SUPPLEMENT ARTICLE

Lack of Marburg Virus Transmission From Experimentally Infected to Susceptible In-Contact Egyptian Fruit Bats

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Egyptian fruit bats (*Rousettus aegyptiacus*) were inoculated subcutaneously (n = 22) with Marburg virus (MARV). No deaths, overt signs of morbidity, or gross lesions was identified, but microscopic pathological changes were seen in the liver of infected bats. The virus was detected in 15 different tissues and plasma but only sporadically in mucosal swab samples, urine, and fecal samples. Neither seroconversion nor viremia could be demonstrated in any of the in-contact susceptible bats (n = 14) up to 42 days after exposure to infected bats. In bats rechallenged (n = 4) on day 48 after infection, there was no viremia, and the virus could not be isolated from any of the tissues tested. This study confirmed that infection profiles are consistent with MARV replication in a reservoir host but failed to demonstrate MARV transmission through direct physical contact or indirectly via air. Bats develop strong protective immunity after infection with MARV.

Keywords. experimental infection; Marburg virus; Egyptian fruit bat; shedding; horizontal transmission.

The Egyptian fruit bat (*Rousettus aegyptiacus*) is implicated as a natural reservoir host for Marburg virus (*Marburg marburgvirus*; MARV) [1–8]. However, the mechanisms by which the virus is transmitted in this species remain elusive. In a previous study, oronasal inoculation of *R. aegyptiacus* with MARV did not result in infection, but bats became infected after subcutaneous inoculation, albeit without demonstrable virus shedding [9]. The MARV used in this first experimental study [9] was subjected to a high passage number, thus increasing the potential for confounding effects of adaptive mutations accumulated through passage in cell culture. To address these shortcomings, Amman et al [10] used a low-passage, bat-borne MARV to inoculate subcutaneously captive-bred juvenile *R. aegyptiacus* bats. All bats became infected, and there was evidence of MARV oral and fecal shedding, with intensive replication at the inoculation site.

Here, we report on an attempt to transmit low passage MARV horizontally from infected to naive *R. aegyptiacus* bats. We studied the infection kinetics in 15 major tissues from days 3 to 14 and in the plasma from days 3 to 42 after infection. MARV was sporadically detected in oral and vaginal secretions, feces, and urine. Despite close physical or indirect contact, there was no evidence for horizontal transmission of the virus from infected to fully susceptible *R. aegyptiacus* during a period of 42 days. Immune bats rechallenged on day 48 were protected against MARV infection.

MATERIALS AND METHODS

Animal ethics clearance for establishing and maintaining breeding colonies of African fruit bats (AEC 136/12) and experimental inoculation of *R. aegyptiacus* with

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MARV (AEC 139/13) was obtained from the Animal Ethics Committee of the National Health Laboratory Service.

Source of *R. aegyptiacus*

Rousettus aegyptiacus were captured at Mahune Cave in the Matlapitsi Valley, Limpopo province, South Africa, using standard trapping procedures [4]. Captured bats were transported to the animal facility in temporary cages and placed under biosafety level (BSL) 3 quarantine for 4 weeks. During the quarantine, bats were kept and fed as described elsewhere [9]. All ectoparasites were removed from the bats and tested for the presence of MARV. Blood samples were taken at 3-weekly intervals to confirm that the bats remained serologically stable for antibodies against filoviruses or rabies-related lyssaviruses before moving to a flight cage for housing. Colony conditions were maintained as described elsewhere [11].

Accommodation and Handling of *R. aegyptiacus* in the BSL4

Bats were housed in groups of 6 animals per cage in the same type of cage used for the quarantine period, with internal dimensions of 40 × 42 × 42 cm (long, wide, and high, respectively; Figure 1*A*). Cages were isolated under negative pressure in ventilated, high-efficiency particulate arrestance (HEPA)-filtered cabinets within the BSL4 containment, where environmental conditions were maintained as described elsewhere [9]. Experimental bats were acclimatized to the BSL4 environment for 1 week before the experimental procedures started.

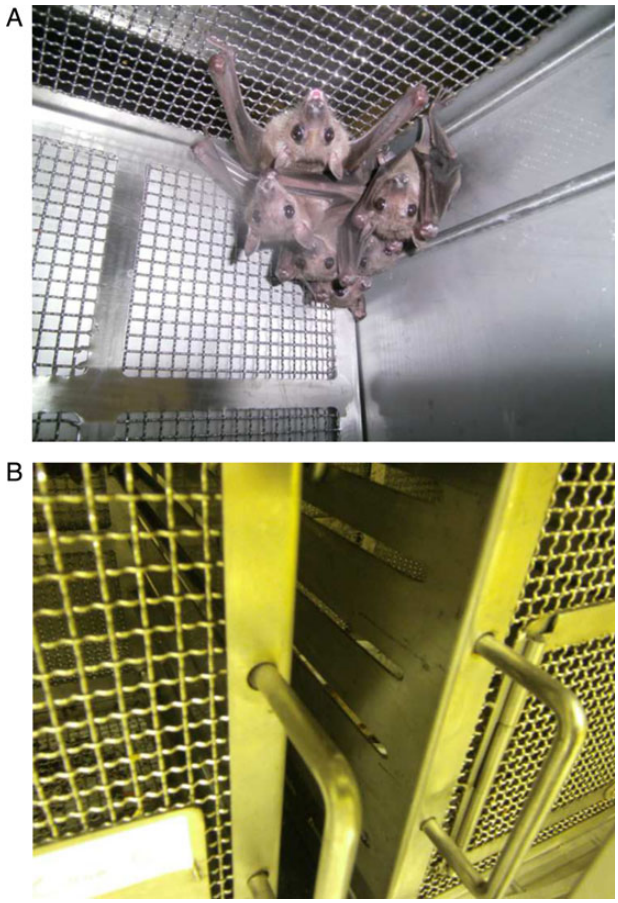

Figure 1. Custom-made experimental bat cages. *A*, Cage housing 6 Egyptian fruit bats (*Rousettus aegyptiacus*). *B*, Cages with ventilation slots allowing for directional air flow between cages.

