3-1-2018

Ebola Virus Localization in the Macaque Reproductive Tract during Acute Ebola Virus Disease.

Donna L. Perry  
*National Institute for Allergy and Infectious Diseases, NIH*

Louis M. Huzella  
*National Institute for Allergy and Infectious Diseases, NIH*

John G. Bernbaum  
*National Institute for Allergy and Infectious Diseases, NIH*

Michael R. Holbrook  
*National Institute for Allergy and Infectious Diseases, NIH*

Peter B. Jahrling  
*National Institute for Allergy and Infectious Diseases, NIH*

*See next page for additional authors*

Let us know how access to this document benefits you

Follow this and additional works at: [https://jdc.jefferson.edu/mifp](https://jdc.jefferson.edu/mifp)

Part of the [Infectious Disease Commons](https://jdc.jefferson.edu/mifp)

**Recommended Citation**


[https://jdc.jefferson.edu/mifp/104](https://jdc.jefferson.edu/mifp/104)
SHORT COMMUNICATION

Ebola Virus Localization in the Macaque Reproductive Tract during Acute Ebola Virus Disease

Donna L. Perry,* Louis M. Huzella,* John G. Bernbaum,* Michael R. Holbrook,* Peter B. Jahrling,*† Katie R. Hagen,* Matthias J. Schnell,† and Reed F. Johnson†

Accepted for publication November 16, 2017.

Address correspondence to Donna L. Perry, D.V.M., Ph.D., Integrated Research Facility, National Institute for Allergy and Infectious Diseases, NIH, 8200 Research Plaza, Frederick, MD 21702. E-mail: perrydl@niaid.nih.gov.

Sexual transmission of Ebola virus (EBOV) has been demonstrated more than a year after recovery from the acute phase of Ebola virus disease (EVD). The mechanisms underlying EBOV persistence and sexual transmission are not currently understood. Using the acute macaque model of EVD, we hypothesized EBOV would infect the reproductive tissues and sought to localize the infection in these tissues using immunohistochemistry and transmission electron microscopy. In four female and eight male macaques that succumbed to EVD between 6 and 9 days after EBOV challenge, we demonstrate widespread EBOV infection of the interstitial tissues and endothelium in the ovary, uterus, testis, seminal vesicle, epididymis, and prostate gland, with minimal associated tissue immune response or organ pathology. Given the widespread involvement of EBOV in the reproductive tracts of both male and female macaques, it is reasonable to surmise that our understanding of the mechanisms underlying sexual transmission of EVD and persistence of EBOV in immune-privileged sites would be facilitated by the development of a nonhuman primate model in which the macaques survived past the acute stage into convalescence. (Am J Pathol 2018, 188: 550–558; https://doi.org/10.1016/j.ajpath.2017.11.004)
interstitial or supporting connective tissues of the male and female reproductive organs, with minimal associated tissue immune response or organ pathology.

Twelve macaques, seven rhesus (*Macaca mulatta*; one female and six males) and five cynomolgus (*Macaca fascicularis*; three females and two males), ranging in age from 35 to 58 months, were challenged with 1000 plaque-forming units i.m. of EBOV Makona C05. Macaques were euthanized after meeting end point criteria between 6 and 9 days after EBOV challenge. Each macaque underwent a complete postmortem examination when predetermined end point criteria for EVD were reached. Reproductive tissues were collected from all macaques for routine histology, immunohistochemistry (IHC) for EBOV viral protein 40 (VP40) matrix protein and/or glycoprotein (GP), and transmission electron microscopy examination. Immuno-histochemistry for EBOV VP40 and/or GP was positive in the reproductive organs of all 12 macaques. Transmission electron microscopy of the reproductive tissues was performed in 11 of 12 macaques that succumbed to EVD, and widespread EBOV infection of the stromal connective tissues, including endothelial cells, was observed in all macaques examined. A summation of findings can be found in Table 1.

