Role of Key Infectivity Parameters in the Transmission of Ebola Virus Makona in Macaques

Marc-Antoine de La Vega,¹ Gary Wong,² Haiyan Wei,² Shihua He,² Alexander Bello,¹ Hugues Fausteur-Bovendo,¹ Jonathan Audet,² Kevin Tierney,² Kaylie Tran,² Geoff Soule,³ Trina Racine,³ James E. Strong,³ Xiangguo Guo,¹ and Gary P. Kobinger¹,2

¹Département de microbiologie-infectiologie et d’immunologie, Université Laval, Québec, Québec, Canada, ²Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada, ³Institute of Infectious Disease, Henan Center for Disease Control and Prevention, Zhengzhou, Henan, China, ⁴Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, and ⁵Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Many characteristics associated with Ebola virus disease remain to be fully understood. It is known that direct contact with infected bodily fluids is an associated risk factor, but few studies have investigated parameters associated with transmission between individuals, such as the dose of virus required to facilitate spread and route of infection. Therefore, we sought to characterize the impact by route of infection, viremia, and viral shedding through various mucosae, with regards to intraspecies transmission of Ebola virus in a nonhuman primate model. Here, challenge via the esophagus or aerosol to the face did not result in clinical disease, although seroconversion of both challenged and contact animals was observed in the latter. Subsequent intramuscular or intratracheal challenges suggest that viral loads determine transmission likelihood to naive animals in an intramuscular-challenge model, which is greatly facilitated in an intratracheal-challenge model where transmission from challenged to direct contact animal was observed consistently.

Keywords: Ebola virus; rhesus macaque; route of infection; transmission; viral load.

Although a considerable number of advances have been made regarding specific prophylactic and therapeutic options against Ebola virus (EBOV) [1], as well as post-EBOV disease syndrome [2, 3], various aspects of pathogenesis and transmission remain to be defined. For example, the term “superspreader” was widely used during the West African outbreak and defines contagious individuals who go on to infect a high number of contacts, resulting in multiple secondary infections [4, 5]. Although numerous environmental and behavioral factors can partially account for superspreading events, it has been hypothesized that these individuals can shed higher amounts of virus and/or for an extended period of time, thereby facilitating infection of others [6]. Whether this is virus-dependent, host-dependent, or both remains to be clarified. Detection of EBOV viral RNA from humans has been described in blood, saliva, urine, aqueous humor, breast milk, semen, stool, and amniotic and cerebrospinal fluid, as well as conjunctival, vaginal and skin swabs, whereas laboratory culture of these samples only reported the presence of infectious particles in the first 6 types of samples [7]. Viral shedding has also been characterized in various animal models following EBOV infection including guinea pigs, ferrets, pigs, and nonhuman primates (NHPs). Shedding was found to increase with disease progression and has been reported from the oral, nasal, and rectal cavities in all 4 models [8–11]. However, the role of key infectivity parameters, such as dose and route of infection, are not well understood. A study in guinea pigs has shown that animals infected intranasally with guinea pig–adapted EBOV (GA-EBOV) were more contagious to their naive counterparts compared with animals that were infected intraperitoneally. Indeed, intranasally infected animals shed GA-EBOV from their nasal cavity earlier than intraperitoneally infected animals and had a delayed time to death, prolonging the exposure of naive animals [8]. This suggests that, in this model at least, route of infection and time of exposure are factors that may influence disease progression and viral transmission. However, current small animal models, which include mice, hamsters, and guinea pigs, poorly mimic clinical Ebola virus disease (EVD) in humans. These hosts also require infection with a host-adapted variant of EBOV. As such, timely evaluation of novel EBOV isolates can be problematic in rodent models [12]. Adaptation of the virus to the host also generates mutations, a bias toward its naturally occurring counterpart. The NHP remains the most biologically relevant animal model for pathogenesis studies due to its ability to replicate most human hallmarks of EVD and virus adaptation is not...
required. Infection of NHPs with EBOV in the laboratory has mostly been performed by intramuscular (i.m.) injection. Currently, it is controversial whether these i.m.-infected animals are contagious to their naive counterparts without direct contact. Although i.m. infection of rhesus NHPs with EBOV-Mayinga has been reported to have resulted in the infection of naive rhesus animals in the absence of contact [13], a more recent study did not observe transmission of EBOV-Kikwit from i.m.-inoculated rhesus to cynomolgus macaques [14].

