Ebola virus transmission in guinea pigs

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Abstract

Ebola virus (EBOV) transmission is currently poorly characterized and thought to occur primarily by direct contact with infectious material; however transmission from swine to nonhuman primates via the respiratory tract has been documented. To establish an EBOV transmission model for performing studies with statistical significance, groups of six guinea pigs (gps) were challenged intranasally (IN) or intraperitoneally (IP) with 10,000 x LD$_{50}$ of gp-adapted EBOV, and naïve gps were then introduced as cage-mates for contact exposure at 1 day post-infection (dpi). Animals were monitored for survival and clinical signs of disease, and quantitated for virus shedding post-exposure. Changes in contact duration of naïve gps with infected animals were evaluated for impact on transmission efficiency. Transmission was more efficient from IN compared to IP-challenged gps, with 17% versus 83% of naïve gps surviving exposure, respectively. Virus shedding was detected beginning at 3 dpi from both IN- and IP-challenged animals. Contact duration positively correlated with transmission efficiency, and the abrogation of direct contact between infected and naïve animals through the erection of a steel mesh is effective at stopping virus spread, provided that infectious animal bedding was absent in the cages. Histopathological and immunohistochemical findings show that IN-infected gps display enhanced lung pathology and EBOV antigen in the trachea, which support increased virus transmission from these animals. The results suggest that IN-challenged gps are more infectious to naïve animals than their systemically-infected counterparts, and that transmission occurs through direct contact with infectious materials, including those transported through air movement over short distances.
Importance

Ebola is generally thought to be spread between humans through infectious bodily fluids. However, a study has shown that Ebola can be spread from pigs to monkeys without direct contact. Further studies have been hampered, because an economical animal model for Ebola transmission is not available. To address this, we established a transmission model in guinea pigs, and determined the mechanisms behind virus spread. The survival data, in addition to microscopic examination of lung and trachea sections, show that mucosal infection of guinea pigs is an efficient model for Ebola transmission. Virus spread is increased with longer contact times to an infected animal and is possible without direct contact between an infected and naïve host, but can be stopped if infectious materials were absent. These results warrant consideration for the development of future strategies against Ebola transmission, and a better understanding of the parameters involved with virus spread.
Introduction

Ebola virus (EBOV) is a zoonotic pathogen that causes sporadic outbreaks localized to the humid, remote rainforests of sub-Saharan Africa. Infected individuals develop symptoms similar to many other common pathogens including fever, nausea, diarrhea and general malaise before progressing to specific signs characterized by hemorrhagic symptoms as well as multi-organ failure and a syndrome resembling septic shock, leading to death within 6 to 10 days after the onset of symptoms (7). While EBOV is the most virulent with mortality rates of up to 90% in humans, Reston virus is currently the most widespread, with serological evidence of infection amongst pigs in the Philippines (3), orangutans in Indonesia (13) as well as bats in China (23) and Bangladesh (14). The erratic nature of these outbreaks, in addition to isolated occasions when the virus can be accidentally introduced into non-endemic areas from the import of infected nonhuman primates (NHPs) (1) (2) (9), have caused concern amongst public health authorities. The natural reservoir of EBOV is currently unknown; however fruit bats have been postulated as a potential culprit due to evidence of asymptomatic infection in wild populations (12). Past experimental inoculation of fruit and insectivorous bats with EBOV resulted in extensive viral replication in bat tissues and high viremia with no apparent clinical symptoms or fatalities associated with disease, but the shedding of live virus was only successfully recovered from the feces of one infected fruit bat (18).

Direct contact with infected materials is generally accepted as the primary mode of transmission, as outbreaks can occasionally be traced back to an index case where the individual has handled infected bush meat (15). Amplification and transmission of virus between humans is subsequently made possible via contact with infected tissue, bodily fluids and improper use of
needles (5), either in a nosocomial setting or during burial rituals. Past reports have also described successful lethal infection of NHPs with aerosolized EBOV (10), where initial infection occurred in the respiratory lymphoid tissues before spread to regional lymph nodes by infected dendritic cells and macrophages (19). Multiple organs, including the liver and spleen are then infected following extensive viral replication in the lymph nodes. Clinical disease occurs within 7 to 10 days of infection and include hallmarks of EBOV disease, such as decreasing lymphocyte and platelet levels, elevated liver enzymes culminating in coagulation disorders and shock (19). In addition, recent studies have demonstrated that pigs can be infected mucosally with EBOV and then transmit virus to other naïve pigs (11) and to naïve NHPs (22). The establishment of interspecies transmission without direct contact, in addition to past epidemiological observations (17) supports the notion that airborne EBOV transmission could potentially be contributing, albeit at a low level, to the spread of disease during an outbreak.