Serology

An indirect immunoglobulin (Ig) G enzyme-linked immunosorbent assay (ELISA) was based on purified recombinant MARV (Musoke) glycoprotein antigen (Integrated BioTherapeutics). Positive control serum was from *R. aegyptiacus* infected with MARV in a previous study [9]. Negative control serum was pooled from 6 captive-bred *R. aegyptiacus*.

ELISA plates were coated with 100 µL/well of stock antigen (0.6 mg/mL; diluted 1:1500 in phosphate-buffered saline [PBS]; pH 7.2) and then incubated overnight at 4°C. Plates were washed 3 times with PBS (pH 7.2) containing 0.1% Tween 20. The same washing procedure followed each subsequent stage of the assay. The coated plates were then blocked with 10% fat-free milk powder in PBS and incubated for 1 hour at 37°C. After washing, 100 µL/well of control and test serum samples diluted 1:100 in PBS containing 2% milk powder was added to the plates. Next, after 1-hour incubation at 37°C, plates were washed, and a 100-µL volume of a 1:2000 dilution of the anti–bat immunoglobulin–horseradish peroxidase conjugate (Bethyl) was added to each well. After incubation for 1 hour at 37°C, plates were washed and 100 µL of 2,2′-azinodiethylbenzothiazoline–sulfonic acid substrate was added to each well. Plates were incubated in the dark for 30 minutes at room temperature. Reactions were stopped by the addition of 100 µL per well of a 1% sodium dodecyl sulfate solution, and optical densities were measured at 405 nm. The mean optical density readings were converted to a percentage of positive (PP) control serum, as described elsewhere [12].

Determination of Cutoff Value

The cutoff value for the ELISA was determined by monitoring passive humoral IgG antibody levels in 15 *R. aegyptiacus* pups born in captivity to MARV-seropositive mothers during a period of 3–10 months after birth.

Virus Stock

The SPU 148/99/1 isolate of MARV (second passage in Vero cells) used to inoculate bats was isolated from the serum of a patient who contracted a fatal Marburg virus disease in 1999 in Watsa, Democratic Republic of Congo [1].

Experimental Infections

Experiment 1

A week before any inoculation procedure started, 36 bats, aged 8–12 months, were bled to reconfirm their MARV-negative

serological status. Twenty-two MARV-seronegative *R. aegyptiacus* were inoculated subcutaneously with 100 μL of tissue culture supernatant containing $10^{5.3}$/mL median tissue culture infective dose ($TCID_{50}$) of MARV, and 14 MARV-seronegative bats were mock inoculated subcutaneously with 100 μL of Eagle's minimal essential medium. Bats were subdivided in 6 cages (A–F). Cages A–D contained 4 infected and 2 uninfected bats each and had solid side walls (Figure 1*A*). Cages E and F, containing 6 infected and 6 uninfected bats, respectively, were spaced 10 cm apart from each another and had ventilation slots on both sides, allowing for directional air flow from cage E to cage F (Figure 1*B*) in the HEPA-filtered cabinets. Bats were sampled on days 0, 3, 5, 7, 9, 12, 14, 21, 28, 35, and 42 after inoculation.

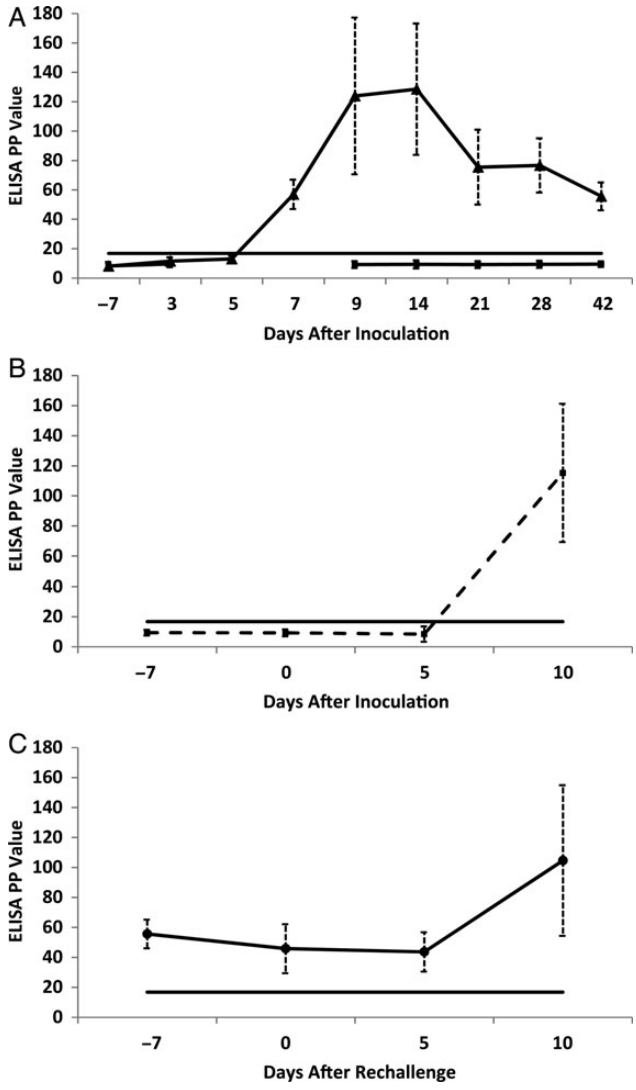

Figure 2. Mean immunoglobulin (Ig) G levels (with standard deviations) in Egyptian fruit bats (*Rousettus aegyptiacus*) inoculated with Marburg virus (MARV). Results for anti-MARV IgG antibody by enzyme-linked immunosorbent assay (ELISA) are shown as the percentage positivity (PP) relative to positive internal control serum; solid line denotes ELISA cutoff value of 16.78 PP. *A*, Bats inoculated with MARV (*triangles*) and MARV-seronegative bats in-contact with MARV-infected bats (*squares*), both in experiment 1. *B*, MARV-seronegative in-contact bats from experiment 1 inoculated with MARV in experiment 2. *C*, Immune bats from experiment 1 rechallenged with MARV in experiment 3.

