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Evaluation of transmission risks associated with *in vivo* replication of several high containment pathogens in a biosafety level 4 laboratory

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Containment level 4 (CL4) laboratories studying biosafety level 4 viruses are under strict regulations to conduct nonhuman primate (NHP) studies in compliance of both animal welfare and biosafety requirements. NHPs housed in open-barred cages raise concerns about cross-contamination between animals, and accidental exposure of personnel to infectious materials. To address these concerns, two NHP experiments were performed. One examined the simultaneous infection of 6 groups of NHPs with 6 different viruses (Machupo, Junin, Rift Valley Fever, Crimean-Congo Hemorrhagic Fever, Nipah and Hendra viruses). Washing personnel between handling each NHP group, floor to ceiling biobubble with HEPA filter, and plexiglass between cages were employed for partial primary containment. The second experiment employed no primary containment around open barred cages with Ebola virus infected NHPs 0.3 meters from naïve NHPs. Viral antigen-specific ELISAs, qRT-PCR and $TCID_{50}$ infectious assays were utilized to determine antibody levels and viral loads. No transmission of virus to neighbouring NHPs was observed suggesting limited containment protocols are sufficient for multi-viral CL4 experiments within one room. The results support the concept that Ebola virus infection is self-contained in NHPs infected intramuscularly, at least in the present experimental conditions, and is not transmitted to naïve NHPs via an airborne route.

Conducting non-human primate (NHP) experiments in containment level 4 (CL4) laboratories are difficult because of the complex logistics required to comply with all biosafety and animal care regulations. NHPs require a large amount of space due to their size, and are therefore housed singly or paired in large open-barred cages. The use of enclosed cage systems with negative pressure and independent HEPA filtration to prevent cross-contamination and increase containment of infectious agents is relatively easy to implement for rodents. However, installing primary containment around NHP cage systems is challenging because of the large area requiring containment, and more importantly the daily animal care. The small rodent cages can be changed in a biosafety cabinet or other contained aseptic field, thereby maintaining primary containment relatively easily. However, NHP physical examinations and daily husbandry breaks primary containment several times per day. In addition, primary containment isolates these social animals and makes manipulations more cumbersome for workers, possibly increasing the risks of exposure.

Experimental cross-contamination or infection of personnel depends on providing appropriate containment but also upon the viruses under investigation. A variety of CL4 viruses are utilized, with transmission occurring through direct contact or through the airway via aerosols or large droplets. Transmission for the bunyaviruses Rift Valley Fever Virus (RFV) and Crimean-Congo Hemorrhagic Fever virus (CCHFV), which have a case fatality rate (CFR) of 1–2% and 5–80%, respectively[1–5], is primarily via arthropods, or contact with infected fluids or tissues. However, aerosol infection of NHPs with RFV resulted in mild disease with no fatalities in cynomolgus and rhesus

Table 1 | Dosage and inoculation routes for experiment #1

Virus	Dose (IFU* in 1 ml PBS)	Inoculation Route**	Number of subjects
CCHFVor RFV	Twice 10^5	im and iv	2 per virus
NiV	Twice 10^5	im and iv, im and sc	1 per route
HeV	Twice 10^5	im and iv	2
JUNV or MACV	10^5	im	2 per virus

*IFU = infectious units.
**im = intramuscular; iv = intravenous; sc = subcutaneous.

macaques, but was lethal in marmosets and African green monkeys (AGM)[6]. For the arenaviruses Machupo (MACV) and Junin (JUNV) which have a human CFR up to 30%, human-to-human transmission is rare (reviewed in[7–9]), but is mainly through inhalation of aerosolised body fluids or excretions of infected rodents (reviewed in[10,11]). JUNV and MACV are lethal in marmosets, with MACV lethal in AGM, but only partially lethal in rhesus and cynomolgus macaques[12–15]. *Paramyxoviridae* family members Nipah virus (NiV) and Hendra virus (HeV) have a CFR of 38–100% and 57%, respectively[16–19]). For NiV, humans are infected via respiratory secretions, aerosols[20,21], contact with fluids from sick domestic animals, or eating contaminated food[22,23]. Human-to-human transmission is believed to be responsible for 51% of the cases in Bangladesh between 2001 and 2007[22]. In contrast to hundreds of NiV infections, there have only been 7 human HeV infections all arising through interaction with infected horses (reviewed in[19]). Both NiV and Hev are lethal in the AGM model, but have not been tested in cynomolgus macaques[19]. One of the best studied CL4 virus is Filoviridae family member Ebola virus (EBOV) with a human CFR up to 90%. In humans EBOV infection requires contact with infected bodily fluids into an open wound or mucous membrane, however, aerosol infection has been demonstrated in NHPS under experimental conditions using aerosol dispersion chambers[24,25]. One experiment reported contact free transmission between infected NHPs to one uninfected NHP although cross-contamination due to husbandry practices could not be ruled out with certainty[26]. Interestingly, EBOV infected swine transmitted the virus to naïve NHPs over a 0.3 meter buffer zone that prevented direct contact between the 2 species[27]. Overall, all four virus families have demonstrated the capacity to be transmitted via the air in different experimental protocols. However, airborne transmission in natural outbreaks cannot be a common occurrence and is possibly insignificant by the account of several reports[4,9,28–30].