Transmission studies in swine and NHPs require the use of precious animal species and necessitate extensive coordination and resources, making them impractical and possibly unsuitable to support scientific progresses, such as the early advancement of vaccines and treatments. Previous sequential passaging of EBOV in guinea pigs (gps) has yielded a gp-adapted EBOV variant (GA-EBOV), which is lethal to these animals (6) (21). GA-EBOV has previously been extensively utilized in pathogenesis, vaccine and treatment studies. The objective of this study was to investigate whether GA-EBOV transmission from direct contact between systemically or mucosally infected gps to naïve animals is possible. Factors which may impact transmission, such as the length of contact between the two gps, as well as the prevention of direct physical contact with an infectious animal or material were also investigated. Survival,
weight loss, virus shedding patterns and evidence of transmission were monitored in this study, providing insight into the factors behind EBOV spread. In addition, the pathology of infected animals was also examined to compare the nature of infection between gps infected systemically, mucosally or via transmission.
Materials and Methods

Ethics statement. All infectious experiments were carried out in the Biosafety Level 4 (BSL-4) laboratory at the National Microbiology Laboratory (NML) at the Public Health Agency of Canada (PHAC) in Winnipeg, Canada. All procedures involving live animals were approved by the Canadian Science Center for Human and Animal Health – Animal Care Committee (CSCHAH-ACC), following the guidelines of the Canadian Council on Animal Care. All protocols were designed to minimize animal discomfort. The approval documentation used for this study was animal use document (AUD) # H-11-007.

Study design. The objective of this study was to establish gps as a useful small animal model for EBOV shedding and transmission studies. Groups of 6 challenged gps were first infected intranasally (IN) or intraperitoneally (IP) with 1,000 or 10,000 x LD$_{50}$ GA-EBOV (diluted in 0.5 mL total volume for IN, and 1 mL total volume for IP challenges), before the addition of 6 naïve contact gps 24 hours after infection, in which one naïve animal was pair-housed with one infected animal in an isolated cage, and monitored for survival, weight loss and clinical symptoms. Shedding patterns were characterized via RT-PCR and infectivity assays on oral, nasal and rectal swabs collected at 3, 5 and 7 dpi on IN and IP-challenged gps, whereas the same samples in addition to blood were collected at 13-14 and 28 dpe on IN and IP-contact gps and analyzed in the same manner. The length of contact time with the infectious animal will be shortened in a subsequent experiment as contact animals will be added at 3, 5 and 7 dpi to the challenge animals in a similar manner as described above, and monitored for survival and weight loss. To determine whether virus transmission could occur without direct physical contact, GA-EBOV infected gps were separated into groups of three and placed in one half of a single
ventilated rabbit/ferret cage separated by two steel meshes spaced 5 cm apart. Groups of three naïve gps were then placed into the other half of the cage, downwind of the infected animals without animal bedding. The rabbit/ferret cage experiment was then replicated under the same challenge and exposure conditions in the presence of gp bedding (BioFresh, USA) to investigate whether contact with infectious materials is sufficient for virus transmission. Finally, to compare between systemic and mucosal infection of gps with GA-EBOV the lungs, nasal passages and trachea of infected animals were harvested at 6 and/or 12 dpi and stained to analyze the pathology and the presence of EBOV VP40 antigen.

Ventilated caging system. A negative ventilated rabbit/ferret housing cage system was used to conduct the non-direct contact transmission experiments in gps (Allentown, USA). The isolators were retro-fitted with stainless steel wire mesh to equally divide the compartment of each unit within the isolator. Briefly, guide rails were bolted to both the top and bottom of the metal cage and two 4x4 (¼” x ¼” square) stainless steel inserts slid into place generating a 1 inch space between to the halves of each of the six units generating 12 compartments in total. Infected animals were housed in the outer half of each unit while the naïve animals occupied the inner half of each unit. Directional airflow of approximately 35 cubic feet per minute was directed over the shedding animals and across naïve animals as it exited the unit.

Animals and challenge. Outbred 4 to 8 week old female Hartley strain gps (Charles River) were used for these studies. Animals were infected IN or IP with 1,000 or 10,000 x LD<sub>50</sub> GA-EBOV, strain Mayinga (Ebola virus VECTOR/C.porcellus-lab/COD/1976/Mayinga-GPA,
Genbank accession number AF272001.1) (6) and monitored every day for 28-29 days for survival, weight and clinical symptoms.