In the current study, we sought to characterize the role of viral load, shedding, and route of infection in the likelihood of intraspecies EBOV transmission, within the context of a rhesus macaque model. A series of independent studies was carried out, in which experimentally challenged animals were inoculated via the intraesophageal, aerosol, i.m., or intratracheal (i.t.) routes, and then placed in direct contact with naive animals. Viremia and viral shedding were monitored throughout the course of the experiments and transmission events were recorded to characterize virus spread.

METHODS

Ethics Statement
The experiments described in this study were carried out at the National Microbiology Laboratory as described in the animal use document number H-14-011, and were approved by the Animal Care Committee of the Canadian Science Center for Human and Animal Health, in accordance with the guidelines provided by the Canadian Council on Animal Care.

Viruses
The virus used for challenge in NHPs was passage 1A of Ebola virus/Homo sapiens-wt-GIN/2014/Makona-C05 (EBOV-Makona; GenBank accession number KT013254; order Mononegavirales, family Filoviridae, species Zaire ebolavirus).

Animal Studies
A total of 24 NHPs (rhesus macaques [Macaca mulatta]) were used. The 6 animals used for the aerosol and the first i.m. challenge experiments were purchased from Primus Bio-Resources Inc and were males weighing between 3.6 and 6.0 kg. The 18 animals used for the intraesophageal, repeat i.m., and i.t. challenge experiments were purchased from PrimGen and included both males and females, weighing between 3.1 and 4.3 kg. Animals were fed standard monkey chow, fruits, vegetables, and treats ad libitum. NHPs were challenged either i.m., intraesophageally, i.t., or by aerosol with a targeted dose of 1000 × median tissue culture infectious dose (TCID₅₀) of EBOV-Makona. The virus was prepared in Dulbecco’s modified Eagle medium (DMEM) for all challenges. The animals were then scored daily for observable signs of disease, in addition to changes in food and water consumption. All challenges and sampling were performed following i.m. injection of 6–8 mg/kg of ketamine. Blood was taken for serum biochemistry, complete blood counts, and quantification of viremia. Oral, nasal, and rectal swabs were taken to quantify levels of virus shedding. Aerosol challenge was performed using an in-house nebulizer inside a biosafety cabinet. In brief, the nebulizer was attached into 1 end of a tube about 10 cm in diameter and 30 cm in length, with a breathing mask on the other end, which covered the mouth and nose of NHPs. Animals were given 2 mists of 500 µL each at 2.5-minute intervals in the presence of continuous oxygen. After 5 minutes, the face of the animals was wiped down with a towel sprayed with 70% ethanol. Animals challenged i.m. were given 1 injection of 500 µL in each thigh while intraesophageal and i.t. challenges were performed using a tracheal tube. In brief, the sedated animals were laid on their back, and the tube was inserted about 15 cm, either in the esophagus or the trachea, using a laryngoscope. The 4-mL virus inoculum was then slowly added in the tube via the use of a syringe. Due to logistical constraints in the number of available cages inside the biosafety level 4 laboratory, challenged animals had to be pair-housed with contact animals on the same day of challenge.