Experiment 2

Fourteen bats that remained seronegative after 42 days in-contact exposure to infected bats were inoculated as in experiment 1. Bats were sampled on days 4, 5, 7, and 10 after inoculation.

Experiment 3

Four immune bats from experiment 1 were rechallenged 48 days after inoculation, as described for experiment 1. Samples were obtained days 0, 4, and 10 after challenge. Bats were anesthetized before inoculation and specimen collection, as described elsewhere [9], and they were monitored daily for the development of clinical signs as well as for food intake. Oral, nasal, vaginal, penile, urine, and rectal swab samples were collected at regular intervals with sterile cotton swabs and immediately transferred to 0.5 mL of Eagle's minimal essential medium. Occasionally, urine samples were collected directly into sterile tubes or from the bladder by aspiration with a syringe. Postmortem tissues were processed as described elsewhere [9].

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction and Virus Isolation

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and virus isolation were performed as described elsewhere [9]. Samples with cycle threshold values ≤40 were regarded as positive. RNA copy numbers detected in samples were converted into $TCID_{50}$ equivalents per milliliter of plasma and urine or per gram of tissue [9].

Histopathological and Immunohistochemical Analyses

Paraffin-embedded blocks were prepared from 10% formalin-fixed tissues using standard methods [13, 14]. Sections from paraffin-embedded tissue samples were concurrently examined with samples from an uninfected seronegative bat. Initially, specific immune reactivity was detected using primary rabbit antibody against MARV VP40 protein at a 1:4000 dilution for 30 minutes. There was some nonspecific background staining with this antibody, making interpretation inconclusive on many of the tissues tested. Therefore, anti-MARV mouse monoclonal antibody at a 1:250 dilution for 30 minutes was also used [15]. The tissues were processed for immunohistochemistry using the Dako Autostainer. The secondary antibody used was biotinylated goat anti–rabbit IgG (Vector Labs BA-1000) or goat anti–mouse IgG (Vector Labs BA-1000), both used at 1:200 dilution for 30 minutes, followed by Dako LSAB2 streptavidin–horseradish peroxidase (K1016) for 15 minutes. Slides were developed with Dako 3,3′-Diaminobenzidine

Table 1. Quantitative Reverse-Transcription PCR and VI Results in Plasma and Tissues of Egyptian Fruit Bats (*Rousettus aegyptiacus*) Inoculated Subcutaneously with Marburg Virus: Experiment 1

	Days After Inoculation[a]						
Bat IDs and Sample Type	3 (n = 3)[b,c]	5 (n = 4)[c]	7 (n = 3)[c]	9 (n = 3)	12 (n = 3)	14 (n = 2)	21, 28, 35, and 42 (n = 4)
Bat IDs (sex)	1 (F), 9 (M), 15 (F)	12 (F), 25 (F), 31 (M), 32 (M)	4 (M), 11 (F), 16 (F)	5 (M), 22 (M), 34 (M)	26 (F), 28 (M), 36 (F)	29 (M), 41 (F)	6 (F), 18 (M), 24 (M), 37 (F)
Sample type							
Plasma	3/3	4/4	3/3	2/3	1/3	0/2	0/4
Liver	3/3; VI: 3/3	4/4; VI: 4/4	3/3; VI: 3/3	1/3; VI: 0/3	1/3; VI: 0/3	1/2; VI: 0/2	NS
Spleen	3/3; VI: 3/3	4/4; VI: 4/4	3/3; VI: 3/3	1/3; VI: 0/3	3/3; VI: 0/3	2/2; VI: 0/2	NS
Kidney	0/3	0/4	1/3; VI: 1/3	0/3	1/3; VI: 0/3	0/2	NS
Lung	0/3	2/4; VI: 2/4	0/3	0/3	0/3	0/2	NS
Heart	0/3	1/4; VI: 1/4	0/3	0/3	0/3	0/2	NS
Stomach	1/3; VI: 1/3	0/4	2/3; VI: 2/3	0/3	0/3	0/2	NS
Small intestine	0/3	2/4; VI: 2/4	1/3; VI: 1/3	1/3; VI: 0/3	2/3; VI: 0/3	0/2	NS
Large intestine	0/3	1/4; VI: 0/4	2/3; VI: 1/3	0/3	0/3	0/2	NS
Rectum	0/3	2/4; VI: 2/4	1/3; VI: 1/3	0/3	1/3; VI: 0/3	0/2	NS
Bladder	0/3	1/4; VI: 1/4	0/3	0/3	0/3	0/2	NS
Reproductive tract	1 (F)/3; VI: 1/3	1 (M)/4; VI: 1/4	1 (F)/3; VI: 1/3	0/3	1 (M)/3; VI: 0/3	0/2	NS
Salivary glands	0/3	0/4	2/3; VI: 2/3	0/3	2/3; VI: 2/3	1/2; VI: ½	NS
Skin	0/3	0/4	1/3; VI: 1/3	1/3; VI: 0/3	0/3	0/2	NS
Muscle	0/3	0/4	1/3; VI: 1/3	0/3	0/3	0/2	NS
Brain	0/3	0/4	0/3	0/3	0/3	0/2	NS

Abbreviations: F, female; ID, identification number; M, male; NS, not sampled; PCR, polymerase chain reaction; VI, virus isolation.