**Histopathology and immunohistochemistry.** For histopathology, lung tissue samples were collected from gps and fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin. For immunohistochemistry, paraffin tissue sections were quenched for 10 minutes in aqueous 3% H2O2 then pre-treated with proteinase K for 10 minutes. The primary antibody was a rabbit polyclonal anti-EBOV VP40 and was used at a 1:1000 dilution for one hour. They were then visualized using a horseradish peroxidase labelled polymer, Envision® + system (anti-rabbit) (Dako, USA), reacted with the chromogen diaminobenzidine (DAB). The sections were then counter stained with Gill’s hematoxylin.

**Virus titrations.** Oral, nasal and rectal swab samples were collected into 1mL of DMEM and the amount of virus present was quantitated by both direct titration onto VeroE6 cells, or quantitative reverse transcriptase (RT-qPCR). For titration of live virus, samples were inoculated in 10-fold serial dilutions of DMEM on VeroE6 cells with 3 replicates per sample. Plates were scored 10-14 days after infection for cytopathic effect, TCID$_{50}$ titers were calculated using the Reed-Muench method (16) and expressed as TCID$_{50}$/mL of sample. For detection of GA-EBOV RNA, total RNA was extracted from samples with the QIAamp Viral RNA Mini Kit (Qiagen), and then quantified with the LightCycler 480 RNA Master Hydrolysis Probes Kit (Roche) and oligos targeting the GA-EBOV RNA polymerase gene. The sequences are: EBOVLF2 (CAGCCAGCAATTTCTTCCAT), EBOVLR2 (TTTCGGTTGCTGTTTCTGTG) and EBOVLP2 FAM (FAM-
ATCATTGGCGTACTGGAGGAGCAG-MBG). RT-qPCR was performed on a Step One Plus RT thermocycler (Applied Biosystems) under the following reaction conditions: 63°C for 3 min, 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec and 60°C for 40 sec. Results were presented as genome equivalents per mL of sample (GEQ/mL).

Serology. Sera were collected from naïve animals and heat inactivated at 56°C for 45 minutes. An enzyme-linked immunosorbent assay (ELISA) was used to quantitate glycoprotein (GP)-specific IgG levels. Plates were coated overnight with 30μg/well of His-tagged EBOV GP (BioTherapeutics Inc.) and the assay was performed after blocking with each sample in triplicate, first diluted to 1:50, and then 2-fold serially in 2% skim milk/PBS/0.05% Tween 20. Washes were performed with TBS/0.1% Tween 20 and the HRP-conjugated goat anti-gp IgG (KPL) was used as a detection antibody. Plates were read using a VMax Kinetic ELISA Microplate Reader (Molecular Devices) at an optical density of 405 nm and the data was analyzed using the CellMaxPro software.

Statistical analysis. Statistical significance for survival curves were calculated using the Log-rank Mantel-Cox test. Values of p<0.05 were considered significant, p<0.01 were considered highly significant, and p<0.001 were considered extremely significant. Error bars on graphs, where applicable, indicate one standard deviation from the mean. All analyses were performed on GraphPad Prism v.5.01 software.
Results

Survival and clinical symptoms of GA-EBOV-infected and naïve contact animals. To establish whether EBOV transmission can occur from infected to naïve gps through close contact, a naïve gp was pair-housed with an experimentally infected animal in a single cage, 24 hours after challenge. Initially, challenge animals were infected via either the IN or IP route with 1,000 or 10,000 x LD<sub>50</sub> of GA-EBOV. Animals challenged IP (n = 6) or IN (n = 6) with 10,000 x LD<sub>50</sub> succumbed to GA-EBOV infection at an average time to death of 7.3 ± 0.5 or 8.7 ± 1.0 days post-infection (dpi), respectively (Figure 1A). Rapid weight loss was observed by 5 dpi (Figure 1B) in addition to a fever spike of above 40°C preceding hypothermia two days before death in both groups (Figure 1C). One of six naïve gps introduced to IP-challenged animals lost weight starting 11 days after initial exposure (dpe) and succumbed to disease at 14 dpe, whereas five of six naïve gps introduced to IN-challenged animals lost weight starting at 7 dpe and succumbed to disease with an average time to death of 12.6 ± 1.1 dpe (p = 0.01). Surviving naïve contact gps did not lose weight or exhibit significant changes in body temperature despite being in close contact with a terminally ill animal (Figures 1B and 1C). To determine if these transmission results are reproducible, and whether survival rate of naïve contact animals mimics that of human infections, survival of naïve contact gps from repeat experiments (n = 27) were pooled together under identical conditions with the exception that the animals were challenged with 1,000 x LD<sub>50</sub> GA-EBOV. It was found that naïve animals exposed to IP and IN-challenged gps resulted in fatality rates of 17% (2 of 12) and 80% (12 of 15), respectively, with an average time to death of 16.5 ± 4.9 days and 15.2 ± 4.9 dpe (p=0.001), respectively (Figure 1D). Similar survival rates were observed from naïve gps pair-housed with animals challenged at 1,000 x LD<sub>50</sub> or 10,000 x LD<sub>50</sub>, however the time to death of naïve gps was less variable at the higher
challenge dose (Figures 1A and 1D), indicating that more uniform transmission occurred at 10,000 x LD$_{50}$. Therefore, 10,000 x LD$_{50}$ was selected for subsequent experiments, with naïve gps introduced 24 hours following infection.