### Materials and Methods

#### Animal Ethics Statement

This study was performed in strict adherence to the *Guide for the Care and Use of Laboratory Animals* of the NIH, the Office of Animal Welfare, and the US Department of Agriculture. All work was approved by the US National Institute of Allergy and Infectious Diseases, Division of Clinical Research Animal Care and Use Committee, and performed at the US National Institute of Allergy and Infectious Diseases Research Facilities. Procedures were performed after animals had been anesthetized by trained personnel under the supervision of veterinary staff. Food and water were available ad libitum.

#### Animals

This study includes 12 macaques that served as controls in three separate studies that were designed to evaluate the efficacy of a therapeutic agent intended to protect against the development of EVD. The first two experiments are described by Johnson et al. The seven rhesus macaques (*M. mulatta*) and five cynomolgus macaques (*M. fascicularis*) ranged in age from 35 to 58 months, with a

---

**Table 1** Signalment, EBOV in Peripheral Blood, and EBOV Distribution in the Reproductive Tract of Female and Male Macaques

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Macaque species</th>
<th>Age, months</th>
<th>Necropsy after inoculation, days</th>
<th>EBOV vRNA, copy/mL (log10)</th>
<th>EBOV IHC results</th>
<th>TEM results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cynomolgus</td>
<td>46</td>
<td>7</td>
<td>10.6</td>
<td>Stromal/vascular</td>
<td>Stromal/EC</td>
</tr>
<tr>
<td>2</td>
<td>Cynomolgus</td>
<td>53</td>
<td>8</td>
<td>9.96</td>
<td>Stromal/vascular</td>
<td>Stromal</td>
</tr>
<tr>
<td>3</td>
<td>Cynomolgus</td>
<td>53</td>
<td>7</td>
<td>10.1</td>
<td>Stromal/vascular</td>
<td>Stromal/EC</td>
</tr>
<tr>
<td>4</td>
<td>Rhesus</td>
<td>35</td>
<td>7</td>
<td>10.4</td>
<td>Stromal/vascular</td>
<td>Stromal</td>
</tr>
<tr>
<td>5</td>
<td>Cynomolgus</td>
<td>48</td>
<td>6</td>
<td>10.3</td>
<td>Stromal/vascular</td>
<td>Stromal/EC</td>
</tr>
<tr>
<td>6</td>
<td>Cynomolgus</td>
<td>44</td>
<td>8</td>
<td>10</td>
<td>Stromal/vascular</td>
<td>Stromal/EC</td>
</tr>
<tr>
<td>7</td>
<td>Rhesus</td>
<td>44</td>
<td>9</td>
<td>8.83</td>
<td>Stromal/vascular</td>
<td>NP Stromal/EC</td>
</tr>
<tr>
<td>8</td>
<td>Rhesus</td>
<td>48</td>
<td>7</td>
<td>9.78</td>
<td>Stromal/vascular</td>
<td>Stromal/EC</td>
</tr>
<tr>
<td>9</td>
<td>Rhesus</td>
<td>58</td>
<td>8</td>
<td>9.95</td>
<td>Stromal/vascular</td>
<td>Stromal/EC</td>
</tr>
<tr>
<td>10</td>
<td>Rhesus</td>
<td>56</td>
<td>7</td>
<td>10.2</td>
<td>Stromal/vascular</td>
<td>Stromal/EC</td>
</tr>
<tr>
<td>11</td>
<td>Rhesus</td>
<td>35</td>
<td>7</td>
<td>10.1</td>
<td>Stromal/vascular</td>
<td>Stromal/EC</td>
</tr>
<tr>
<td>12</td>
<td>Rhesus</td>
<td>38</td>
<td>6</td>
<td>10.6</td>
<td>Stromal/vascular</td>
<td>NP</td>
</tr>
</tbody>
</table>

EBOV, Ebola virus; EC, endothelial cell; ID, identification; IHC, immunohistochemistry; NP, not performed; TEM, transmission electron microscopy; vRNA, viral RNA.
total of four females (three cynomolgus and one rhesus) and eight males (two cynomolgus and six rhesus). These 12 macaques were randomized to control groups before EBOV i.m. challenge with EBOV (Zaire species) in the lateral head of the right triceps muscle using the Makona C05 EBOV isolate at a target dose of 1000 plaque-forming units. All macaques succumbed to EVD between 6 and 9 days after EBOV challenge.