[a] Unless otherwise specified, data represent No. of bats with positive samples/No. tested. Results are PCR results unless otherwise indicated; VI results are given only for PCR-positive tissues, because only these tissues were subjected to VI.

[b] Sample sizes in column heads represent No. of bats tested post mortem.

[c] Bats in these columns had tissues subjected to both histopathological and immunohistochemical examinations.

chromogen (K3468) for 5 minutes and counterstained with Harris hematoxylin for 1 minute. Selected tissues from bats sampled on days 3–7 after inoculation were subjected to immunohistochemical analyses. Rhesus macaque liver tissue infected with MARV virus was used as a positive control for immunohistochemistry, with cytoplasmic staining in hepatocytes as an expected positive result.

Statistical Analysis

The cutoff value for the ELISA was determined as the mean plus 3 standard deviations (SDs) of the results observed in 5–10 month old *R. aegyptiacus* pups. The statistical significance of differences in humoral immunity and viral load levels were calculated by using the Fisher *F* test and Kruskal–Wallis 1-way analysis of variance.

RESULTS

There was a statistically significant difference ($P < .001$; Fisher *F* test) between mean (SD) PP ELISA values recorded in *R. aegyptiacus* pups 5–10 months after birth (8.4 [2.5]), compared with those recorded 3 months after birth (50.0 [29.1]). The mean levels of IgG in serum samples collected from pups 5, 8, and 10 months after birth remained at similar levels. These results demonstrate that the maternal immunity was lost 3–5 months after birth; thus, the ELISA readings recorded in serum samples 5–10 months after birth represent the assay background noise rather than the measurement of specific levels of anti-MARV IgG. Consequently, the ELISA cutoff was determined as 16.78 PP, equivalent to the mean PP value plus 3 SDs recorded in 60 serum samples collected from pups during a period 5–10 months after birth.

Experiment 1

All bats remained clinically well, maintained normal food uptake, and no gross disease was identified. No fighting or other behavioral changes were observed. None of the bats sustained apparent skin or mucous wounds or injury during the duration of the experiments.

The first seroconversions were detected on day 7 after inoculation, and by day 14 all the infected bats had seroconverted. The highest mean IgG antibody level was recorded 2 weeks after inoculation, followed by a sharp decrease by day 42 (Figure 2*A*). Fifteen of 22 bats (68.2%) were viremic on day 3

Table 2. Quantitative Reverse-Transcription PCR and VI Results in Plasma and Tissues of Egyptian Fruit Bats (*Rousettus aegyptiacus*) Inoculated Subcutaneously With Marburg Virus: Experiment 2

	Days After Inoculation[a]			
Sample Type and Bat IDs	4 (n = 3)[b,c]	5 (n = 2)[c]	7 (n = 2)[c]	10 (n = 7)
Bat IDs (Sex)	8 (M), 10 (F), 14 (F)	30 (M), 33 (F)	19 (F), 21 (M)	17 (M), 38 (M), 39 (M), 40 (M), 44 (F), 46 (F), 717 (F)
Sample type				
Plasma	3/3	2/2	2/2	4/7
Liver	3/3; VI: 3/3	2/2; VI: 2/2	2/2; VI: 2/2	4/7; VI: 1/7
Spleen	3/3; VI: 3/3	2/2; VI: 1/2	2/2; VI: 1/2	7/7; VI: 4/7
Kidney	1/3; VI: 1/3	0/2; VI: 0/2	0/2; VI: 0/2	3/7; VI: 1/7
Lung	3/3; VI: 2/3	1/2; VI: 0/2	0/2; VI: 0/2	1/7; VI: 1/7
Heart	0/3	0/2	0/2	0/3
Stomach	0/3	0/2	0/2	0/3
Small intestine	0/3	0/2	0/2	1/3; VI: 0/3
Large intestine	1/3; VI: 0/3	0/2	1/2; VI: 1/2	0/3
Rectum	1/3; VI: 1/3	1/2; VI: 1/2	0/2	1/3; VI: 0/3
Bladder	0/3	1/2 VI: 1/2	0/2	0/3
Reproductive tract	0/3	0/2	0/2	0/3
Salivary glands	1/3; VI: 1/3	1/2; VI: 1/2	0/2	0/3
Skin	0/3	0/2	1/2; VI: 1/2	0/3
Muscle	0/3	0/2	0/2	0/3
Brain	0/3	0/2	0/2	0/3

Abbreviations: PCR, polymerase chain reaction; VI, virus isolation.

[a] Unless otherwise specified, data represent No. of bats with positive samples/No. tested. VI results are given only for PCR-positive tissues, because only these tissues were subjected to VI.

[b] Sample sizes in column heads represent No. of bats tested post mortem.

[c] Bats in these columns had tissues subjected to both histopathological and immunohistochemical examinations.

after inoculation, including those euthanized on that day. Blood samples of 7 bats that were negative on day 3 were all positive on day 7, including those obtained from bats euthanized on that day. MARV was not detectable in the blood on days 14–42 after inoculation (Table 1). The average level of equivalent viremia ranged from $10^{0.92}$ $TCID_{50}$/mL on day 9 after inoculation to $10^{3.6}$ $TCID_{50}$/mL on day 5 (Figure 3*A*). Neither seroconversion (Figure 2*A*) nor viremia was detected in any of the in-contact bats.