Shedding patterns of IN versus IP-challenged animals and exposed naïve animals. Levels of GA-EBOV shedding from challenged gps (n = 6 per group) were measured from oral, nasal and rectal swabs sampled on 1, 3, 5 and 7 dpi, and then analyzed for the presence of virus using quantitative RT-PCR (RT-qPCR) specific to the GA-EBOV polymerase gene. Live virus titrations were performed as confirmation of the PCR results. GA-EBOV virus could not be detected in any swab samples at 1 dpi. In IP-challenged animals, GA-EBOV could be detected at 3 dpi in oral swabs at $\sim 10^4$ genome equivalents per mL (GEQ/mL) and in rectal swabs at $\sim 10^6$ GEQ/mL. Amongst IN-challenged gps, GA-EBOV could be detected at 3 dpi in nasal swabs at $\sim 10^5$ GEQ/mL (Figure 2A). At 5 dpi, GA-EBOV could be detected in the oral, nasal and rectal swabs of IP-challenged animals at levels of $\sim 10^6$, $\sim 10^7$, and $\sim 10^4$ GEQ/mL, respectively. Amongst IN-challenged gps, GA-EBOV was detected in the oral and nasal swabs at $\sim 10^4$ and $\sim 10^5$ GEQ/mL, respectively (Figure 2B). At 7 dpi, GA-EBOV could be detected in the oral, nasal and rectal swabs of IP-challenged animals at $\sim 10^6$, $\sim 10^6$ and $\sim 10^7$ GEQ/mL, respectively. Amongst IN-challenged gps, GA-EBOV was detected in the oral, nasal and rectal swabs at $\sim 10^6$, $\sim 10^5$, and $\sim 10^7$ GEQ/mL, respectively (Figure 2C). Live GA-EBOV levels from swabs, expressed in TCID$_{50}$/mL, were overall lower but found to correlate in relation to observed RT-qPCR data (Figures 2A, 2B and 2C).
Amongst contact animals, high levels of GA-EBOV was detected only in non-surviving naïve contact IP (n = 1) and IN (n = 5) gps at the time of death (between 13 to 14 dpe). Non-surviving animals exposed to IP-challenged gps had detectable levels of GA-EBOV in the blood as well as oral, nasal and rectal swabs, at ~10^6, ~10^4, ~10^3, and ~10^3 GEQ/mL, respectively. Non-surviving animals exposed to IN-challenged gps had comparatively higher levels of virus in the blood as well as oral, nasal and rectal swabs, at ~10^7, ~10^5, ~10^5, and ~10^5 GEQ/mL, respectively (Figure 2D). At the termination of the experiment at 28 dpe, low levels of GA-EBOV was detected in the oral and nasal swabs of the lone surviving naïve contact IN animal at ~10^4 and ~10^2 GEQ/mL, respectively, however live virus was not recovered. Despite the lack of clinical symptoms, GA-EBOV was surprisingly detected in the blood as well as oral, nasal and rectal swabs from 3 of 5 naïve contact IP gps at ~10^4, ~10^2, ~10^3, and ~10^2 GEQ/mL, respectively (Figure 2E).