Blood Collection

Nine pre-EBOV challenge blood draws were performed in each macaque, the last at 15 days before EBOV challenge (0 day). After EBOV challenge, blood draws were performed every other day starting at 2 days until 8 days after EBOV challenge. The last blood draw was performed immediately before necropsy. Complete blood cell counts, select serum chemistries, and real-time quantitative PCR for viremia were performed on these samples.

Viremia by Quantitative RT-PCR

Whole blood was inactivated in TRIzol LS (Thermo Fisher Scientific, Waltham, MA) in accordance with established methods for inactivation of risk group 4 pathogens. Briefly, total RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD) using 700 µL of TRIzol LS inactivated sample that was added to 280 µL of Qiagen Buffer AVL containing carrier RNA. The sample was eluted in 70 µL of Buffer AVE, aliquoted, and frozen until assayed. Whole blood viral load was measured using BEI Resources Critical Reagents Program (Manassas, VA) EZ1 quantitative RT-PCR kit assay, in accordance with manufacturer’s instructions, and reported as viral RNA copies (log10) per mL of sample.

Tissue Processing and Immunohistochemistry

Reproductive tissue collected from females (uterus and ovaries) and males (testes, prostate, seminal vesicles, head, and tail of the epididymides) at necropsy was fixed in 10% neutral-buffered formalin in preparation for routine histopathology and immunohistochemistry. Ebola virus immunohistochemistry was performed on routinely processed tissue with an anti-EBOV VP40 (catalog number 0201-016; IBT Bioservices, Rockville, MD) or anti-EBOV GP antibody (catalog number 0301-015; IBT Bioservices), followed by a biotinylated anti-mouse (catalog number 2.0% uranyl acetate, tissues were dehydrated in a series of graded ethanolos and infiltrated and embedded in Spurr’s plastic resin (E.M. Sciences). Embedded blocks were divided into sections using a Leica UC7 ultramicrotome (Leica, Wetzlar, Germany). Sections (70 to 80 nm thick) were collected on 200 mesh copper grids and poststained with Reynold’s lead citrate. Samples were examined in an FEI Tecnai Spirit Twin transmission electron microscope (FEI Company, Hillsboro, OR), operating at 80 kV.

Results

Ebola Virus Infects Mesenchymal Cells in the Ovary

Histopathological lesions in the ovary secondary to EVD were not observed in the macaques examined. Inclusion bodies could be seen in the mesenchymal thecal cells surrounding secondary and tertiary ovarian follicles. Immunohistochemical staining for EBOV GP in the ovary was multifocally positive in the thecal cells surrounding secondary and tertiary ovarian follicles and in the vascular endothelium in macaques that succumbed to EVD (Figure 1). Immunohistochemical positivity in the granulosa cells was rare. Ultrastructural examination of the ovary confirmed viral infection of ovarian stromal cells and endothelial cells (Figure 1). Fimbrial epithelial cells of the salpinx (Fallopian tube or oviduct) stained positive multifocally for EBOV VP40 and GP (data not shown).

Ebola Virus Infects the Endometrial Stromal Cells and Smooth Muscle Cells of the Myometrium

Histopathological lesions in the uterus secondary to EVD were not observed in the macaques examined. Immunohistochemical staining for EBOV VP40 revealed widespread positivity in the vasculature, endometrial stromal cells, and smooth muscle cells of the myometrium in macaques that succumbed to EVD (Figure 1). Ultrastructural examination...
of the uterus confirmed viral infection of endometrial stromal cells and smooth muscles cells of the myometrium (Figure 1).