EBOV Titration by TCID₅₀ and Reverse-Transcription Quantitative Polymerase Chain Reaction
Titration of live EBOV was determined by adding 100 µL of 10-fold serial dilutions of whole blood or swab sample, in DMEM, to VeroE6 cells, with 3 replicates per dilution. The plates were scored for cytopathic effect at 13 days postinfection (dpi), and titers were calculated with the Reed-Muench method. For titers measured by reverse-transcription quantitative polymerase chain reaction (RT-qPCR), total RNA was extracted from whole blood or DMEM from swab samples with the QIAamp Viral RNA Mini Kit (Qiagen). EBOV was detected with the LightCycler 480 RNA Master Hydrolysis Probes (Roche) kit, with the RNA polymerase (nucleotides 16472 to 16538, AF086833) as the target gene. The reaction conditions were as follows: 63°C for 3 minutes, 95°C for 30 seconds, and cycling of 95°C for 15 seconds, 60°C for 30 seconds for 45 cycles on the ABI StepOnePlus. The lower detection limit for this assay is 86 genome equivalents (GEQ)/mL. The sequences of primers used were as follows: EBOVLF2 (CACCGCAGCAATTTCTTCCAT), EBOVLR2 (TTTCGGTTGCTGTTTCTGTG), and EBOVL2FAM (FAM-ATCATTGGCGGCTACGAGGACAG-BHQ1).

Enzyme-Linked Immunosorbent Assay
Immunoglobulin M (IgM) and immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) to determine preexisting antibodies against EBOV-Makona was performed as described previously [15], using EBOV-GPΔTM (IBT BioServices) as a capture antigen. Each sample was assayed in triplicate. A titer was considered to represent a positive result if the average value was 7.733 standard deviations above background.
RESULTS

Intraesophageal Challenge Results in Neither Disease Nor Seroconversion
To investigate virus replication and transmission likelihood after ingestion of EBOV, 3 NHPs (A1–A3) were experimentally infected intraesophageally with a target dose of $1000 \times \text{TCID}_{50}$ of EBOV-Makona. Each of these animals was then co-housed with a naive animal (A4–A6) immediately after infection to assess the transmission potential of the virus. At 28 dpi, none of the challenged or contact animals succumbed to infection or developed any clinical signs of disease. Furthermore, none of the animals became viremic or seroconverted, as assessed by the absence of viral loads, and anti-EBOV IgM and IgG antibodies (Supplementary Table 1). This suggests that gastric exposure does not facilitate infection with EBOV in macaques.

Facial Aerosol Exposure With EBOV in NHPs Resulted in Subclinical Infection
To assess the impact of aerosol exposure to the face on EBOV transmissibility, animals B1, B2, and B3 were challenged via a spray to the face with a target dose of $1000 \times \text{TCID}_{50}$ of EBOV-Makona. Following inoculation, their faces were wiped, the animals were pair-housed with a naive animal (B4, B5, and B6, respectively), and all were monitored for survival and clinical signs. Surprisingly, challenged animals did not succumb to infection or demonstrate any observable signs of disease (Supplementary Figure 1). Viremia was not detected by RT-qPCR, raising the possibility that the aerosolization process to the face may not be efficient for delivering virus to a susceptible host. Interestingly, EBOV-specific IgM and IgG were detected from all animals by 21 dpi. For IgM, all challenge and contact animals exhibited seropositivity to EBOV-Makona, with endpoint titers peaking between $3 \times 10^3$ and $1 \times 10^5$. For IgG, the challenged animal B1 along with contact animals B4 and B6 were shown to be seropositive for EBOV, with endpoint dilution titers ranging from $1 \times 10^4$ to $3 \times 10^5$ (Figure 1). These results indicate that an infection with EBOV may have occurred, resulting in antibody seroprevalence, but clinical signs (if any) were subclinical. As such, evaluating transmission was difficult with facial aerosol challenge.

High Viral Loads, but not Preexisting Immunity, Impact EBOV Transmission
The goal of these pilot experiments was to evaluate routes of EBOV infection that are more commonly encountered in a natural outbreak setting [16]. However, symptomatic disease could not be easily achieved in these animals and therefore more typical routes known to cause clinical EVD were

