Detection of Ebola Virus RNA Through Aerosol Sampling of Animal Biosafety Level 4 Rooms Housing Challenged Nonhuman Primates

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Ebola virus disease is a serious illness of humans and non-human primates (NHPs). Direct contact has been shown to be the primary source of Ebola (EBOV) transmission. We used a high-volume air sampler to determine whether EBOV could be detected during 3 independent studies with EBOV-challenged NHPs. Viral RNA was recovered during days 9 and 10 of Study I and days 7 and 8 of Study III. Viral RNA levels were below limits of detection during all other collections. The results demonstrate that the biosafety level 4 (BSL-4) suit protects workers from aerosols in a BSL-4 environment using proper engineering and administrative controls.

Keywords. nonhuman primate, Ebola, aerosol transmission, BSL-4, animal model, aerosol sampling

Ebola virus disease is a serious illness of humans and nonhuman primates (NHPs) caused by the Ebola virus (EBOV). Five strains (Bundibugyo, Reston, Sudan, Tai Forest, and Zaire) of EBOV have been isolated around the world [1, 2]. Disease progression is rapid; early stages that may be limited to fever and rash progress to more advanced stages that may include vomiting, diarrhea, severe hemorrhage, multiorgan failure, and ultimately death. Because no US Food and Drug Administration (FDA)–approved vaccines or therapeutics are currently available, case management and strict infection control measures must be put in place to limit disease transmission.

Transmission of EBOV primarily has occurred among close contacts providing medical care for affected individuals [2, 3]. Nosocomial infections have been documented among healthcare workers relying on varying levels of personal protective equipment [1–3]. Although direct contact has been shown to be the primary source of EBOV transmission, the role of aerosol transmission in human disease is unclear. Humans have been shown to harbor EBOV in body fluids, including saliva, during peak viremia and could potentially expel infectious aerosols through periods of vomiting, diarrhea, or hemoptysis [2]. However, there is little evidence of aerosol transmission of EBOV outside of animal models [2]. Environmental sampling in hospitals has shown that EBOV is stable on surfaces in the presence of blood, but standard barrier protection has been effective at preventing nosocomial infections, provided that strict infection control procedures are followed [4].

Transmission of EBOV has occurred between challenged and naïve NHP populations under animal biosafety level 4 (ABSL-4) conditions [5]. Researchers discovered pathological findings within the pulmonary tract of the affected NHPs, supporting the potential of aerosol transmission occurring, and a recent study demonstrating pig-to-NHP transmission of EBOV generated similar findings [5, 6]. Neither study was able to rule out fomite or direct contact during husbandry practices as a possible source of transmission [5, 6]. Aerosol sampling conducted during the pig-to-NHP transmission study did not recover live virus but did detect viral RNA [6]. Attempts to replicate the study conditions using NHP-to-NHP transmission models were unsuccessful [6].

Because of the inconsistent evidence of EBOV aerosol transmission and the presence of infectious aerosols during EBOV-challenged NHP studies, we used a high-volume air sampler to determine whether EBOV viral RNA could be detected over the course of an NHP EBOV study. Previous studies evaluating the efficacy of aerosol sampling have shown little difference between the all-glass impinger and membrane filter when similar sampling rates are used [7]. By using a high-volume air sampler with sampling rates up to 2800 L/min compared with air samplers that use sampling rates of 5–50 L/min, we expected to enhance our ability to detect any potential aerosolized EBOV RNA within a challenged NHP room under ABSL-4 conditions [7, 8]. Air sampling was conducted during three separate NHP studies across three distinct ABSL-4 suites with independent husbandry staffs for each study. Corridor sampling also occurred to determine whether the combination of engineering and administrative controls relied on during BSL-4 operations are sufficient to contain any aerosolized EBOV RNA.

