	3.8	6.5	3.1	3.8	<3.4	2.9	<2.6	<2.8	3.1	<2.8

[a] Values preceded by "<" indicate that expression in the sample was below the lower limit of detection. TBLN, tracheal bronchial lymph node; Olf., olfactory; NA, not analyzed.

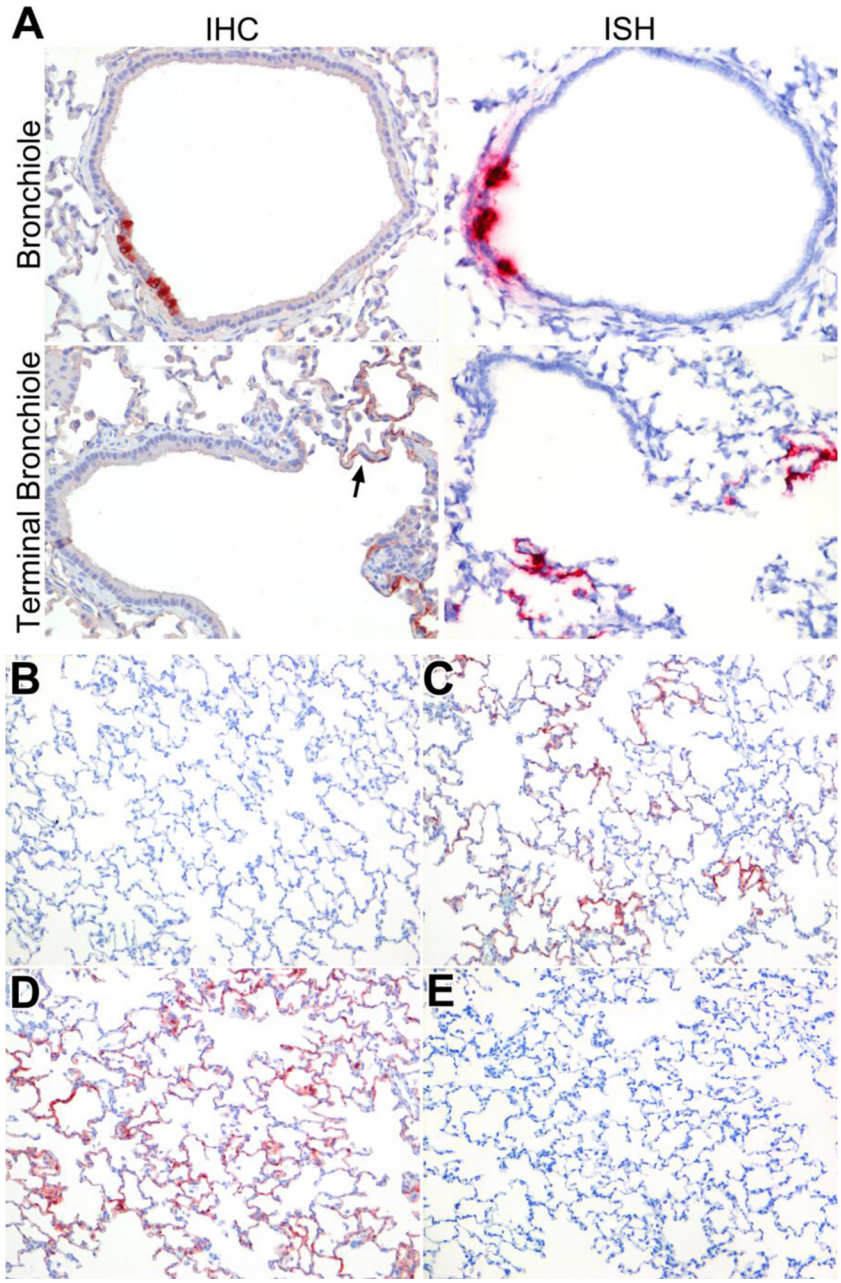

FIG 2 Detection of virus-infected cells in the respiratory tract of rabbits inoculated with MERS-CoV. (A) MERS-CoV antigen detected by immunohistochemistry (IHC [brown]) and MERS-CoV RNA by *in situ* hybridization (ISH [red]) is present in epithelial cells of bronchioles and terminal bronchioles on 3 dpi. (B to E) Overview of antigen expression by IHC at 4 dpi with a monoclonal antibody, showing, respectively, areas in the lung with no expression (B), multifocal little expression (C), and multifocal to coalescing marked expression (D) and an isotype control of a sequential slide of panel D (E).