The current study evaluated shedding and transmission of several CL4 viruses in NHPs in the absence of, or presence of partial primary containment. The viruses selected were the JUNV, MACV, NiV, HeV, CCHFV, RFV, and EBOV, representing four distinct families of CL4 viruses. This study brings data to help develop rationally based decisions in regards to primary containment of NHPs in the CL4 laboratory as well as associated risks.

Results

Two separate experiments were conducted to study the potential for cross-contamination with a variety of CL4 viruses. Infectivity $TCID_{50}$ assays, qRT-PCR and ELISA assays were utilized on the NHP sera, and rectal, oral and nasal swabs to determine whether the uninfected subjects had been exposed to a virus from nearby infected subjects.

NHP Experiment #1. The first experiment used partial containment protocols around each of the cages while simultaneously infecting 6 groups of 2 NHPs with 6 different CL4 viruses, including HeV, NiV, CCHFV, RFV, JUNV, and MACV according to table 1. Based on previous NHP data, a moderate dose for each virus was chosen in order to induce disease but not enough to cause a rapid progression to death, thereby allowing sufficient time for hypothetical transmission to other NHPs. All animals were housed in quads spaced 0.9 meters apart at right angles to each other within the same room (Figure 1). Partial containment protocols included plexiglass between cages within a quad, and a 3-sided biobubble with a HEPA filter. CL4 suits were decontaminated through a chemical shower between handling of each NHP group to prevent cross-contamination due to husbandry.

Disease progression was documented in each animal and found to be mild to moderate before a full recovery with the exception of the two HeV subjects which were terminated at 7 and 8 days post-infection (dpi). Nasal, oral, rectal swabs, and blood were collected on the exam dates as indicated in tables 2 and 3. qRT-PCR was conducted on these samples to determine viral levels for each of the subjects (Table 2). Variable levels (1.2–5.3 $\log_{10}$ genome copies/ml) of either CCHFV, RFV, NiV, HeV, JUNV, and MACV were found in the blood of CCHFV-1 and -2, RFV-1 and -2, NiV-1 and -2, HeV-1 and -2, and JUNV-2 infected NHPs, respectively. Homologous virus was also found in the nasal swabs of CCHFV-1 and -2; oral and nasal swabs of RFV-1 and -2; oral, nasal and rectal swabs of NiV-1 and -2, and HeV-1 and -2; the nasal and rectal swab of JUNV-2; and the rectal swab of MACV-2. Neighbouring NHPs within the same quad were also tested for the virus of their infected neighbours. There was no CCHFV detected in the RFV NHPs and vice versa, nor any JUNV detected in the MACV NHPs and vice versa, nor NiV in the HeV or vice versa. For additional evaluation of possible exposure between groups of NHPS in the quad, virus specific IgM and IgG levels were determined (Table 3). Only the day with the highest antibody titre for each NHP is shown. Each group of NHPs were either IgM (range 1 : 100 to 1 : 1600) or IgG (range 1/400 to 1/6400) positive for the viruses they were infected with but were negative for the viruses of the neighbouring NHPs. Overall, cross contamination due to viral transmission between neighbouring groups could not be detected.

NHP Experiment #2. The second experiment, which did not utilize any physical containment protocols was designed to examine whether uninfected NHPs could become infected via ambient air when placed in cages next to NHPs infected with Ebola virus. EBOV infections by aerosol have been demonstrated utilizing aerosol chambers for infecting NHPs[24]. To examine the possibility of transmission between an EBOV infected NHP and nearby naïve NHPs, a quad containing two EBOV infected rhesus macaques (EBOV-1 and EBOV-2) were placed in close proximity to another quad containing two uninfected cynomolgus macaques (Cyno-1 and Cyno-2) (Figure 2).