MARV RNA was detected on days 9–14 after inoculation in the liver but replicating virus was only detected on days 3, 5, and 7 (Table 1). Mean virus concentrations ranged from $10^{2.6}$ $TCID_{50}$/g tissue on day 12 after inoculation to $10^{3.7}$ $TCID_{50}$/g tissue on day 5 (Figure 3*B*). Similarly, MARV RNA and the replicating virus was detected in the spleen of all bats on days 3, 5, and 7 after inoculation, and MARV RNA only on days 9–14 (Table 1). Mean virus concentrations ranged from $10^{2.95}$ $TCID_{50}$/g tissue on day 9 to $10^{3.89}$ $TCID_{50}$/g tissue on day 5 (Figure 3*C*).

MARV RNA and live virus was detected in the salivary glands of 5 of 22 bats on days 7, 12, and 14 after inoculation, with viral loads ranging from $10^{2.6}$ to $10^{4.1}$ $TCID_{50}$/g tissue. MARV RNA was detected in the small intestines of 5 of 22 bats on days 5–12, of which those obtained on days 5 and 7 were also positive by virus isolation; viral loads ranged from $10^{2.6}$ $TCID_{50}$/g tissue to $10^{3.7}$ $TCID_{50}$/g tissue. MARV RNA was detected in the rectums of 4 of 22 bats on days 5, 7, and 12 after inoculation, of which those obtained on days 5 and 7 were also positive by virus isolation (Table 1); viral loads ranged from $10^{2.0}$ to $10^{3.91}$ $TCID_{50}$/g tissue.

Of the 11 female and 11 male bats, 2 of each sex had MARV RNA–positive reproductive tissues, representing pooled samples of uteri and ovaries collected on days 3 ($10^{3.3}$ $TCID_{50}$/g) and 7 ($10^{4.2}$ $TCID_{50}$/g) after inoculation, and pooled samples of testicular and epididymal tissue collected on days 5 ($10^{3.3}$ $TCID_{50}$/g) and 12 ($10^{2.8}$ $TCID_{50}$/g), respectively. In all but 1 bat (a male sampled on day 12 after inoculation), qRT-PCR–positive reproductive tissues were also positive at virus isolation. Less frequently, MARV was detected in the stomachs and colons (3 of 22 bats) or the kidneys, lungs, and skin (2 of 22 bats), with viral loads ranging from $10^{2.9}$ to $10^{3.42}$ $TCID_{50}$/g tissue. The virus was detected in the heart and the bladder of 1 of 22 bats on day 5 after inoculation and in the muscle tissue of 1 of 22 on day 7, with viral loads ranging from $10^{2.1}$ to $10^{3.1}$ $TCID_{50}$/g of tissue. All brain tissues were negative by qRT-PCR (Table 1).

MARV RNA was detected in a single oral swab sample on day 7 after inoculation, in pooled vaginal swab samples on days 14