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Figure 1. Humoral response of challenged and contact nonhuman primates challenged in the context of aerosol delivery of Ebola virus (EBOV)–Makona. Endpoint titers of immunoglobulin M (IgM, A) and immunoglobulin G (IgG, B) antibodies against the glycoprotein of EBOV throughout the course of the experiment are shown.
investigated. To this end, the same animals from the facial aerosol challenge were reused, as none of them succumbed to challenge nor presented clinical manifestations. At 21 dpi of the facial aerosol challenge experiment, animals that were in the contact group (B4*, B5*, and B6*) were challenged i.m. with a target dose of $1000 \times \text{TCID}_{50}$ whereas animals that were challenged in the previous experiment (B1*, B2*, and B3*) became the direct contact group (Supplementary Figure 2). The challenged NHPs all succumbed to infection at 7 or 8 dpi, despite detection of preexisting immunity against EBOV in B4* and B6*, which developed following mucosal exposure to EBOV (Figure 2). Regarding contact animals, B2* and B3* both succumbed throughout the course of the experiment; however, B2* did not have detectable viremia at the time of death. Since symptoms in this animal started following anesthesia and were not consistent with EVD, the cause of death was attributed to an unknown cause, possibly an adverse event due to the anesthetic procedure. As for B3*, it succumbed to EVD on 14 dpi, thus the timeline is consistent with this animal being infected by its terminally ill cagemate (B6*). Interestingly, B6*, which was the only animal to transmit EBOV to its contact cagemate, displayed the highest viremia and viral shedding. Indeed, this animal exhibited a peak viremia of $>1 \times 10^8 \text{TCID}_{50}$/mL, which was over the limit of detection of the assay ($2.8 \times 10^7 \text{GEQ}/mL), while the other macaques did not exceed $6.8 \times 10^7 \text{TCID}_{50}$/mL ($7.3 \times 10^5 \text{GEQ}/mL) (Figure 3A; Supplementary Figure 3A). Viral shedding through the oral, nasal, and rectal cavities followed a similar trend, in which the transmitting challenged animal exhibited peak shedding of $0, 1.5 \times 10^1, \text{and } 1.5 \times 10^1 \text{TCID}_{50}$/mL ($7.6 \times 10^3, 5.7 \times 10^3, \text{and } 8.2 \times 10^3 \text{GEQ}/mL), respectively, whereas nontransmitting challenged animals peaked at an average of $1.6 \times 10^2, 7.3 \times 10^0, \text{and } 0 \text{ TCID}_{50}$/mL ($5.2 \times 10^4, 5.2 \times 10^4, \text{and } 1.8 \times 10^4 \text{GEQ}/mL), respectively (Figure 3B–D; Supplementary Figure 3B–D). The contact NHP (B1*) that survived exposure to its infected cagemate was coincidentally the animal exhibiting the highest levels of preexisting immunity, as measured by endpoint IgG titers (Figure 1B). However, due to the low number of animals used and because preexisting immunity may have interfered with our hypothesis that high viral shedding positively influences viral transmission rates, a repeat experiment was necessary.

