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

David E. Harbourt[1]*, Sara C. Johnston[1], James Pettitt[2], Travis K. Warren[1] and William R. Dorman[1]

[1]United States Army Medical Research Institute of Infectious Diseases (USAMRIID)
1425 Porter Street
Ft. Detrick, MD 21702

[2]Integrated Research Facility
8200 Research Plaza
Ft. Detrick, MD 21702

*corresponding author
David.e.harbourt.civ@mail.mil
301 619 9875
301 619 4768
Author Information:
Sara C. Johnston[1]: 301-619-4695; Sara.c.johnston2.ctr@mail.mil
James Pettitt[2]: 301-631-7269; James.pettitt@nih.gov
Travis K. Warren[1]: 301-619-3414; Travis.k.warren.ctr@mail.mil
William R. Dorman[1]: 301-619-3318; William.r.dorman4.civ@mail.mil

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1425 Porter Street
Ft. Detrick, MD 21702
(301) 619-9875 Office
(301) 619-4768 Fax
David.e.harbourt.civ@mail.mil

To whom it may concern,

My colleagues and I are submitting the attached manuscript, *Detection of Ebola Virus RNA through Aerosol Sampling of ABSL-4 Rooms Housing Challenged Nonhuman Primates* for publication in an edition of The Journal of Infectious Disease. This manuscript has not been submitted for publication in any other journal and all authors have fulfilled the criteria for authorship. No writing assistance was provided to the authors during the preparation of this manuscript. We have chosen the "Brief Report" format as the best fit for this article. There is one figure and one table covering the experimental setup and results that could be call out boxes or sidebars or simply figures embedded in the texts. This is the first report demonstrating detection of Ebola virus RNA from animal rooms housing infected nonhuman primates and the first report utilizing the Dry Filter Unit 1000(DFU 1000) high volume air sampler for detection of aerosolized viral RNA under BSL-4 conditions. The results obtained in this report can continue to add the discussion topic of potential aerosol transmission of Ebola virus and be of considerable interest to your publication's readers. Five potential reviewers for this paper would be:

- Dr. Heinz Feldmann, Director of NIH Rocky Mountain Laboratories, heinrich.feldmann@nih.gov, Dr. Feldmann is an internationally recognized expert on Ebola and other BSL-4 viruses
- Dr. Jens Kuhn, Virologist at NIH Integrated Research Facility at Ft. Detrick, MD, jens.kuhn@nih.gov, Extensively published expert on Ebola genealogy and filoviruses
- Dr. Peter Jahrling, Director of NIH Integrated Research Facility at Ft. Detrick, MD, peter.jahrling@nih.gov, Former USAMRIID virologist and Ebola expert
- Dr. Arthur Goff, Supervisory Microbiologist at USAMRIID, arthur.j.goff.civ@mail.mil, Expert on Ebola virus disease and NHP animal models of filovirus infections
- Dr. Robert Hawley, Private Consultant, bobhawley226@gmail.com, Former Biosafety Officer and Director of Safety at USAMRIID who is a board certified expert in biosafety and applied biosafety research

Please contact me with any questions about the content or format of the paper. We look forward to your reply.
Sincerely,

David Harbourt

ABSTRACT (259 words)

Ebola Virus Disease is a serious illness of humans and nonhuman primates (NHPs) caused by the Ebola Virus (EBOV). Historically, transmission of EBOV has occurred primarily among close contacts and family members providing medical care for affected individuals. While direct contact has been shown to be the primary source of EBOV transmission, there is limited evidence of aerosol transmission among humans. Due to the inconsistent evidence of EBOV aerosol transmission, we utilized a high volume air sampler to determine if EBOV viral RNA could be detected over the course of a study with NHPs challenged with EBOV. Air sampling was conducted during three separate NHP studies across three distinct ABSL-4 suites with independent husbandry staffs for each study. Initial proof of concept studies demonstrated EBOV viral RNA recovery from filters placed within a Class III BSC during a sham spray. Viral RNA was recovered during day 9 and 10 of Study I and day 7 and 8 of Study III. Viral RNA levels were below limits of detection during all other sample collection time points. C_T values for positive samples were between 34.43 and 39.79 indicating a small amount of viral RNA was recovered from the filters despite continuous sampling. The combination of the low levels of viral RNA observed during the studies combined with the protection factor of the BSL-4 positive pressure encapsulating suit (in excess of 10^5 even in the event of a breach) demonstrate that BSL-4 workers are protected from potentially infectious aerosols in a BSL-4 environment provided that proper engineering and administrative controls are implemented.