Efficiency of GA-EBOV transmission is dependent on length of contact exposure. The effect of contact duration between naïve and infected gps on GA-EBOV transmission rates was then evaluated. Animals (n = 18) were challenged IN with 10,000 x LD_{50} of GA-EBOV, with naïve gps (n = 6 per group) introduced as cage mates at 3, 5, or 7 dpi. IN-challenged gps succumbed to infection between 7 to 10 dpi with an average time to death of 8.6 ± 0.9 dpi, in which weight loss and clinical symptoms corresponding to GA-EBOV disease were observed (Figures 3A and 3B). Naïve contact gps introduced at 3, 5 and 7 dpi resulted in survival rates of 33%, 50% and 83%, respectively, with non-survivors succumbing to disease with a mean time to death of 14.3 ± 2.0, 13.0 ± 2.0 and 12 ± 0.0 dpe, respectively (p = 0.24, p = 0.20, and p = 0.04, respectively).
comparing to the group of naïve gps introduced at 1 dpi (Figure 3A). Surviving gps did not significantly lose weight (Figure 3B) nor exhibit clinical signs of disease.

Direct contact with infectious materials is needed for GA-EBOV transmission. A study was then conducted in order to explore the mechanism of GA-EBOV transmission and whether the presence of infectious materials could have played a role in enhanced virus spread. Using a ventilated caging system, gps (n = 6) were separated equally into two different cages and challenged IN with 10,000 x LD$_{50}$ of GA-EBOV. At 1 dpi, naïve gps (n = 6) were separated equally into two cages downwind of the cages housing the infected animals. Also present in both sides of the cages was animal bedding that was sufficiently small enough to be able to pass through the holes in the steel mesh barrier. While all infected gps succumbed to GA-EBOV with an average time to death of 8.0 ± 0.6 dpi, one out of two naïve gp cages also lost weight starting at 11 dpi and succumbed to disease with an average time to death of 16.7 ± 3.5 dpe (Figures 4D and 4E). The three surviving naïve animals in the remaining cage tested seronegative for GA-EBOV at the termination of the experiment (data not shown). The experiment was then repeated without animal bedding inside the cages of the infected and naïve animals. Animals (n = 9) were first separated equally into three different cages and each were challenged IN with 10,000 x LD$_{50}$ of GA-EBOV. At 1 dpi, naïve gps (n = 9) were separated equally into three different cages downwind of the cages housing the infected animals, with two steel meshes spaced 1 inch apart physically separating the infected gps from the naïve animals (Figure 4A). While IN-challenged gps succumbed to infection between 7 and 9 dpi with an average time to death of 8.1 ± 0.8 dpi, all naïve animals survived the infection without significant weight loss (Figures 4B and 4C).
The surviving animals were shown to be seronegative for GA-EBOV by IgG ELISA (data not shown), indicating that transmission did not occur.

**Histopathology and immunohistochemistry of infected gos show evidence of respiratory infection.** The lungs, nasal passages and tracheas of IP and IN-challenged gos at 6 dpi, as well as IP and IN-transmission animals at 6 and 12 dpi were harvested to establish whether any pathological differences exist between a systemic and a mucosal GA-EBOV infection. One infected animal was co-housed with one naïve gp in a single cage for these studies. In the IP-challenged group, observed lung lesions were consistent with a pulmonary interstitial reaction and were characterized by expansion of alveolar septa by edema fluid, inflammatory cells (primarily macrophages and granulocytes) and scattered degenerating cells with pyknotic nuclei (Figures 5A and 5B). In the IN-infected group, patchy areas of alveolar inflammation were similar to those observed for the IP-infected group; however, there were also multifocal, locally extensive areas of severe bronchointerstitial pneumonia (Figure 5C). Bronchioles contain neutrophils, and there was evidence of a loss of bronchiolar epithelial cells (Figure 5D). Alveolar walls were expanded due to hyperplasia of type II pneumocytes as well the presence of edema, macrophages, granulocytes and degenerating cells with pyknotic nuclei. Alveolar air space was largely replaced by alveolar macrophages, fibrin, necrotic debris, and granulocytes, of which many were degenerating (Figure 5D). In the IN-transmission group at 12 dpi, several of the lung sections had lesions similar to the severe bronchointerstitial pneumonia described for the IN-challenged group (Figure 5E). However, this type of lesion was only observed in 2 of 3 animals and on 2 of 15 observed lung sections. This was in contrast to the IN-challenged group, in which the lesions were observed on all slides. The rest of the lung sections for the IN-
transmission group showed an interstitial reaction similar to those observed in the IP-infected group. Significant lung lesions were not observed with the IP-transmission group at 12 dpi (Figure 5F), nor were they observed with the IP- and IN-transmission groups at 6 dpi.