EBOV-1 and -2 showed the typical signs of viral hemorrhagic fever, such as fever, macular rashes, lethargy and unresponsiveness, associated with an EBOV infection, and were terminated on day 6. In contrast Cyno-1 and -2 showed no signs of illness for the entire 28 day period. Nasal, oral, and rectal swabs, and blood were collected on 0, 3, and 6 dpi for EBOV-1 and -2, as well as 0, 3, 7, 15, and 28 dpi for Cyno-1 and -2 (table 4). At 3 and 6 dpi EBOV-1 and EBOV-2 had 3.6–7.1 $\log_{10}$ EBOV genome copies/ml in their blood. At 6 dpi 4.2–5.3 $\log_{10}$ genome copies/ml EBOV were seen in the oral swabs of EBOV-1 and -2, nasal swab of EBOV-1 and rectal swab of EBOV-2 demonstrating the possibility for shedding and transmission of EBOV. However, EBOV could not be detected in Cyno-1 and -2

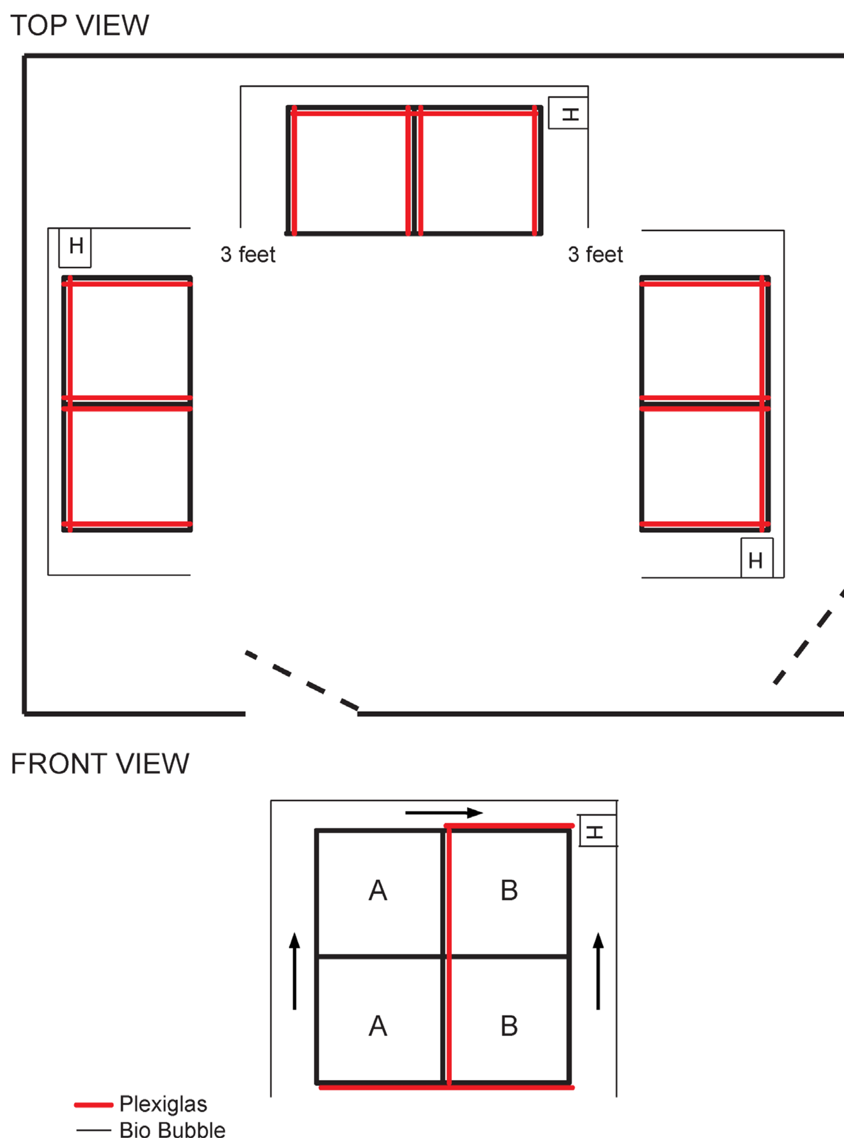

Figure 1 | Cage Arrangement for Experiment #1: Multi-virus Transmission. Cages were setup in a manner that prevented any cross-contamination of viruses within the "biobubble". The biobubble was a floor to ceiling plastic curtain (grey lines) with a HEPA filter in the top right hand corner (H). There was no curtain in front of the cage. Three mm plexiglass panels (red lines) were placed between cages within a quad to control the flow of air (arrows) towards the HEPA filter. Each quad housed one virus family (ie Arenaviridae) grouped such that the (A) top and bottom left side contained one virus (ie MACV) and the (B) top and bottom right side housed the other virus (ie JUNV) from the same family.