Ebola Virus Infects Interstitial Mesenchymal Cells in the Testis

Histopathological lesions in the testis were uncommon but included the presence of spermatid giant cells and oligospermia; however, macaque species are seasonal breeders and sperm density must be interpreted in the context of age, social rank, recent changes in social housing, and photoperiod or season. Uncommonly, thrombosis with infarction of testicular stroma occurred secondary to EVD-induced coagulopathy, resulting in areas of ischemic necrosis of the testicular tissue. Immunohistochemistry for EBOV VP40 and GP in the testis revealed widespread positivity in the interstitial connective tissues, including endothelial cells (Figure 2). Ultrastructural examination of the testis confirmed viral infection of interstitial connective tissues, including endothelial cells (Figure 2).

Ebola Virus Infects Interstitial Mesenchymal Cells in the Accessory Male Sex Glands (Prostate Gland, Seminal Vesicle, and Epididymis)

In the prostate gland and seminal vesicle, histopathological lesions secondary to EVD were not observed, despite widespread immunohistochemical positivity for EBOV VP40 and
GP in the stromal connective tissues (Figures 2 and 3). Epithelial cell positivity for EBOV by IHC in these organs was rare. In the head and tail of the epididymis, histopathological lesions secondary to EVD were nonspecific, but included interstitial edema and, rarely, vascular thrombosis. Similar widespread multifocal positivity for EBOV VP40 and GP was detected in the interstitial connective tissue supporting the tubular epithelium of the epididymis in acutely EVD-affected macaques (Figure 3). Ultrastructural examination of the prostate gland, epididymis, and seminal vesicle confirmed viral infection of interstitial mesenchymal cells, including endothelial cells (Figures 2 and 3). Mature EBOV particles were seen within the disrupted membranes of an expanded endoplasmic reticulum; however, the virus was only observed to bud from the cell surface or plasma membrane. Ebola virus GP is known to accumulate in the endoplasmic reticulum of infected cells, which may partially explain the marked expansion of endoplasmic reticulum membranes observed occasionally in EBOV-infected cells. Within the ductus deferens, epithelial positivity for EBOV VP40 protein was observed (data not shown).

Discussion

Using the macaque model of acute EVD, we have demonstrated early and widespread infection of the male and female reproductive tract after i.m. EBOV challenge. Ebola
virus replication occurs predominantly within the mesenchymal or supporting stromal cells of the reproductive tract. The presence of the virus in the ovary and uterus in the macaque model of EVD has not been reported previously. However, detection of EBOV in the thecal mesenchymal cells surrounding ovarian follicles and IHC positivity associated with inflammation in necrosis in the uterus have been demonstrated in the guinea pig model of EVD. The effect of EBOV infection of thecal cells on the denouement of secondary and tertiary ovarian follicles in macaques is unknown. The effect of EBOV infection of smooth muscle cells in the uterus is also unknown; however, in pregnant women with EVD, an average maternal death rate of 82% and a fetal death rate of 100% have been reported. Ebola virus is able to infect the fetus. In the placenta, fetal-origin syncytiotrophoblasts can be infected with the Sudan and Bundibugyo viruses. The only known congenitally infected infant to survive EBOV infection was treated on 2, 5, 8, and 11 days of life with the monoclonal antibody cocktail, ZMapp, and received an i.v. transfusion of leukocytes from an EBOV survivor on day 11 of life; beginning on day 19 of life, this infant was treated for 12 days with the nucleotide prodrug, GS-5734. Ebola virus is shed in breast milk, and epidemiologically linked transmission from an asymptomatic mother to a 9-month-old child through breast milk has been reported. The presence or absence of EBOV persistence in women is understudied, as are the effects of EVD in pregnant women and their offspring.