In the lungs of IP-challenged gps, moderate amounts of viral antigen were detected within alveolar walls and bronchiolar associated lymphoid tissue (BALT) (Figure 6A). Positive immunostaining was observed within macrophages, endothelial cells, and occasional pneumocytes as well as free within capillaries and vessels (Figure 6B). Viral antigen was not observed within bronchiolar epithelial cells, but was detected in occasional cells within the lumen (likely macrophages) as well as within the bronchiolar submucosal tissue. With the IN-challenged animals, most sections show areas of intense and extensive positive immunostaining which correlate with the presence of lesions and often appear to be centered on bronchioles and associated arteries (Figure 6C). Positive immunostaining was occasionally observed within bronchial and bronchiolar epithelial cells (Figure 6D). Within the interstitium, the staining was so intense that it was difficult to identify individual cells, however staining was observed to be present in pneumocytes by double immunolabeling (Figure 6E) as well as within macrophages, endothelial cells, BALT, free within capillaries and vessels as well as in association with alveolar exudate. In a few areas, the staining pattern was similar to that described for the IP-challenged group. In the IN-transmission group at 12 dpi, most of the sections showed a staining pattern similar to that described for IP-challenged animals. However, in the 2 sections that show lesions of bronchointerstitial pneumonia, the staining pattern is similar to that described for the IN-challenged group with intense focal staining (Figure 6F).
In samples taken from nasal passages of the IP-, IN-challenged (at 6 dpi) and IN-transmission

groups (at 12 dpi), viral antigen was detected within the endothelial cells of submucosal vessels;
however, no antigen was detected within epithelial cells (Figure 7A). In the trachea of the IN-
challenged group, viral antigen could be detected within the epithelium as well as the submucosa
(Figure 7B). Within tracheas of the IP-challenged and IN-transmission groups, antigen was only
detected within the submucosa (Figure 7C). No viral antigen was detected in any tissues from
the IP-transmission group at 12 dpi.
Discussion

Direct and indirect parameters contributing to transmission of EBOV between animals, animal-to-human and human-to-human are currently largely undefined. It is therefore important to elucidate and characterize the mechanisms behind EBOV transmission and in cases where exposure to virus has occurred, to evaluate the effectiveness of specific countermeasures. A better understating of EBOV transmission is also important to maximize rational-based decisions when managing an outbreak.

Since both mucosal and systemic inoculation of NHPs with EBOV cause fatal disease highlighted by similar pathogenesis hallmarks typical of filoviral disease (8) (10), gps were infected IN or IP to determine whether they were susceptible to both routes of infection. While both methods of inoculation resulted in fatal disease for all challenge animals within a comparable timeframe, characterized by similar levels of virus isolated from oral, nasal and rectal swabs at the time of death, there is a marked difference between the transmission properties of mucosal and systemic infection in gps. Naïve contact gps caged with IN-challenged gps fared significantly worse than those housed with IP-challenged animals, as seen by lower rates of survival. Interestingly, the overall mortality rate of 80% observed among the contact gps of IN-challenged animals in our studies (Figure 1D) mimics the case fatality rate (CFR) for all documented EBOV outbreaks amongst humans, which stands at 1084 deaths out of 1381 total cases (CFR = 78.5%) (4), excluding the ongoing 2014 outbreak in West Africa for which final numbers are not available due to the evolving situation.
The observed difference in transmission efficiency between the IN and IP-challenged gp groups could be explained by the higher levels of GA-EBOV shedding from the nasal route of the IN-challenged animals early after contact as seen at 3 dpi. Another parameter possibly playing a role is that IP-challenged gps succumb to disease more quickly compared to IN-challenged animals maybe due to a route of infection that more directly promotes systemic virus circulation. Since animals that succumb to disease are removed from their cage-mates immediately after death, it is possible that a comparatively longer exposure time between IN-infected and naïve animals resulted in increased transmission. Naive gps introduced to infected mates at progressively later times to decrease the contact time spent with a terminally ill animal showed increased survival when the time of contact with an infected animal was decreased, and this difference became statistically significant between naïve gps exposed on 1 versus 7 dpi. These results suggest that the length of contact time between a naïve and an infected animal plays an important role. It is possible that both the exposure to a higher virus load early after contact together with a longer time of exposure is leading to more transmission from the IN-challenged group. A third possibility that is currently being evaluated in the laboratory is whether immune components from the infected animals (e.g. antibodies) are present at higher levels in the IP-challenged animals and are interfering with the transfer of infectious particles to the naïve animals. This would explain the sublethal infections documented in the surviving animals (Figure 2E), which has never been described in gps by limiting dilutions of the challenge virus.