on 3, 7, 15, and 28 dpi indicating that no productive viral transmission occurred. As confirmation that the rhesus macaques were not shedding virus, a $TCID_{50}$ assay was performed on the 6 dpi swabs and blood of EBOV-1 and -2. There was no infectious virus found on any of the oral, rectal or nasal swabs. In comparison the 6 dpi blood sample had a titre of 3.2×10^4 and 6.8×10^5 $TCID_{50}$/ml for EBOV-1 and -2, respectively. To further document possible exposure of the naïve animals the antibody response was examined utilizing an EBOV-GP-specific ELISA. An IgM or IgG response to EBOV could not be detected in Cyno-1 or -2 for up to 28 days after infection of the EBOV-1 and -2 challenged animals nearby. EBOV-1 and -2 NHPs were also negative likely because 6 dpi is not sufficient to develop a detectable antibody response as previously reported[31,32].

Discussion

The use of open-barred NHP caging systems can limit the ability to conduct simultaneous experiments using multiple viruses. This study demonstrates by qRT-PCR and ELISA that multiple viruses can be used simultaneously in one room without transmission to neighbouring cages, with the use of simple barriers and containment protocols. One consideration is how the viruses are transmitted. In human cases, NiV (Malaysian strain), HeV, CCHFV and RFV are generally spread via direct contact with infected tissues or fluid[1–3,16,18,20,22,33]. Although the primary route of infection for NiV is by contact with infected fluids or by ingestion of contaminated food[23], airborne transmission has been suggested to be possible in human to human transmission via respiratory secretions[20,34]. Also, AGM and marmosets were highly susceptible to aerosolized RFV when delivered via a nebulizer[6]. CCHFV and RFV can also spread through arthropods, which is not a factor in this study. Human infection to JUNV and MACV can be acquired by aerosolized body fluids or excretions of infected rodents, in addition to contact with infected fluids or tissues[7–9]. Overall, these studies indicate that airborne transmission in the current experiments was a theoretical possibility.

The ability to detect virus nucleic acid in the oral, nasal and rectal swabs also indicates the potential for viral shedding and transmission existed. A factor which could account for the lack of cross-contamination is that either no virus was detected, or the viral loads were

Table 2 | Viral RNA detection and cross-reactivity by qRT-PCR. The value is $\log_{10}$ genome copies/ml of sample tested. The gene/segment targeted is listed after the virus

		Oral	Nasal	Rectal	Blood	Oral	Nasal	Rectal	Blood
Animal	dpi	CCHFV S	CCHFV S	CCHFV S	CCHFV S	RFV S	RFV S	RFV S	RFV S
CCHFV-1	3	-	2.4	-	2.2	-	-	-	-
	6	-	2.7	-	3.5	-	-	-	-
	9	-	2.6	-	2.7	-	-	-	-
CCHFV-2	3	-	-	-	1.4	-	-	-	-
	6	-	1.7	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
RFV-1	3	-	-	-	-	2.0	2.9	-	2.6
	6	-	-	-	-	2.6	3.7	-	2.0
	9	-	-	-	-	-	4.0	-	1.9
	29	-	-	-	-	-	-	-	3.9
RFV-2	3	-	-	-	-	1.9	3.6	-	3.9
	6	-	-	-	-	1.9	3.9	-	3.1
	9	-	-	-	-	3.5	3.5	-	3.3
Animal	dpi	NiV L	NiV L	NiV L	NiV L	HeV L	HeV L	HeV L	HeV L
NiV-1	3	-	-	2.4	2.0	-	-	-	-
	6	2.7	-	-	2.6	-	-	-	-
	9	2.3	2.2	2.4	3.1	-	-	-	-
NiV-2	3	-	-	-	-	-	-	-	-
	6	2.5	-	-	2.3	-	-	-	-
	9	2.5	1.5	2.5	3.4	-	-	-	-
HeV-1	3	-	-	-	-	2.7	4.8	-	3.7
	6	-	-	-	-	3.9	-	2.9	5.3
HeV-2	3	-	-	-	-	-	-	-	-
	6	-	-	-	-	3.6	-	2.6	3.6
	8	-	-	-	-	-	-	-	4.7
Animal	dpi	JUNV L	JUNV L	JUNV L	JUNV L	MACV L	MACV L	MACV L	MACV L
JUNV-1	3	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
JUNV-2	3	-	-	-	-	-	-	-	-
	6	-	-	1.3	1.2	-	-	-	-
	9	-	1.3	3.5	2.4	-	-	-	-
MACV-1	3	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
MACV-2	3	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	3.6	-