METHODS

Direct Inoculation
Membrane gel filters (47 cm) combined with polyester felt filters (1 µm, 1 7/8” diameter) were directly inoculated with 1 mL of...
EBOV (Kikwit95, 4 \times 10^6 pfu/mL) within a Class II biological safety cabinet (BSC) in a BSL-4 suit laboratory. Inoculated filters (n = 3) were then placed inside 50-mL conical tubes containing 25 mL of minimal essential media (MEM). Tubes were shaken then placed in a 37°C incubator for 1 hour. Samples were then stored at \(-80^\circ\)C until inactivation. Samples were inactivated with TRIzol LS Reagent as previously published and removed from the BSL-4 laboratory for RNA extraction and real time polymerase chain reaction (RT-PCR) analysis [9, 10].

Class III BSC Sham Spray
Filters were placed inside an aerosol exposure chamber housed within a Class III BSC contained in a BSL-4 laboratory. The Class III BSC was maintained under negative pressure. A sham spray using a 200-pfu/mL aerosol of Ebola Sudan (SUDV) virus was created by a 3-jet Collison nebulizer and controlled by the automated bioaerosol system. Total spray time was 10 minutes. Filters were then transferred to the BSL-4 suit laboratory for incubation and inactivation.

Study I
Research at USAMRIID was conducted under an Institutional Animal Care and Use Committee–approved protocol in compliance with the Animal Welfare Act, Public Health Service policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals. Twenty experimentally naïve rhesus macaques (Macaca mulatta; 5.1–10.0 kg; aged 4.9–9.4 years) were challenged with 1000 pfu of EBOV following acclimation in the BSL-4 laboratory. The ABSL-4 room temperature was maintained at 25°C with a relative humidity (RH) of 50% during all three studies. High-volume air samplers (Dry Filter Unit [DFU] 1000) were placed both within the laboratory and in the adjacent corridor. Samplers housed the membrane gel and polyester felt filters and ran continuously at a sampling rate of 2800 L/min throughout the study. Samples were collected for a 24-hour period on days 0, 1, 5, 6, 7, 8, 9, 10, 14, and 28 of the study. Samples were then placed in MEM, incubated, and held at \(-80^\circ\)C until inactivation, extraction, and PCR analysis.

Study II
Twelve experimentally naïve, Asian-origin, adult cynomolgus macaques (Macaca fascicularis; \(\geq 22.9\) kg; aged 3 to 6 years) were acclimated and then challenged with 1000 pfu of EBOV (Kikwit95 8 U variant) through either the intramuscular or aerosol route as previously reported [11]. The DFU 1000 containing both the membrane and polyester filters was placed within the animal room and run continuously at a sampling rate of 2800 L/min through the duration of the study. Samples were collected within the animal room at identical time points to Study I. Samples were then placed in MEM, incubated, and held at \(-80^\circ\)C until inactivation, extraction, and PCR analysis.

Viral Extraction
An aliquot of 70.0 µL of each TRIzol-inactivated sample was obtained, and nucleic acid was extracted using the QIAamp Viral RNA Mini Kit following the manufacturer’s protocol (spin protocol).

Polymerase Chain Reaction Analysis
RNA was detected with an EBOV glycoprotein-specific reverse transcriptase RT-PCR assay [12]. All assays were analyzed with an Applied Biosystems 7500 Fast Dx RT-PCR Instrument according to the manufacturer’s operating instructions and assay conditions described in the original literature [12].

RESULTS

Proof of Concept
All of the filters that were either directly inoculated or placed within the aerosol exposure chamber tested positive for EBOV (direct inoculation) or SUDV (aerosol chamber) RNA.

Study I
Twelve of twenty challenged NHPs survived until the end of the study. Eight NHPs had viral RNA detected in their blood and were euthanized by day 10. All animal room corridor samples were below limits of detection (LOD; 584 copies/PCR reaction) by PCR. Samples taken within the animal room on day 9 and day 10 of the study were positive for EBOV by PCR (Table 1).

Study II
Four of twelve challenged NHPs survived until the end of the study. The remaining eight NHPs were euthanized by study day 10 due to disease progression and clinical scoring criteria. No aerosol sampling was conducted in the adjacent corridor due to...
safety considerations (Figure 1). All animal room samples collected were below LOD for EBOV by PCR (Table 1).