Key words: nonhuman primate, Ebola, aerosol transmission, BSL-4

INTRODUCTION (Total manuscript word count: 1996)

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, multi-organ failure and ultimately death. Because no FDA-approved vaccines or therapeutics are currently available for treatment, 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 [2-5]. Nosocomial infections have been documented among healthcare workers relying on varying levels of personal protective equipment [1-5]. While direct contact has been shown to be the primary source of EBOV transmission, the role of aerosol transmission in human disease is debatable. Humans have been shown to harbor EBOV in body fluids during peak viremia including saliva, 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 proven effective at preventing nosocomial infections, provided that strict infection control procedures are followed [6].

Transmission of EBOV has occurred between challenged and naive NHP populations under animal biosafety level 4 (ABSL-4) conditions [7]. 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 [7, 8]. Neither study was able to rule out fomite nor direct contact during husbandry practices as a possible source of transmission [7, 8]. Aerosol sampling conducted during the pig-to-NHP transmission study did not recover live virus but did detect viral RNA (8). Attempts to replicate the study conditions utilizing NHP-to-NHP transmission models were unsuccessful (8).

Due to the inconsistent evidence of EBOV aerosol transmission and the presence of infectious aerosols during EBOV-challenged NHP studies, we utilized a high volume air sampler to determine if EBOV viral RNA could be detected over the course of a NHP EBOV study. Previous studies evaluating the efficacy of aerosol sampling have shown little difference between the all glass impinger (AGI) and membrane filter when similar sampling rates are employed [9]. By using a high volume air sampler with sampling rates up to 2800 L/min compared with standard air samplers that utilize sampling rates between 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 [9, 10]. 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 if 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, Sartorius AG, Goettingen, Germany) combined with polyester felt filters (1µm, 1 7/8" diameter) were directly inoculated with 1mL of EBOV (Kikwit95, $4*10^6$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; Gibco Life Technologies, Carlsbad, CA). Tubes were shaken then placed in a 37ºC incubator for one hour. After incubation, samples were stored at -80ºC until inactivation. Samples were inactivated with TRIzol LS Reagent (Sigma-Aldrich, St. Louis, MO) as previously published and removed from the BSL-4 laboratory for RNA extraction and RT-PCR analysis (11, 12).

Class III BSC sham spray

Membrane gel filters combined with polyester felt 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 utilizing a 200pfu/mL aerosol of Ebola Sudan (SUDV) virus was created by a 3-jet Collison nebulizer (BGI Incorporated, Waltham, MA) and controlled by the automated bioaerosol system. Total spray time was ten minutes. Following the

spray, the filters were transferred to the BSL-4 suit laboratory for incubation and inactivation.

Study I

Research at USAMRIID was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS 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*, National Research Council, 2011. Twenty experimentally naïve rhesus macaques (*Maccaca mulatta*, 5.1-10.0 kg, 4.9-9.4 years of age) were challenged with 1000pfu of EBOV following acclimation in the BSL-4 laboratory. The ABSL-4 room temperature was maintained at 25ºC with a relative humidity (RH) of 50% during all three studies. High volume air samplers (Dry Filter Unit (DFU) 1000, US Army, Ft. Detrick, MD) were placed both within the laboratory and in the adjacent corridor. Samplers housed the membrane gel filter and polyester felt filter in combination and were run continuously at a sampling rate of 2800L/min throughout the study. Samples were collected for a 24h period on days 0, 1, 5, 6, 7, 8, 9, 10, 14, and 28 of the study. After collection samples were placed in MEM, incubated and held at -80ºC until inactivation, extraction and PCR analysis.

Study II

Twelve experimentally naive, adult cynomolgous macaques (*Macaca fascicularis*) of Asian origin, mixed male and female, weighing ≥ 2.9 kg were acclimated then

challenged with 1000pfu of EBOV (Kikwit95 8U variant) through either the IM or aerosol route as previously reported [13]. The DFU 1000 containing both the membrane and polyester filters was placed within the animal room and run continuously at a sampling rate of 2800L/min through the duration of the study. Samples were collected within the animal room at identical time points to Study I. Following collection, samples were placed in MEM, incubated and held at -80ºC until inactivation, extraction and PCR analysis.