(-) Represents a negative value where no virus was detected.

very low in the blood or oral, nasal and rectal swabs of the arenaviruses, The inability of virus to replicate efficiently in these NHPs resulted in lower viral titres which likely lowered the capacity of the viruses to shed and transmit to other animals. This multi-virus experiment used incomplete primary containment in the form of plexiglass barriers inserted into the open-barred cages, and surrounding the bank with a plastic curtain on three sides with a HEPA filter at one corner to direct airflow. Additionally, CL4 personnel decontaminated their suits between groups of NHPs. These observations indicate that a completely closed NHP caging system is not required to prevent cross-contamination with these viruses. The possibility exists for environmental viral contamination on the cages themselves which could result in a productive NHP infection (through fomites). The cages were not swabbed to test for neighbouring viruses. However, the fact that there was no transmission detected between groups indicates that fomites did not play a role under these conditions. Even if this were to occur, the virus from the same quad would most likely not provide cross-protection to the neighbouring NHP infected with a different virus, even if from the same family. All the NHPs were infected simultaneously suggesting that by the time the virus titres in the NHP were high enough for shedding to occur, the immune response would be at an early stage and likely not protective against a heterologous virus. The experiment with EBOV using 2 naïve animals to detect transmission further supports that infectious virus did not cross-contaminate neighbouring animals under these conditions.

The second experiment examining the transmission of EBOV used the open-barred cages without any protective barriers. In natural settings, humans become infected through contact with infected bodily fluids, mainly following direct interactions with infected individuals or animals. Experimentally, one early study described transmission between infected NHPs to a naive NHP that occurred without direct contact, presumably due to close proximity of the animals[26]. This study raised the possibility of airborne transmission between primates although transmission due to husbandry practices could not be completely ruled out. Another study using the open-barred cage system demonstrated that pigs infected with EBOV could transmit the virus to four nearby uninfected NHPs without the possibility of direct contact between the 2 species[27]. In the current study, two NHPs were lethally infected with EBOV, and no EBOV virus or antibodies to EBOV GP were detected in the neighbouring uninfected NHPs for up to 28 days after the challenge date. At 6 dpi the EBOV-1 and -2 infected NHPs had high viral titres of infectious particles in the blood, however, only non-infectious particles could

Table 3 | Antibody end point titration and cross-reactivity of neighbouring NHP. NHP sera were assayed for virus- specific antibody by ELISA where the dilution value is the limit of detection where the net OD of the last dilution is considered positive

Animal	Dpi	Antibody	Antigen & Sera Dilution	Antigen & Sera Dilution
			CCHFV	**RFV**
CCHFV-1	9	IgM	1/400	-
	29	IgG	1/6400	-
CCHFV-2	10	IgM	1/1600	-
	10	IgG	1/400	-
			CCHFV	**RFV**
RFV-1	14	IgM	-	1/1600
	14	IgG	-	1/6400
RFV-2	9	IgM	-	1/400
	29	IgG	-	1/6400
			NiV	**HeV**
NiV-1	11	IgM	1/1600	N/D
	11	IgG	1/400	N/D
NiV-2	9	IgM	1/400	N/D
	29	IgG	1/6400	N/D
			JUNV	**MACV**
JUNV-1	39	IgG	1/1600	-
JUNV-2	14	IgM	1/100	-
	14	IgG	-	-
			JUNV	**MACV**
MACV-1	9	IgM	-	*
	38	IgG	-	1/1600
MACV-2	14	IgM	-	*
	14	IgG	-	-

*Western Blot Positive (-) Not Detected N/D Not Determined.

be detected in the oral, nasal and rectal swabs. The presence of transmission in the pig-NHP experiment and not the NHP-NHP experiment, both performed under similar conditions and environments, could be explained by the fact that EBOV disease in pigs is respiratory in nature with high amounts of infectious particles present in the oro-nasal cavities in the symptomatic phase of the disease which provided an opportunity for release into the environment[35]. On the receiving end, NHPs are known to be susceptible to lethal EBOV infection through the respiratory tract[24,27,31] putting the onus of the transmission on the ability of the source to shed infectious particles. Interestingly, infectious EBOV can also be found in significant amounts in the mucosa of the NHPs challenged through the airway[24,27]. Eventually, it will be important to assess the possibility of transmission between mucosally infected NHPs and naïve animals.