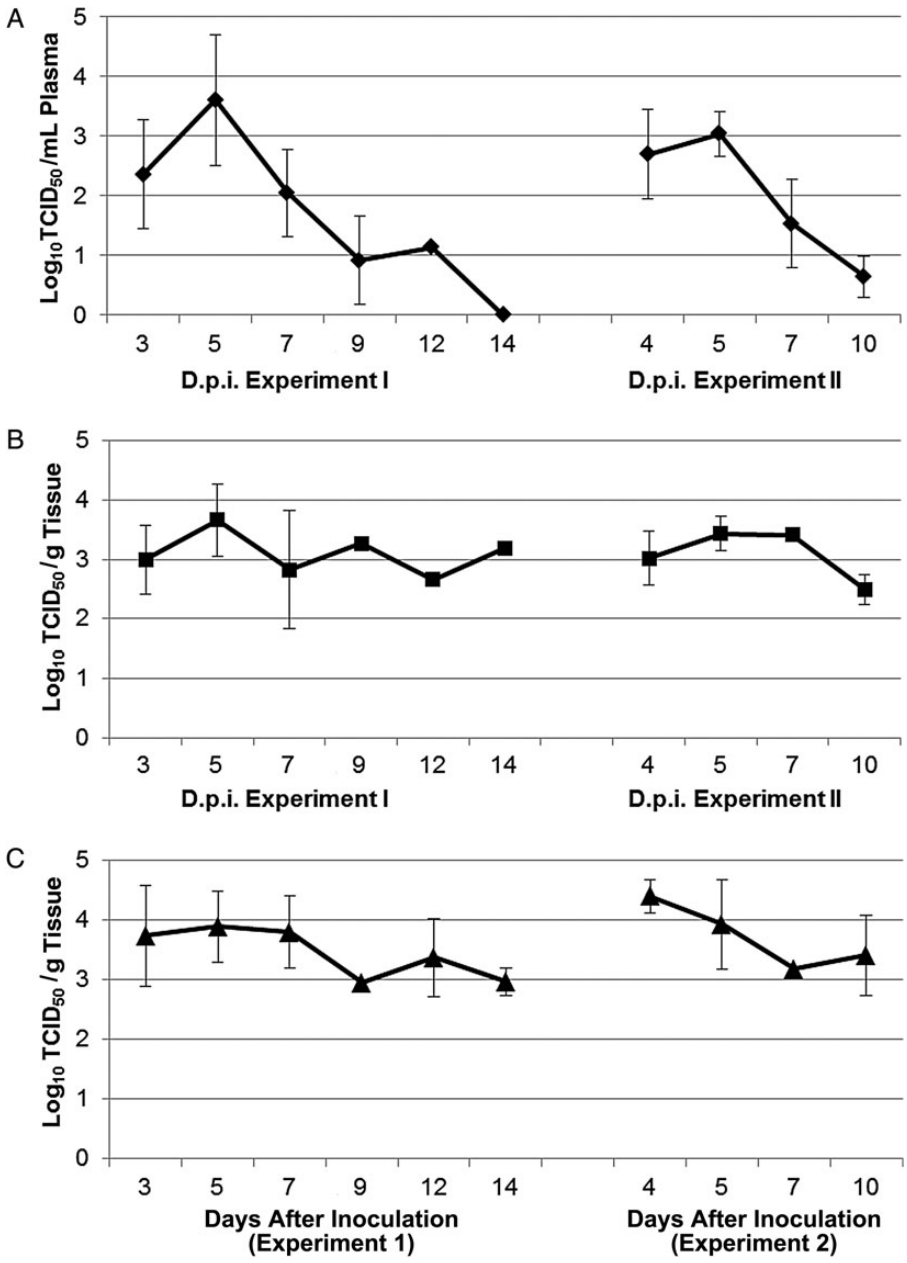

Figure 3. Mean Marburg virus replication levels (with standard deviations) in the plasma (*A*), liver (*B*), and spleen (*C*) of Egyptian fruit bats (*Rousettus aegyptiacus*) inoculated with the virus in experiments 1 (n = 22) and 2 (n = 14). RNA levels measured with quantitative reverse-transcription polymerase chain reaction are given as the equivalent of $\log_{10}$ median tissue culture infective dose ($TCID_{50}$) per milliliter of plasma or gram of tissue.

and 21, in pooled rectal swab samples on day 3, and in individual urine samples on days 7 and 9. The PCR-positive swab samples and urine samples were negative by virus isolation (Table 3).

Experiment 2

All the bats in experiment 2 remained clinically well and maintained normal food uptake, and no gross disease was identified. These bats seroconverted by day 10 after inoculation (Figure 2*B*). Likewise, serological, virological (Table 2 and Figure 3), and histopathological responses were similar to those recorded in experiment 1, confirming their MARV-naive status before and after in-contact exposure to bats infected in experiment 1. MARV RNA was detected in 2 oral swab sample samples on day 10 after inoculation and a single vaginal swab sample on day 4. The PCR-positive swab samples were negative by virus isolation (Table 4).

Kruskal–Wallis 1-way analysis of variance showed no statistically significant differences ($P > .05$) between viral loads in the plasma, livers, and spleens collected from bats at corresponding days after inoculation during the first and the second

Table 3. Quantitative Reverse-Transcription PCR and VI Results in Swab and Urine Samples Collected From Egyptian Fruit Bats (*Rousettus aegyptiacus*) After Subcutaneous Inoculation With Marburg Virus: Experiment 1

	Days After Inoculation[a]						
Sample Type	3	5	7	9	12	14	21
Oral swab	0/3	0/4	1/3 (1.08) VI: 0/1	0/3	0/3	0/2	0/4
Nasal swab	0/3	0/4	0/3	0/3	0/3	0/2	0/4
Vaginal swab	NS	NS	0/3	0/3	0/3	P (0.74); VI: 0/P	P (1.57); VI: 0/P
Penile swab	NS	NS	0/3	0/3	0/3	0/2	0/4
Rectal swab	P (1.44); VI: 0/P	0/4	0/3	0/3	0/3	0/2	0/4
Urine swab	NS	NS	0/3	0/3	0/3	0/2	0/4
Urine	NS	NS	1/1 (0.53); VI: 0/1	1/1 (1.29); VI: 0/1	0/1	0/1	NS

Abbreviations: NS, not sampled; P, pooled sample; PCR, polymerase chain reaction; VI, virus isolation.

[a] Data represent No. of positive samples/No. tested. VI results are given only for PCR-positive tissues, because only these tissues were subjected to VI. Parenthetical values indicate extrapolated $\log_{10}$ median tissue culture infective dose from RNA levels measured with PCR.

experiments. A comparison of the mean viral loads in the plasma, livers, and spleens of bats infected during the first and the second experiments is shown in Figure 3.

Microscopic Examination

The major histopathological and immunohistochemical findings are shown in Figure 4*A*–*H*. In 10 bats examined during the first experiment, the most significant lesions were noted in 7 individuals. Hematoxylin-eosin–stained sections of the livers from these bats showed a multifocal hepatitic process centered on either individual or groups of 2 or 3 hepatocytes, resulting in hepatocelluar necrosis and hepatocyte dropout (Councilman-like bodies). The dominant inflammatory cells were lymphohistiocytic (Figure 4*A*). The inflammatory changes identified in bats euthanized at 3 days after inoculation had progressed in bats euthanized at 7 days, as evidenced by larger inflammatory foci and greater hepatocellular necrosis (Figure 4*B*).

Other changes included mild sinusoidal leukocytosis, characterized by a predominance of lymphocytes and hyperplasia of Kupffer cells. Hepatocellular microvaculoar change, most consistent with glycogen deposition, was also present in 9 of the 10 bats. Positive immunolabeling for anti-MARV antigen in the liver was noted in 4 bats, this being punctuated within the cytoplasm of hepatocytes and sinusoidal mononuclear cells colocalized with hepatic inflammatory aggregates. Punctuate positive immunolabeling was also noted within the cytoplasm of mononuclear cells in the splenic white pulp in 5 bats. Hepatocyte and mononuclear labeling tended to be limited to individualized cells (Figure 4*C*–*H*). Six of the 10 specimens had no immunolabeling identified. No significant pathological changes were identified in other tissues.

In 7 bats examined during the second experiment, the findings were similar to those observed in the bats sampled during the first experiment. Mild lymphoid follicular hyperplasia and prominent macrophages with rare erythrophagocytic cells, located at the periphery of the germinal center and within marginal zones, were noted in 1 of the 7 bats (Figure 4*G*). Positive immunolabeling for anti-MARV antigen was noted in 6 bats. No significant lesions were seen with hematoxylin-eosin staining of liver, lung, spleen, and kidney tissues collected from a negative control that tested negative for anti-MARV immunolabeling.