Figure 3  Epididymis (A, C, and E) and seminal vesicle (B, D, and F). A: Section of epididymis immunohistochemically (IHC) stained for Ebola virus (EBOV) viral protein 40 (VP40) matrix protein, shows multifocal positive cytoplasmic staining (brown) of the interstitial mesenchymal cells of the epididymis. B: Section of seminal vesicle IHC stained for EBOV VP40 matrix protein shows multifocal cytoplasmic positivity (brown) in the mesenchymal cells of the seminal vesicle. C and E: The black boxed area in A reveals the anatomical orientation seen in transmission electron micrographs (TEMs). C: Four intracytoplasmic EBOV inclusion bodies are present within mesenchymal cells adjacent to the basement membrane (BM) of the epididymal epithelium (Ep). E: Higher magnification of the red boxed area in C. Filamentous EBOV nucleocapsid proteins form an intracytoplasmic inclusion body in an interstitial mesenchymal cell adjacent to the BM of the Ep. D and F: The black boxed area in B represents the anatomical orientation of the TEMs. D: An intracytoplasmic EBOV inclusion body is present in a mesenchymal cell adjacent to the BM of the Ep of the seminal vesicle. F: Higher magnification of the red boxed area in D. An intracytoplasmic EBOV inclusion body demonstrates EBOV nucleocapsid formation in longitudinal and cross section [in cross section appearing as crystalline arrays (CAs)] adjacent to the cell nucleus (top left). Scale bars: 200 μm (A and B); 2 μm (C and D); 500 nm (E and F).
In men, temporal evidence and sequence analysis of EBOV samples from the 2014 to 2016 West Africa outbreak demonstrated sexual transmission of EBOV to women from 6 months to >1 year after recovery from EVD.7,8 A recent report from Fischer et al described the longest interval, 965 days, between onset of EVD and the detection of EBOV RNA in semen. The prevalence of detection was significantly higher in older men (median age, 41.8 years), and detection was intermittent. On the basis of our findings, we hypothesize that persistence is established in the interstitial tissues of the reproductive tract and tissue macrophages are the route by which EBOV is transmitted from stromal replication sites in the seminal vesicle, epididymis, prostate gland, and testis into the seminal fluid. Because this study used tissue collected adjunctively at necropsy from ongoing studies, the collection and examination of seminal fluid was not possible, and no fresh tissue was collected for RNA sequencing.

Infection of monocytes and macrophages facilitates systemic spread of the EBOV but neutrophils and B, T, and natural killer cells, critical for both the innate and humoral immune response, are not infected.26 Although we have demonstrated active EBOV infection of endothelial cells in reproductive tissues, endothelial cells exhibit a high degree of heterogeneity, and endothelial cell infection within one tissue type likely does not translate to permissivity of viral infection in another.27 Although the macaque model of human EVD is thought to have a high fidelity, it is unknown if the distribution of the virus is similar in the reproductive tract of affected humans or why sexual transmission in convalescent human populations is rare. Martines et al reported positivity for EBOV antigens by IHC in testicular endothelium and focally within the seminiferous tubular epithelium.9,10 Although several RNA viruses that establish persistence in infected hosts may provide valuable insight into EBOV persistence,55,56 the production of defective interfering particles.39,40 A viral RNA that binds circulating neutralizing antibodies directs against EBOV GP on cell surfaces.38 In vitro, EBOV establishes noncytolytic persistent infections through the production of defective interfering particles.39,40 A nonhuman primate EBOV survival model would be required to study the host immunomodulatory mechanisms that facilitate the development of EBOV persistence in immune-privileged sites and to determine how this highly pathogenic virus attenuates itself to persist in host cells.

Acknowledgments

We thank Amanda Bonilla and Randy Hart for assisting with tissue collection and performing the immunohistochemistry on the tissues examined; Jiro Wada for assisting with the generation of the image plates; Dr. Timothy Cooper and David Liu for critically reviewing the manuscript; Lisa E. Hensley, Marisa St. Claire, Danny Ragland, Russ Byrum, Kurt Cooper, and the entire Emerging Viral Pathogens Section and Integrated Research Facility staff for contributions to these studies.

References