The ability to conduct multiple NHP experiments with different viruses in one room in a CL4 lab is a viable option. The current study demonstrates that airborne transmission of EBOV between NHPs does not occur readily, and it suggests that the route of exposure may impact shedding and the subsequent opportunity for transmission. However, many parameters must be examined in order to determine the level of barriers required. These include, the ability of the virus to become airborne, which would increase transmissibility; the infective dose used, as low NiV doses tend to cause a neurological infection while higher doses result in respiratory infections[19]; the route of infection can result in a more severe, or attenuated disease; the virulence or pathogenicity of the virus strains used; and the animal species used as a particular virus will not cause disease in all NHPs. Additionally, in conducting multi-virus experiments the potential arises for segmented viruses to reassort[36,37]. Reassortment in bunyaviruses is considered a rare event, although phylogenetic analysis suggests it can occur. However, it is more probable with closely related viruses; and in the insect which has the greatest likelihood of being co-infected due to multiple blood meals (reviewed in[38]). To date there is no direct evidence of reassortant bunyaviruses emerging

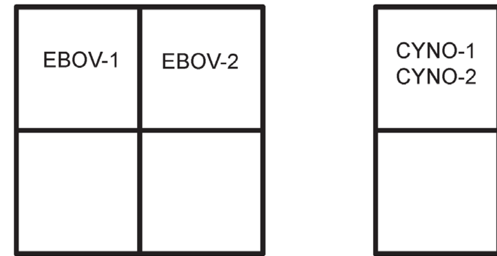

Figure 2 | Cage arrangement for Experiment #2: EBOV Transmission. The EBOV quad was approximately 0.3 m from the Cyno cages. A biobubble was not used therefore airflow was not directed with the use of Plexiglas panels and the use of the HEPA filtered exhaust. Subjects EBOV-1 and EBOV-2 were the EBOV infected rhesus macaques, and the cyno-1 and cyno-2 were the uninfected cynomolgus macaque controls.

due to co-infection in an animal. As for arenaviruses, despite reassortment occurring *in vitro*, the risk of recombining segments is low, possibly due to superinfection exclusion; and as yet no natural reassortants have been detected for the arenaviruses[39,40]. The current study shows that viral transmission was not detected between NHP groups infected with segmented viruses of the same family (RVF/CCHFV and LASV/MACV) therefore minimizing the possibility for reassortment. Overall, depending upon the virus being used and the disease it causes in the particular NHP species, local risk assessments may be an efficient way to determine the appropriate level of barriers, if any, to put in place in order to perform work and meet all expectations within a high biocontainment laboratory.

Methods

Viruses. The *Bunyaviridae* family was represented by Rift Valley Fever virus (RFV) strain Kenya and Crimean Congo Hemorrhagic Fever virus (CCHFV) strain IbAR10200; The *Arenaviridae* family was represented by Machupo virus (MACV) strain Carvallo and Junin virus (JUNV) strain XJ-13; *Paramyxoviridae* family included Nipah virus (NiV) strain Malaysia and Hendra virus (HeV). The *Filoviridae*

Table 4 | Viral RNA detected by qRT-PCR targeting the L gene of EBOV. The reported value is $\log_{10}$ genome copies/ml of sample tested

	dpi	EBOV-1	EBOV-2	Cyno-1	Cyno-2
Oral Swab	0	-	-	-	-
	3	-	-	-	-
	6/7*	5.3	4.2	-	-
	15	N/D	N/D	-	-
	28	N/D	N/D	-	-
Nasal Swab	0	-	-	-	-
	3	-	-	-	-
	6/7*	5.1	-	-	-
	15	N/D	N/D	-	-
	28	N/D	N/D	-	-
Rectal Swab	0	-	-	-	-
	3	-	-	-	-
	6/7*	-	5.2	-	-
	15	N/D	N/D	-	-
	28	N/D	N/D	-	-
Blood	0	-	-	-	-
	3	3.6	5.1	-	-
	6/7*	6.3	7.1	-	-
	15	N/D	N/D	-	-
	28	N/D	N/D	-	-

*EBOV-1&-2 were sampled on day 6 whereas CYNO-1 &-2 were sampled on day 7.
N/D Not Determined as the EBOV-1 & -2 died at 6 dpi.
(-) Not detected.