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**Figure 2.** Survival and clinical parameters of challenged and contact nonhuman primates in the context of intramuscular delivery of the Makona variant of Ebola virus in animals exhibiting preexisting immunity. A, Survival. B, Clinical score. C, Temperature. D, Body weight percentage change.
To this end, 3 additional naive NHPs (C1, C2, and C3) were infected i.m. with a target dose of 1000 × TCID\textsubscript{50} of EBOV-Makona. All animals died from infection on 6, 7, and 8 dpi, respectively. These animals were found to be viremic, shed virus, and displayed clinical symptoms typical of EVD (Supplementary Figure 4). Immediately following infection, each challenged animal was paired with a naive NHP (C4, C5, and C6, respectively) to evaluate transmission. All 3 contact animals not only survived for the duration of the experiment, but they also did not become viremic or seroconvert (Supplementary Figure 5). The lack of transmission in the absence of preexisting immunity suggests that high viral loads in the blood from an i.m. exposure do not necessarily lead to transmission. In the previous i.m.-challenge experiment, the transmitting animal reached a peak viremia of >1 × 10\textsuperscript{8} TCID\textsubscript{50}/mL (2.8 × 10\textsuperscript{7} GEQ/mL), while peak oral, nasal, and rectal shedding were 0, 1.5 × 10\textsuperscript{5}, and 1.5 × 10\textsuperscript{5} TCID\textsubscript{50}/mL (7.6 × 10\textsuperscript{4}, 5.7 × 10\textsuperscript{5}, and 8.2 × 10\textsuperscript{5} GEQ/mL), respectively. In the second experiment, the peak viremia of C1 and C2 was similar to those of NHPs from the first i.m. experiment, >1 × 10\textsuperscript{8} and 3.16 × 10\textsuperscript{7} TCID\textsubscript{50}/mL (average of 1.76 × 10\textsuperscript{7} GEQ/mL), respectively, while C3 failed to reach similar levels (3.16 × 10\textsuperscript{7} TCID\textsubscript{50}/mL; 6.83 × 10\textsuperscript{6} GEQ/mL) (Figure 4A; Supplementary Figure 6A). Furthermore, viral secretions from the oral, nasal, and rectal cavities in challenged animals were not nearly as high as those from the previous experiment. Indeed, average peak shedding for challenged NHPs in the second experiment was 1.1 × 10\textsuperscript{2} TCID\textsubscript{50}/mL (2.4 × 10\textsuperscript{3} GEQ/mL) for oral, 0 TCID\textsubscript{50}/mL (3.6 × 10\textsuperscript{3} GEQ/mL) for nasal, and 0 TCID\textsubscript{50}/mL (1.2 × 10\textsuperscript{3} GEQ/mL) for rectal swabs (Figure 4B–D; Supplementary Figure 6B–D). This suggests that viral loads including from mucosal shedding determine transmission likelihood in an i.m.-challenge model.

**Figure 3.** Viremia and shedding from challenged and contact nonhuman primates in the context of intramuscular delivery of the Makona variant of Ebola virus in animals exhibiting preexisting immunity. Viral loads are measured by median tissue culture infectious dose (TCID\textsubscript{50})/mL in blood (A), oral swabs (B), nasal swabs (C), and rectal swabs (D). The horizontal line in panel A represents a sample that was still positive at the upper limit of the assay.