Table 4. Quantitative Reverse-Transcription PCR and VI Results in Swab Samples and Urine Collected From Egyptian Fruit Bats (*Rousettus aegyptiacus*) After Subcutaneous Inoculation With Marburg Virus: Experiment 2

	Days After Inoculation[a]			
Sample Type	4	5	7	10
Oral swab	0/3	0/2	0/2	2/7 (1.40; 2.19) VI: 0/2
Nasal swab	0/3	0/2	0/2	0/7
Vaginal swab	0/3	1/1 (1.33) VI: 0/1	0/2	0/7
Penile swab	0/3	0/2	0/2	0/7
Rectal swab	1/3	0/2	0/2	0/7
Urine swab	0/3	0/2	0/2	0/7
Urine	0/1	0/1	0/2	0/1

Abbreviations: PCR, polymerase chain reaction; VI, virus isolation.

[a] Data represent No. of positive samples/No. tested. VI results are given only for PCR-positive tissues, because only these tissues were subjected to VI. Parenthetical values indicate extrapolated $\log_{10}$ median tissue culture infective dose from RNA levels measured with PCR.

Experiment 3

In immunologically primed and rechallenged bats, the mean ELISA PP value of 45.79 recorded on day 0 after challenge increased to 104.59 by day 10, indicating an anamnestic humoral response (Figure 2*C*). With the exception of a single positive qRT-PCR result in the spleen on day 4 after challenge (negative by virus isolation), all other tissues tested negative on days 4 and 10 after challenge (results not shown).