The distribution of EBOV antigens in IP-challenged gps suggest that the virus enters the lungs systemically via the bloodstream, whereas in IN-challenged gps the data from the lungs and trachea is consistent with that of an infection from the airway. The lungs of IP-challenged gps...
In contrast, IN-challenged animals displayed a severe inflammatory response in the lungs, in which the alveolar airspace has been replaced by degenerating immune cells, resulting in pneumonia and progression to the systemic disease that is also observed with gps challenged with aerosolized GA-EBOV (20). The earlier release of viral load, combined with the presence of viral antigen in the tracheal epithelium in IN-challenged gps support increased transmissibility from these animals. This is reflected with the survival rates of the naïve contact animals in this study. Naïve gps co-housed with IN-challenged gps died, and evidence of infection through the airway was again observed in the lungs of these animals, albeit at an apparent lower frequency.

It was observed in these studies that GA-EBOV transmission can be abrogated by preventing direct contact between an infected and a naïve gp, provided that no infectious materials could travel to expose the naïve animals. Therefore, the experimental results do not support airborne spread of EBOV, but demonstrate instead that virus transmission can occur through direct contact with infected materials traveling over short distances. Interestingly, the pathology observed in animals infected presumably through contaminated bedding are similar to previous findings with gps infected by aerosolized GA-EBOV, such as severe pneumonia characterized by thickened alveolar septa filled with infected immune cells, as well as infrequent necrosis of bronchiolar epithelial cells (20). In-depth studies will be necessary to further characterize and distinguish the differences in systemic, mucosal and aerosol GA-EBOV infection, and their relation to virus transmissibility.
Although gps do not succumb to infection with wild-type EBOV and an adapted variant has been utilized in its place, the hallmarks of GA-EBOV infection in gps are quite comparable to wild-type EBOV infection in NHPs. The availability of a small animal model capable of transmission studies with statistical significance will serve to accelerate future transmission studies. A better understanding of parameters involved in EBOV transmission should help researchers and medical care professionals to develop better medical countermeasures and protect against all aspects of EBOV outbreaks whether from natural causes or not.
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Figure 1. Survival, weight loss and clinical symptoms of infected and naïve contact gps.
Challenge animals were IN or IP infected with 10,000 x LD₅₀ GA-EBOV and naïve contact gps were introduced at 1 dpi as cage mates. All challenge and contact animals were monitored for changes in (A) survival, (B) weight loss and (C) body temperature. Transmission experiments were repeated for reproducibility and summarized by (D) survival curve of all exposed contact gps, introduced to animals challenged IN or IP with 1,000 or 10,000 x LD₅₀ GA-EBOV IN or IP.

Figure 2. GA-EBOV shedding in infected and naïve contact gps. Oral, nasal and rectal swabs were sampled from challenge gps infected with GA-EBOV IN or IP at (A) 3 dpi, (B) 5 dpi, as well as (C) 7 dpi, then quantitated for levels of virus by RT-qPCR (left y-axis) and virus titration (right y-axis). Oral, nasal and rectal swabs were sampled from contact gps at (D) 13-14 dpe and (E) 28 dpe and quantitated for levels of virus by RT-qPCR. RT-qPCR and virus titration results were expressed in GEQ/mL or TCID₅₀/mL, respectively. Error bars represent ± standard deviation.

Figure 3. Survival and weight loss of infected and naïve contact gps introduced at different times after challenge. Challenge animals were infected with GA-EBOV IN, and naïve contact gps were introduced at 3, 5 or 7 dpi as cage mates. All challenged and contact animals were monitored for changes in (A) survival and (B) weight loss.

Figure 4. Survival and weight loss of infected and naïve gps within a ventilated cage system. (A) A representative ventilated cage with three gps either side of the steel mesh barrier
in the middle. Infected animals are placed on the left hand side, naïve animals are on the right hand side. Airflow is from the left to the right. Challenge animals were given GA-EBOV IN, and naïve gps were introduced into cages downwind of the infected cages, with infected animals prevented from direct with naïve animals by the presence of two steel meshes placed 5 cm apart between the two groups. All challenged and naïve animals were monitored for changes in survival and weight loss. The same experiment was then repeated with the same cage system, but in the presence of gp bedding, which is able to pass through the steel meshes. All challenged and naïve animals were monitored for changes in survival and weight loss.