**Study III**

Ten of eighteen challenged NHPs survived through the duration of the study. Six expired by study day 9. Two additional NHPs were euthanized on study days 10 and 24. Samples taken on day 7 and day 8 within the animal room were positive for EBOV RNA (Table 1), but all other samples taken during the study both in the animal room and the containment corridor were below LOD.

**DISCUSSION**

Since EBOV was first discovered in 1976, there has been limited physical evidence of aerosol transmission among human contacts [2]. Aerosol transmission of EBOV among NHPs in the laboratory setting has been proposed, and NHPs have been effectively challenged through the aerosol route [5, 11]. Aerosol transmission of EBOV from pigs to NHPs has been demonstrated, but similar attempts between NHPs have been unsuccessful [6]. High-volume air samplers have been primarily used within the emergency response setting as a rapid test to determine potential presence of infectious agents. Their combination of durability and ability to continuously collect samples over extended periods makes them ideal for use inside an animal room where high humidity and extensive personnel movement are expected. By sampling at rates up to 500× higher than traditional samplers, we attempted to determine whether aerosols containing EBOV RNA are present during NHP studies under ABSL-4 conditions [7]. In these studies, PCR analysis was used because virtually no intact virus would be recovered due to desiccation.

Sampling and PCR analysis was able to recover EBOV RNA from the animal rooms in Study I and Study III. Air samplers were placed approximately three feet from the nearest animal cage to minimize the potential for EBOV RNA capture through droplets or excretions. Air samplers were also placed outside of any expected foot patterns for staff operations to minimize the potential for fomite collection. Both Study I and Study III relied on intramuscular challenges, whereas Study II contained intramuscular- and aerosol-challenged NHPs. It would be expected that Study II would have generated similar results, but minor variables, including room layout and airflow patterns along with NHP cage placement, could have impacted results. A more likely explanation is that aerosol generation of EBOV by NHPs on study is an infrequent occurrence and is condition dependent.

This explanation is consistent with previously published reports of wide ranges of EBOV transmissibility during human disease outbreaks [2]. This would also explain why aerosol

### Table 1. Results From the Aerosol Sampling Conducted During Studies I, II, and III

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Corridor Sampling was conducted during Studies I and III. Samples were analyzed through polymerase chain reaction, and positives are reported as an average (n = 3) C, Value ± SD. Abbreviations: NC, negative control; ND, not detected; NS, no sampling conducted; PC, positive control; POS, positive.
transmission between challenged and naive NHPs is indicated in some cases but difficult to detect in others [5, 6]. Ebola virus RNA–positive samples were only observed days 7–10 of infection, which lies within the advanced stages of infection with elevated viral shedding [11]. Furthermore, EBOV has been shown to form stable droplet nuclei for up to three hours in laboratory settings, demonstrating that any aerosols generated by NHPs could remain airborne for extended periods [13]. The low observed threshold cycle (Ct) values indicate that aerosol transmission of EBOV does not represent a significant hazard to BSL-4 suit workers but must be taken into consideration for clinicians in outbreak settings outside of the BSL-4 environment. It has been shown previously that even BSL-4 suits with compromised integrity offer workers a protection factor in excess of 10⁵ [14]. This is because inward airflow of 6 cubic feet per minute at 40 psi into the BSL-4 suit greatly exceeds the capacity of the human pulmonary system [15]. The absence of any positive samples within the adjacent corridors during sampling demonstrate that the combination of engineering controls, administrative controls, and the work practices of research and support staff in an ABSL-4 setting can sufficiently contain any aerosols that may be generated during NHP EBOV studies. This study shows that viral RNA can be detected through aerosol sampling during NHP studies. More important, ABSL-4 suits can contain these aerosols, and BSL-4 suits provide sufficient protection from aerosols even if their integrity is compromised.

Notes

Disclaimer. The views, opinions, and/or findings contained herein are those of the authors and should not be construed as an official Department of Army position, policy, or decision unless so designated by other documentation.

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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