family included species *Zaire ebolavirus*, virus Ebola virus(EBOV) strain Kikwit. All viruses were propagated in Vero E6 cells by adding a 1/1000 dilution of the stock virus and incubating at 37°C, 5% CO_2 for 3–4 days. The cells were scraped off the flask, centrifuged at 500 × g for 10 minutes, and the supernatant aliquoted into cryovials and stored at −70°C. EBOV titration was performed using the $TCID_{50}$ assay, described below. For all other viruses the titration was performed using the standard immuno-plaque assay as follows. Media was removed from Vero E6 cells that are 80% confluent in 24-well plates, then 100 ul of a 10-fold serial dilution of the virus in DMEM-2% FBS was added. After a 1 hour incubation, 1.5% carboxymethyl cellulose (CMC)-Eagle-MEM 5% FCS was added, and incubated for 3–4 days, before washing out the CMC with PBS 3 times. The cells were fixed with 10% Formalin, then incubated with 0.05% Triton X-100/PBS for 15 minutes, before blocking with 1% BSA/PBS. After a PBS wash the cells were incubated with viral specific antibody for 30–60 minutes, washed with PBS, then incubated with a secondary anti-IgG-FITC conjugated antibody for 30–60 minutes at room temperature (RT). The cells were washed in PBS and then the foci counted. The following formula was used to calculate the titres infectious immunofluorescent forming unit: IFU/ml = number of foci x10xdilution.

Nonhuman Primate Experiments. Two separate experiments were conducted as indicated below. Animal studies were performed under CL4 conditions and approved by the Canadian Science Centre for Human and Animal Health Animal Care Committee following the guidelines of the Canadian Council on Animal Care. Animals received commercial monkey chow, treats, vegetables and fruit. Husbandry enrichment consisted of commercial toys and visual stimulation. NHPs were acclimatized for 10 days prior to infection.

Experiment #1: consisted of 12 cynomolgus macaques grouped into five groups of two animals, and each group was challenged with either CCHFV, RFV, NiV, HeV, JUNV or MACV. The doses and routes of infection are shown in Table 1. Each bank of NHP cages was a quad with 4 single units arranged two above and two below. Each quad was surrounded by a floor to ceiling plastic curtain "biobubble" around the sides and back, with a HEPA filter at the top right corner for directional air flow (Figure 1). Three mm plexiglass panels were placed between cages to direct flow towards the HEPA filter. The front was left entirely open with no curtains or other barrier. Each quad housed one virus family (ie Arenaviridae) grouped such that the (A) top and bottom left side contained one virus (ie MACV) and the (B) top and bottom right side housed the other virus (ie JUNV) from the same family (Figure 1). During the course of infection animals were sampled at 3, 6, and 9 days post infection (dpi) plus day 29 for RFV. At each time point blood, nasal, oral and rectal swabs were collected and the swab samples tested for viral RNA, and the serum for IgM and IgG antibodies.

Experiment #2: consisted of 2 Rhesus macaques challenged intramuscularly (im) with 3000 $TCID_{50}$ EBOV and 2 uninfected cynomolgus macaques. Rhesus macaques were selected because they survive EBOV challenge for a longer period of time offering more time for transmission while cynomolgus macaques are more sensitive and succumb faster, on average, possibly offering a more sensitive way of detecting transmission. Cages were arranged as a double and a quad with cages each on top and bottom. Plexiglass and floor to ceiling curtains with a HEPA filter were not used with the open-barred cages, thereby allowing a possible spread of virus to occur through the ambient air. During the course of the infection animals were monitored by sampling the Rhesus macaques at 0, 3, and 6 dpi; and the cynomolgus macaques at 0, 3, 7, 15, and 28 dpi. At each time point blood, nasal, oral and rectal swabs were collected and the swab samples were tested for viral RNA and the serum for antibodies. Samples that were positive by RT-PCR were then assayed in a $TCID_{50}$ assay in order to quantitate infectious particles.