Figure 5. Histopathology findings. Lungs from (A, B) IP-challenged, (C, D) IN-challenged, (E) IN-transmission and (F) IP-transmission groups. (A) Alveolar walls appear mildly thickened with increased cellularity. Bar = 200 μm. (B) Alveolar walls are expanded by inflammatory cells. Note the presence of scattered degenerating cells with pyknotic nuclei (arrows). Bar = 20 μm. (C) Note the large area of severe bronchointerstitial pneumonia (*) in contrast to the less affected lung tissue with visible alveolar spaces. Bar = 200 μm. (D) Alveoli are filled with neutrophils and necrotic debris leading to loss of air spaces (*). Bar = 20 μm. Bronchiolar lumina contain neutrophils and there is evidence of degeneration of lining epithelial cells (arrow). Alveolar walls contain hyperplastic Type II pneumocytes (arrowheads). (E) Large areas of severe bronchointerstitial pneumonia (*) were observed on a few lung sections. Bar = 200 μm. (F) There are no significant lesions. Bar = 200 μm.

Figure 6. Detection of GA-EBOV antigen by IHC in lungs. Lungs were harvested from (A, B) IP-challenged, (C, D, E) IN-challenged and (F) IN-transmission groups. (A) Viral antigen
was detected within alveolar walls throughout most of the section (arrows) as well as within the BALT (arrowhead). Bar = 100 μm. (B) Viral antigen detected within endothelial cells (arrow) and within cells that have the morphological appearance of macrophages (arrowheads). Bar = 20 μm. (C) Large areas of the section show intense, widespread immunostaining. Bar = 100 μm. (D) Positive immunostaining in bronchiolar epithelial cells (arrows). Bar = 20 μm. (E) Double immunolabeling shows cytokeratin positive hyperplastic type II pneumocytes (brown, arrows) some of which contain viral antigen (pink, arrowhead). Bar = 10 μm. (F) Viral antigen was observed within occasional bronchiolar epithelial cells (arrow). There is intense staining in the adjacent pulmonary parenchyma and alveolar exudate. Bar = 20 μm.

Figure 7. Detection of GA-EBOV antigen by IHC in the nasal passage and trachea. (A) In sections from nasal passages, viral antigen was detected within endothelial cells of submucosal vessels (arrows) but not in epithelia (arrowhead). (B) In the trachea of the IN-challenged group, viral antigen could be detected within epithelium (arrow) and submucosa (arrowhead). (C) Trachea from IP-challenged group showing viral antigen in submucosa (arrow) but not within epithelium (arrowhead). Bar = 50 μm.
Figure 1. Survival, weight loss and clinical symptoms of infected and naïve contact gps. Challenge animals were IN or IP infected with 10,000 x LD50 GA-EBOV and naïve contact gps were introduced at 1 dpi as cage mates. All challenge and contact animals were monitored for changes in (A) survival, (B) weight loss and (C) body temperature. Transmission experiments were repeated for reproducibility and summarized by (D) survival curve of all exposed contact gps, introduced to animals challenged IN or IP with 1,000 or 10,000 x LD50 GA-EBOV IN or IP.
Figure 2. GA-EBOV shedding in infected and naïve contact gns. Oral, nasal and rectal swabs were sampled from challenge gns infected with GA-EBOV IN or IP at (A) 3 dpi, (B) 5 dpi, as well as (C) 7 dpi, then quantitated for levels of virus by RT-qPCR (left y-axis) and virus titration (right y-axis). Oral, nasal and rectal swabs were sampled from contact gns at (D) 13-14 dpe and (E) 28 dpe and quantitated for levels of virus by RT-qPCR. RT-qPCR and virus titration results were expressed in GEQ/mL or TCID50/mL, respectively. Error bars represent ± standard deviation.
Figure 3. Survival and weight loss of infected and naïve contact groups introduced at different times after challenge. Challenge animals were infected with GA-EBOV IN, and naïve contact groups were introduced at 3, 5 or 7 dpi as cage mates. All challenged and contact animals were monitored for changes in (A) survival and (B) weight loss.
Figure 4. Survival and weight loss of infected and naïve gss within a ventilated cage system. (A) A representative ventilated cage with three gss either side of the steel mesh barrier in the middle. Infected animals are placed on the left hand side, naïve animals are on the right hand side. Airflow is from the left to the right. Challenge animals were given GA-EBOV IN, and naïve gss were introduced into cages downwind of the infected cages, with infected animals prevented from direct with naïve animals by the presence of two steel meshes placed 5 cm apart between the two groups. All challenged and naïve animals were monitored for changes in (B) survival and (C) weight loss. The same experiment was then repeated with the same cage system, but in the presence of gp bedding, which is able to pass through the steel meshes. All challenged and naïve animals were monitored for changes in (D) survival and (E) weight loss.
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