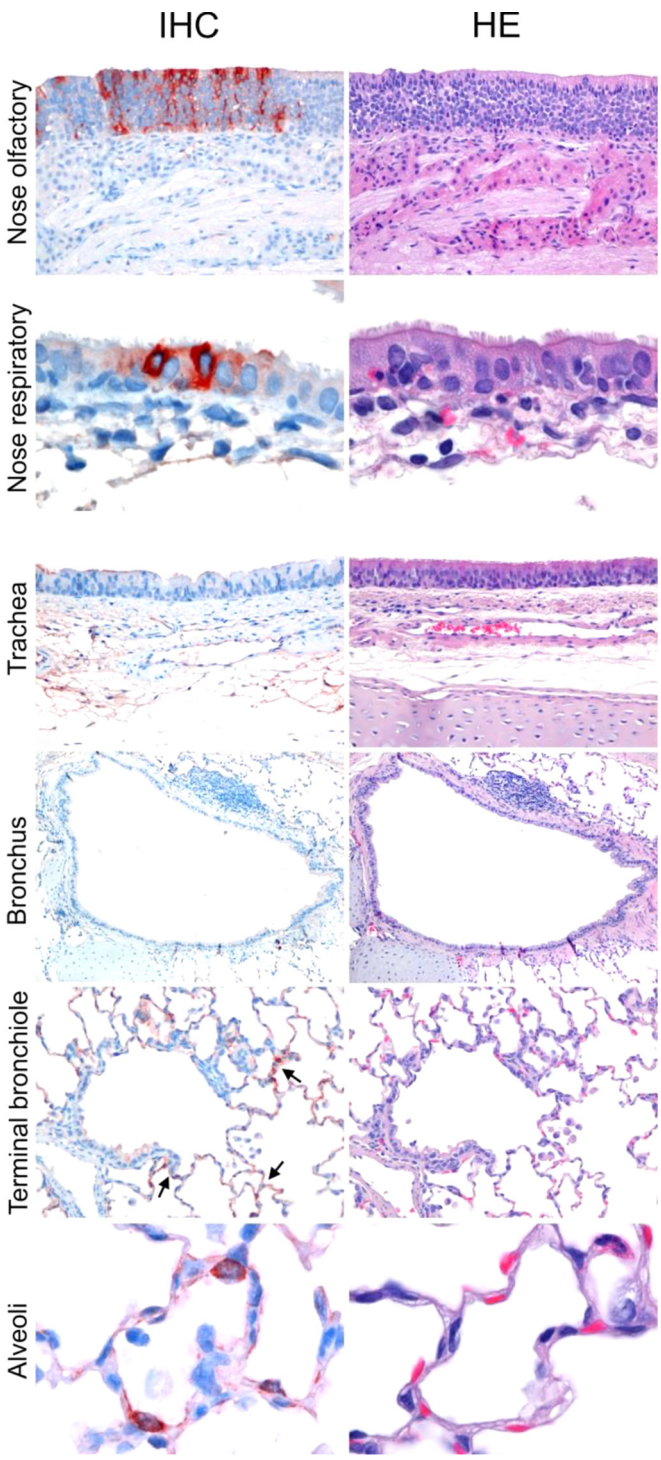

FIG 3 Immunohistochemistry and histopathology of the respiratory tract of rabbits inoculated with MERS-CoV at 4 dpi. MERS-CoV antigen was present in epithelial cells of the olfactory and respiratory part of the nose and in type I and II pneumocytes in alveoli, often centered around the terminal bronchioles. Histopathology of the nose showed focal mild rhinitis with infiltration of few heterophils and mild to moderate necrosis and regeneration. Although there were viral antigen-positive cells in the alveoli, associated lesions were limited or absent.

inoculated with a different human MERS-CoV isolate, England 2 (data not shown). Although these results are in line with those from previous studies in nonhuman primates (14), so far we were not able to reveal the mechanism that caused this apparent discrepancy. By ISH, MERS-CoV RNA was detected in bronchiolar epithelial cells at 3 dpi (Fig. 2), in cells resembling type I and type II pneumocytes in the alveoli at 3 and 4 dpi (Fig. 2 to 3), and in epithelial cells in the nose at 4 dpi (not shown). By immunohistochemistry (IHC), MERS-CoV antigen expression was present in the nose and in the lungs. In the nose, there was focal marked antigen expression in the respiratory epithelium and olfactory epithelium colocalized with lesions at 4 dpi (Fig. 3). In the lungs, there was multifocal antigen expression in predominantly cells resembling type I and II pneumocytes, bronchial and bronchiolar epithelial cells at 3 dpi (Fig. 2), while there was only antigen expression in alveolar epithelial cells at 4 dpi (Fig. 3). Antigen expression colocalized with peribronchiolar lesions, although in some animals, virus was detected more widespread in alveolar epithelium (Fig. 2). No antigen expression was seen in tracheal epithelial cells, bronchus-associated lymphoid tissue, or nonrespiratory tissues.

Mice, ferrets, and hamsters have been shown to resist MERS-CoV infection (9–11), hampering the development of small animal models for MERS. Recent studies have demonstrated that mice can be sensitized to MERS-CoV infection by prior transduction with adenoviral vectors expressing DPP4 (15). In addition, transgenic expression of human DPP4 (hDDP4) in mice allows MERS-CoV to replicate extensively (16). Our study demonstrates that also rabbits can be infected with MERS-CoV. Although the inoculated rabbits did not show overt clinical signs upon MERS-CoV inoculation, this small animal model may be important to test intervention strategies to block MERS-CoV replication *in vivo*. Currently, there are no indications that rabbits may serve as a reservoir for the virus.

Most MERS patients identified thus far have exhibited a severe lower respiratory tract infection that may become fatal, while there is little involvement of the upper respiratory tract (3). In contrast, MERS-CoV has been detected in nasal swabs from dromedary camels (5, 6) in the absence of overt clinical signs, as was observed in our study in experimentally infected rabbits. One could speculate that this phenomenon of subclinical infection also may occur more often in healthy, immunocompetent humans as most MERS patients identified thus far were found to have underlying comorbidities (3). Thus, rabbits may be used as a model to study the pathogenesis of MERS and transmission of MERS-CoV and to test intervention strategies aimed at inhibition of MERS-CoV replication *in vivo*. On the other hand, modulation of host responses (e.g., by immunosuppression) may be needed to reveal the potential of experimental infection of rabbits as a model for more severe MERS.

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