**Intratracheal Infection in NHPs Leads to Efficient Transmission of EBOV-Makona**

To investigate lung involvement in the context of transmission, 3 naive NHPs (D1, D2, and D3) were infected via the i.t. route with a target dose of 1000 × TCID\textsubscript{50} of EBOV-Makona. All animals exhibited preexisting immunity. After challenge, these animals were individually co-housed with a naive NHP (D4, D5, and D6, respectively) to assess transmission. The challenged animals were found to be viremic, shed virus, and displayed clinical symptoms typical of EVD (Supplementary Figure 7). Immediately following infection, each challenged animal was paired with a naive NHP (D4, D5, and D6, respectively) to evaluate transmission. All 3 contact animals not only survived for the duration of the experiment, but they also did not become viremic or seroconvert (Supplementary Figure 8). The lack of transmission in the absence of preexisting immunity suggests that high viral loads in the blood from an i.t. exposure do not necessarily lead to transmission. In the previous i.t.-challenge experiment, the transmitting animal reached a peak viremia of >1 × 10\textsuperscript{8} TCID\textsubscript{50}/mL (1.38 × 10\textsuperscript{7} GEQ/mL), while peak oral, nasal, and rectal shedding were 0, 1.5 × 10\textsuperscript{5}, and 1.5 × 10\textsuperscript{5} TCID\textsubscript{50}/mL (7.6 × 10\textsuperscript{4}, 5.7 × 10\textsuperscript{5}, and 8.2 × 10\textsuperscript{5} GEQ/mL), respectively. In the second experiment, the peak viremia of D1 and D2 was similar to those of NHPs from the first i.t. experiment, >1 × 10\textsuperscript{8} and 3.16 × 10\textsuperscript{7} TCID\textsubscript{50}/mL (average of 1.76 × 10\textsuperscript{7} GEQ/mL), respectively, while D3 failed to reach similar levels (3.16 × 10\textsuperscript{7} TCID\textsubscript{50}/mL; 6.83 × 10\textsuperscript{6} GEQ/mL) (Figure 4A; Supplementary Figure 6A). Furthermore, viral secretions from the oral, nasal, and rectal cavities in challenged animals were not nearly as high as those from the previous experiment. Indeed, average peak shedding for challenged NHPs in the second experiment was 1.1 × 10\textsuperscript{2} TCID\textsubscript{50}/mL (2.4 × 10\textsuperscript{3} GEQ/mL) for oral, 0 TCID\textsubscript{50}/mL (3.6 × 10\textsuperscript{3} GEQ/mL) for nasal, and 0 TCID\textsubscript{50}/mL (1.2 × 10\textsuperscript{3} GEQ/mL) for rectal swabs (Figure 4B–D; Supplementary Figure 6B–D). This suggests that viral loads including from mucosal shedding determine transmission likelihood in an i.t.-challenge model.
succumbed to infection 7, 8, and 7 dpi, respectively, and all 3 contact animals also succumbed to infection 16, 14, and 15 dpi, displaying a typical EVD clinical profile (Supplementary Figure 7). Viremia in challenged animals at the time of death were all above the $1 \times 10^8$ TCID$_{50}$/mL limit of the assay (1.7 $\times$ 10$^7$ GEQ/mL), while peak oral, nasal, and rectal shedding averaged 1.1 $\times$ 10$^3$, 2.3 $\times$ 10$^2$, and 4.9 $\times$ 10$^2$ TCID$_{50}$/mL (6.7 $\times$ 10$^2$, 3.1 $\times$ 10$^3$, and 3.3 $\times$ 10$^4$ GEQ/mL), respectively. Interestingly, viremia from all transmitting animals of the i.t. experiment reached similar levels to that of the transmitting animal from the first i.m. experiment, while shedding was higher regarding the oral (D2), nasal (D3), or rectal (D1) routes (Figure 5; Supplementary Figure 8). While PCR data were lower regarding shedding, live virus titration suggest that an i.t. challenge facilitated viral excretion through oral, nasal, and rectal mucosae, which potentially favored transmission in this context.

**DISCUSSION**

During the West African epidemic, health workers directly involved with care of EBOV patients but without direct contact were, oddly, found to have also been infected, suggesting that either decontamination procedures were not strictly adhered to or that certain factors resulted in opportunities for virus transmission. Early in the outbreak, many first responders and members of the public raised concerns regarding EBOV transmission resulting from eating or drinking from the same plate or glass as an ill family member. Here, we demonstrate that intraesophageal infection with EBOV is unlikely to result in disease or seroconversion, most likely due to the highly acidic content of the stomach [17]. However, this experiment did not account for the possibility of viral entry through the buccal cavity. Others have shown that oral or conjunctival challenge of NHPs with 158 000 plaque-forming units (PFUs) of EBOV-Mayinga resulted in a lethal infection for 75% and 100% of NHPs [18], while a recent study could achieve illness or lethal infection following inoculation by either route with 100 PFUs of EBOV-Makona, but not with 10 PFUs [19]. This suggests that these routes may require higher doses of EBOV to induce EVD, or that differences between EBOV-Makona and EBOV-Mayinga...
It would be interesting to investigate whether higher doses of EBOV could result in EVD in an intraesophageal challenge, in order to understand whether infection of the digestive tract is limited to the buccal cavity. Finally, others have shown that intranasal infection using an atomization device of NHPs with EBOV-Kikwit resulted in a delayed time to death\textsuperscript{20}. It will be interesting to evaluate whether robust transmission can be achieved using this system, as it was previously shown in guinea pigs that transmission of GA-EBOV was dependent on the length of contact exposure. However, similar to our study, the small number of animals used for each type of challenge limits our ability to draw any definite conclusions about transmission.