qRT-PCR. For RNA isolation from blood and swabs for experiment #2 and blood samples from experiment #1, the QIAamp Viral RNA Mini Kit (Qiagen) was used as per manufacturer's protocol. For the swabs in experiment #1 the Nucleospin 96 Virus core kit (Macherey-Nagel) was used with the CAS-1820 X-tractor Gene instrument (Corbett). Detection of RNA was by qRT-PCR using the LightCycler 480 RNA Master Hydrolysis Probe kit (Roche). Reaction conditions were the following; 63°C – 3 minutes, 95°C – 30 seconds and cycling of 95°C – 15 seconds, 60°C – 30 seconds for 45 cycles on the LightCycler 480 (Roche). In house designed primers and probes were designed using the Primer Express 3.0 software (ABI) except for the CCHFV primers which were described by Wölfel[41]. The primers for Ebola were designed to pick up the L gene. The primers are as follows; MACV (Forward-CGATRTGATGAATCTG-GTTAGCAAA, Reverse-TCYCCRTCAAARAGGAATCAA, Probe-FAM-TAYCT-YAATCCTTGTAGAAAGG-MGB), JUNV (Forward-CATCTTCCCCTTCACCC-AAA, Reverse-CTGGATCAGAGGTGCTGATTCA, Probe-FAM-TTGTCTG-GAAAAGTTCCACAGCCATCCT-BHQ-1), RVF (Forward-ATCATRTGCCTT-GGGTATGC, Reverse-TGAGTGGCTTCCTGTCACTG, Probe- ALX532-AGGGGATAGGCCRTCCATGGTDGTC-BHQ-1), HeV (Forward-CTGGGCA-TACGGAGATTCTG, Reverse-ATCAATGTTGACCCCTCTGG, Probe-Alx532-TTGGTATGAGGCTTGGTACTTGGCTTC-BHQ-1), NiV (Forward-CAAAA-CAGAGATGCGAGCAG, Reverse-ATGCATGAATCTGAACGGAA, Probe-FAM-GATCAAGAATTCRCAAAAGCCGAAA-BHQ-1), EBOV (Forward-CAGCCAG-CAATTTCTTCCAT, Reverse-TTTCGGTTGCTGTTTCTGTG, FAM-ATCATT-GGCGTACTGGAGGAGCAG-BHQ-1).

ELISA. *Experiment #1.* Plates were coated overnight at 4°C with 100 ul/well of irradiated cell lysate infected with the respective virus at the following dilutions: 1 : 500 (JUNV, MACV), 1 : 1000 (RFV), or 1 : 2000 (NiV), (CCHFV NP protein provided by Brian Mark). The lysates were removed and the plates blocked with PBS, 5% skim milk, 0.1% Tween 20. NHP test sera were diluted starting at 1 : 100 in a 4 fold dilution series to determine antibody endpoint in blocking buffer. The bound antibody was detected with a secondary goat anti-human IgG or IgM horseradish peroxidise-conjugated antibody (KPL Inc.) along with using 3% ABTS Peroxidase Substrate (KPL Inc.). Plates were read at 405 nm optical density (OD_{405}) and values higher than 1.0 were considered positive for the presence of anti-(specific virus) antibodies.

Experiment #2. High binding polystyrene microtitre half well plates were coated overnight at 4°C with 30 ul 1 ug/ml recombinant EBOVZaire GPΔTM protein (IBT Bioservices). After blocking for 1 hour, 37°C with PBS, 5% skim milk, the block was removed, and 30 ul of the sera, diluted 2 fold in PBS, 2% skim milk, was added and incubated for another 1 hour at 37°C. The plate was washed 6 times with 150 ul PBS, 0.1% Tween 20) before adding 30 ul of a secondary HRP conjugated goat anti-human IgG (KPL) or anti-NHP IgM (Rockland) antibody. After one hour incubation at 37°C the plates were washed again and 30 ul of ABTS + Peroxidase Substrate (KPL) were added for 30 minutes at RT before reading at 405 nm on the Versa Max plate reader. The day 0 uninfected sera for each NHP was used as the negative control, and each serum dilution was subtracted from the equivalently diluted infected serum. A sample was considered positive if it was more than 2 times the standard deviation of the equivalent dilution of the day 0 sample for that NHP. The samples were run in triplicate.

$TCID_{50}$ Assay. Forty ul of blood, or media from swabs were diluted in 360 ul of DMEM, 2% FBS before performing a 10-fold serial dilution. Vero E6 cells were seeded in 96 well flat bottom tissue culture plates the day before so they would be ~90% confluent on the day of the assay. The media was removed from the cells and 100 ul of the diluted sample was added to the well, in triplicate. After an 1 hour incubation at 37°C, 5% CO_2 the inoculum was removed and 100 ul of DMEM, 2% FBS was added. After 14 days the wells showing cytopathic effect were tabulated for each dilution and the $TCID_{50}$ was calculated according to the Spearman and Karber algorithm.

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Author contributions

J.A., A.L. and G.K. wrote the manuscript and prepared figures. J.A., A.L., S.J., J.G., X.Q., L.F., A.G., J.S. and G.K. conducted the NHP experiments. J.A., A.L., S.J., B.B., G.W., U.S., A.G., J.S. and G.K. conducted the assays and data analysis.

Additional information

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