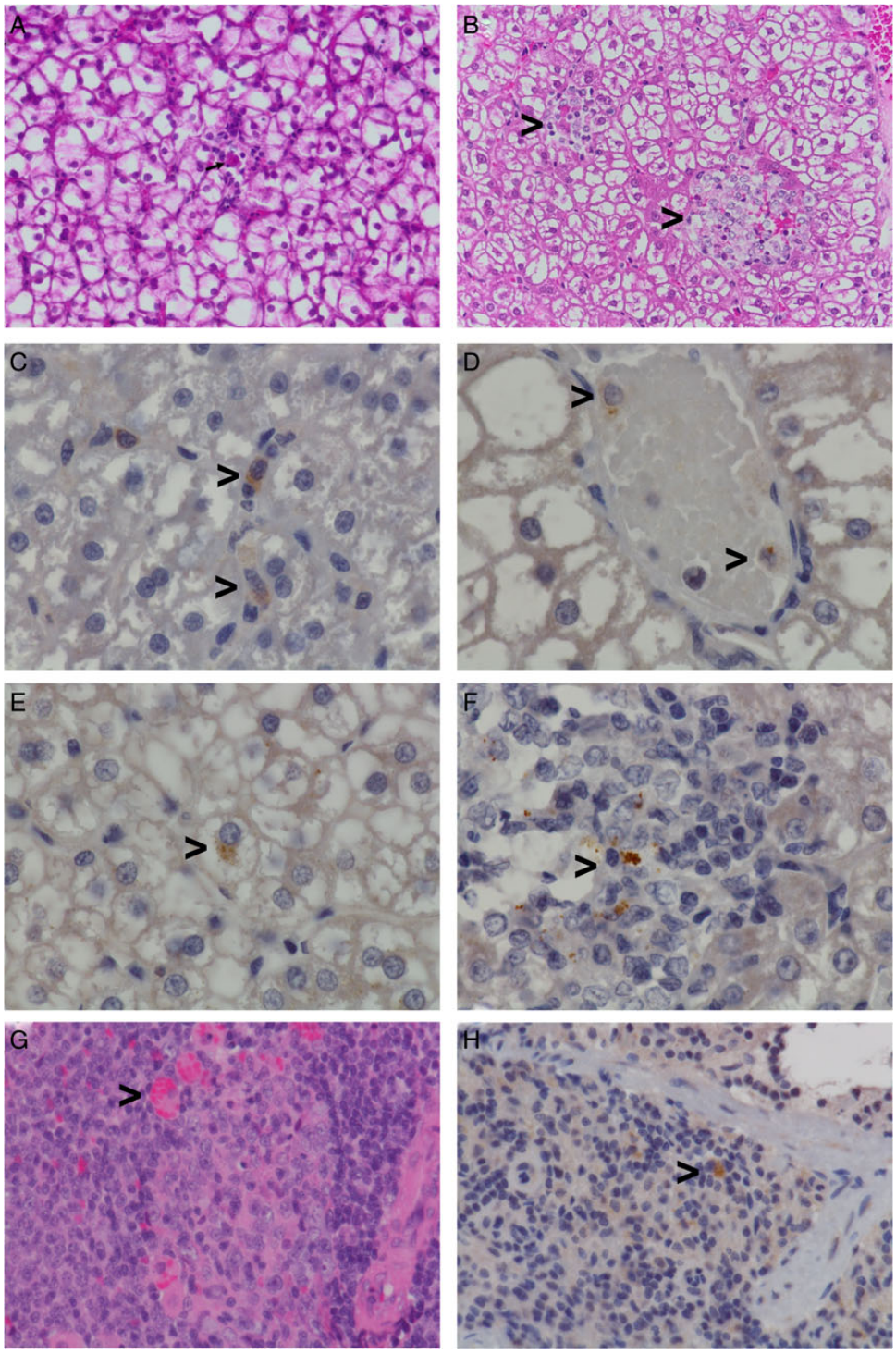

Figure 4. *A–H*, Findings at histopathology (hematoxylin-eosin staining [HE]) and immunohistochemistry (IHC) in Egyptian fruit bats (*Rousettus aegyptiacus*) infected subcutaneously with Marburg virus (MARV). *A, B*, Liver, HE (original magnification ×40). *A*, Section of liver from a bat (bat 32) euthanized on day 5 after inoculation shows a focus of lobular inflammation comprising lymphocytes and histiocytes, with a necrotic hepatocyte (*arrow*) identifiable in its center. *B*, Section of liver from a bat (bat 16) euthanized on day 7 after inoculation shows multiple foci of lobular inflammation exhibiting progression of inflammatory changes associated with the loss of multiple hepatocytes (*arrowheads*). *C–F*, Liver, IHC (original magnification ×100); cytoplasmic immunolabeling for anti-MARV antigen from 2 bats euthanized on day 5 after inoculation. *C,* Mononuclear cells within hepatic sinusoids (*arrowheads*) (bat 25). *D*, Mononuclear cells within hepatic vessels (*arrowheads*) (bat 30). *E*, Hepatocyte (*arrowhead*) (bat 30). *F*, Mononuclear cells colocalized with hepatic inflammatory nodule (*arrowhead*) (bat 30). *G*, Spleen, HE (original magnification ×60); lymphoid follicular hyperplasia and prominent marginal zone macrophages, occasionally erythrophagocytic (*arrowhead*), from a bat (bat 30) euthanized on day 5. *H*, Spleen, IHC (original magnification ×60); cytoplasmic immunolabeling for anti-MARV antigen within mononuclear cells located within splenic white pulp (*arrowhead*), from a bat (bat 30) euthanized on day 5.

DISCUSSION

This study confirms that *R. aegyptiacus* can be consistently infected with MARV by the subcutaneous route without resultant death or overt morbidity. It also evidences the lack of MARV transmission, either by close bat-to-bat contact or via air. Infected *R. aegyptiacus* cleared MARV from all major tissues shortly after seroconversion. The dynamics of immune responses recorded in this study demonstrates that the serological threshold selected for classifying *R. aegyptiacus* as naive to MARV is valid. Except for more intensive oral and fecal shedding and high level of virus replication at the inoculation site, the clinical, serological, and MARV replication patterns in major tissues recorded in our study are similar to those reported by Amman et al [10]. The latter work, however, did not investigate bat-to-bat MARV transmission.

The *R. aegyptiacus* bats inoculated subcutaneously with MARV seroconverted and became viremic, and the virus was detected in various tissues, but animals remained clinically normal and no gross disease was identified. There was evidence of an anamnestic humoral response in the immunologically primed, rechallenged bats, but they did not develop viremia and were protected against virus replication. The lack of overt clinical manifestations is supported by the results of the histopathological examination, which demonstrated only sparsely and localized microscopic lesions in the liver, and no major disease in other tissues.

The regular presence of MARV in the livers and spleens of experimentally infected *R. aegyptiacus* bats correlates with the detection of the virus in tissues of naturally infected bats [2, 4, 7, 8]. MARV replicated to much lower levels in *R. aegyptiacus* compared with other natural and experimental animal models [16–18]. The liver was the only tissue with apparent microscopic lesions, but it was affected to less than described for MARV infection in humans and/or nonhuman primates [11, 19–22]. The lack of severe necrosis in liver parenchymal cells of *R. aegyptiacus* bats is probably due to a low viral load.

Most of the tissues were infectious on days 5 and 7 after inoculation, but liver, spleen, kidney, small intestine, rectum, reproductive organs, and salivary glands were still PCR positive in some individuals on days 9–14. Not all PCR-positive results correlated with virus isolation. This could be because bats by day 12 after inoculation had a high level of IgG, with immune complexes neutralizing and preventing virus replication but with qRT-PCR still able to amplify viral genome fragments.

MARV was detected in tissues that could be implicated in horizontal transmission, (eg, lungs, intestines, kidneys, and bladders), but these did not support virus replication to high titers and for an extended period. Low levels of MARV RNA were sporadically detected in fecal, vaginal, and oral swab samples and in urine samples, but the virus could not be isolated from these specimens, indicating low infectivity.

These findings are supported by unsuccessful horizontal transmission of MARV from infected to susceptible in-contact bats. The roosting behavior of *R. aegyptiacus* means that these bats spend most of the daytime in close contact with other bats. Direct physical contact between infected and susceptible bats was replicated in this study, yet no transmission of MARV occurred. A lack of aerosol transmission could be explained by the transient presence and generally low MARV infectivity in the lungs as well as the absence of the virus in nasal secretions. The previously reported unsuccessful infection of bats by the oronasal route [9] suggests that the respiratory route is not the primary mode of MARV transmission in *R. aegyptiacus* bats.

The duration of viremia in *R. aegyptiacus* after subcutaneous infection suggests the possibility of MARV transmission by hematophagous vectors or by contact with infected blood. We also demonstrated the presence of MARV in the *R. aegyptiacus* male and female reproductive tracts. Both MARV and *Zaire ebolavirus* have been isolated from human seminal fluids months after disease onset and the patient's full clinical recovery [23, 24]. Whether a carrier state can be established in these tissues, at least in some proportion of MARV-infected *R. aegyptiacus* bats, remains unknown.

Our results indicate slower clearance of the virus from salivary glands compared to other tissues tested. The presence of replicating virus in salivary glands might provide insight into another possible mechanism of transmission. Based on observations from sampling of *R. aegyptiacus* bats in the field, there seems to be a fair amount of biting among individuals. Juveniles are the most adversely affected, because as they probably get bitten by older bats displaying dominance.

In conclusion, our results show that close physical contact and the air-borne route are not the primary mechanisms of MARV transmission in *R. aegyptiacus* bats. Although the biology of the *R. aegyptiacus*–MARV association requires further investigations to determine the exact mechanisms of natural transmission cycles, our findings further confirm that entering *R. aegyptiacus* roosting sites and hunting these bats for food carries a danger of infection for humans.

Notes

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Potential conflicts of interest. All authors: No potential conflicts of interest.

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