One limitation of the facial aerosol exposure experiment is that availability of cages inside the biosafety level 4 laboratory was insufficient to allow challenged NHPs to be separated from contact animals for a buffer period, in order to ensure that no virus used during challenge remained on the face of animals, which could be passed on to contact cagemates. This experimental limitation is supported in the current study by serological data showing that contact animals of this particular challenge route developed antibodies at similar timepoints as challenged animals, suggesting that contacts may have been exposed to viral antigens by the remaining virus from the face of the challenged animal, rather than a transmission event following disease development. Although this experimental design may have affected result interpretation, it is most likely closer to the reality of a natural outbreak setting. Indeed, individuals infected throughout the course of an epidemic are more often than not unaware of the moment and route through which they were infected; therefore, it is fair to assume that newly infected individuals do go about their day at work and with their family and friends without self-isolating immediately after an unnoticed exposure.

Previous work has shown that EBOV-Makona, isolate C05, is more virulent than EBOV-Kikwit in rhesus macaques, as evidenced by the higher viremia following an i.m. challenge. EBOV-Makona also demonstrated a higher affinity for the lungs, as shown by the...

Figure 5. Viremia and shedding from challenged and contact nonhuman primates in the context of intratracheal delivery of the Makona variant of Ebola virus in naive animals. Viral loads are measured by median tissue culture infectious dose (TCID\textsubscript{50})/mL in blood (A), oral swabs (B), nasal swabs (C), and rectal swabs (D). The horizontal line in panel A represents a sample that was still positive at the upper limit of the assay. The dotted line represents values obtained for the transmitting animal in the initial intramuscular challenge with animals exhibiting preexisting immunity.
enhanced lung pathology in some NHPs [10]. A previous study in guinea pigs has also shown that the length of exposure time to EBOV plays a bigger role than the exposure dose during successful EBOV transmission [8]. It will be interesting to investigate in NHPs whether the transmission of EBOV is possible without direct contact between the infected and naive contact animals.

These results also demonstrate that subclinical infection with EBOV can be achieved with rhesus macaques in the laboratory, and that this may be dependent on route of infection. Indeed, results obtained in the facial aerosol exposure study have shown that even the contact animals were seropositive, with animal B3 even displaying transient viral loads by RT-qPCR on the last day of this particular challenge. This means that infected animals were definitely exposed with live virus and may have shed low levels of virus that infected contact animals. Previous work by our group has shown, in the guinea pig and ferret models, that seroconversion, but not disease, can occur over short distances without direct contact in naive animals. Interestingly, high prevalence of asymptomatic infection with EBOV has been previously noted from a large-scale study in Gabon. Of 4349 individuals from 220 randomly selected villages, 15.3% were found to be seropositive to EBOV by IgG ELISA, which raises the possibility that these people were possibly previously exposed to the virus via a route such as the mucosa, which led to production of antibodies but without severe clinical disease and death. Leroy and colleagues have also shown that, in humans, asymptomatic infection is possible and these individuals replicate EBOV at very low levels, necessitating a 2-round PCR in peripheral blood mononuclear cells to detect viral RNA [21].

The results of this study show that the induced preexisting antibodies were not always sufficient to protect against EBOV-Makona. Consistent with a past study, nonsurvivors of EVD demonstrated low levels of EBOV-specific IgG antibodies [22]. This again suggests that the quantity and possibly quality of the antibody response is an important factor in predicting survival from EVD.

While the exact mechanisms behind virus transmission from infected to contact animals remain to be fully elucidated, the findings from these studies have substantial implications for EBOV outbreaks, as survivors of EVD, whether from fluid replenishment combined with other supportive therapies or an experimental treatment such as mAb114, REGN-EB3, or ZMapp, may still be susceptible to reinfection if the IgG antibody levels are suboptimal. Antibody levels in vaccine recipients need to be checked over time as well to ensure that immunity is sustained against EBOV. The in-depth characterization of EBOV-Makona will allow us to understand the differences between this novel, divergent virus and its phylogenetic cousins, as well as aid in the effective management and termination of future outbreaks.

Supplementary Data
Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

**Author contributions.** M.-A. D. L. V., G. W., and G. K. designed the experiments and wrote the manuscript. M.-A. D. L. V., G. W., H. W., S. H., A. B., H. F.-B., J. A., K. T., K. T., G. S., T. R., J. E. S., X. Q., and G. K. conducted the experiments.

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