Study III

Sixteen experimentally naïve, Indian origin rhesus macaques (*Macaca mulatta*, 3.4–4.7 kg, 1.5–2.9 years of age) were acclimated then challenged with 1170pfu of EBOV intramuscularly. The DFU 1000 was placed within the animal room and the adjacent corridor and run continuously at a sampling rate of 2800L/min for the duration of the study. Samples were collected at identical time points to Study I and Study II and then placed in MEM, incubated and held at -80ºC until inactivation, extraction and PCR analysis.

Viral extraction

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

PCR Analysis

RNA was detected with an EBOV glycoprotein-specific reverse transcriptase RT-PCR assay [14]. All assays were analyzed with an Applied Biosystems 7500 Fast Dx

RT-PCR Instrument (Thermo Fisher Scientific Inc, Grand Island, NY).according to the manufacturer's operating instructions and assay conditions described in the original literature [14].

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 from 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 related to extension cord usage and trip hazards in the containment corridor (**Figure 1**). All animal room samples collected were below LOD for EBOV by PCR (**Table 1)**.

Study III

Six of eighteen challenged NHPs expired by study day 9. One additional NHP expired on study day 10 while another was euthanized due to disease complications on day 24. The additional ten NHPs survived through the duration of the study. Samples taken on Day 7 and Day 8 from 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, evidence of aerosol transmission among human contacts has remained theoretical with little physical evidence supporting this assertion [2]. Aerosol transmission of EBOV among NHPs in the laboratory setting has been proposed and NHPs have been effectively challenged through the aerosol route [7, 13]. Aerosol transmission of EBOV from pigs to NHPs has been demonstrated but similar attempts at replicating the results between NHPs have been unsuccessful [8]. High volume air samplers have been primarily used within the emergency response setting as a rapid test to determine if infectious agents are potentially located within an affected area. Their combination of durability and ability to continuously collect samples over extended periods make them ideal for use inside an animal room where high humidity and extensive personnel movement are expected. By sampling at rates up to 500X higher than traditional samplers, we attempted to determine if aerosols containing EBOV RNA are present during NHP

studies under ABSL-4 conditions [9]. In these studies, PCR analysis was employed since 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 laboratory and husbandry staff operations to minimize the potential for fomite collection. Both Study I and Study III relied on IM challenges only while Study II contained IM and aerosol challenged NHPs. It would be expected that Study II would have generated similar results to the other studies 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 transmission between challenged and naïve NHPs is indicated in some cases but difficult to detect in others [7, 8]. In addition, EBOV RNA positive samples were only observed between days 7-10 of infection which lies within the advanced stages of infection when viral shedding would be at its highest [13]. The low observed C_T values indicate that aerosol transmission of EBOV does not represent a significant hazard to BSL-4 suit workers, provided that appropriate standard operating procedures and engineering controls are installed. It has been shown

previously that even BSL-4 suits with compromised integrity offer workers a protection factor in excess of 10^5 [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 importantly, ABSL-4 suites can contain these aerosols and BSL-4 suits provide sufficient protection from aerosols even if their integrity is compromised.

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LIST OF TABLES AND FIGURES

STUDY I

Days Post Challenge	Animal Room Sampling Results	Corridor Sampling Results
0	ND[a]	ND
1	ND	ND
5	ND	ND
6	ND	ND
7	ND	ND
8	ND	ND
9	**POS (39.79 ±1.00)**	ND
10	**POS (34.63 ±0.50)**	ND
14	ND	ND
21	ND	ND
28	ND	ND
PC[b]	**POS[d]**	**POS**
NC[c]	ND	ND

STUDY II

Days Post Challenge	Animal Room Sampling Results	Corridor Sampling Results
0	ND	NS[e]
1	ND	NS
5	ND	NS
6	ND	NS
7	ND	NS
8	ND	NS

9	ND	NS
10	ND	NS
14	ND	NS
21	ND	NS
28	ND	NS
PC	**POS**	**POS**
NC	ND	ND

STUDY III

Days Post Challenge	Animal Room Sampling Results	Corridor Sampling Results
0	ND	ND
1	ND	ND
5	ND	ND
6	ND	ND
7	**POS (38.13 ±2.05)**	ND
8	**POS (34.43 ±0.15)**	ND
9	ND	ND
10	ND	ND
14	ND	ND
21	ND	ND
28	ND	ND
PC	**POS**	**POS**
NC	ND	ND

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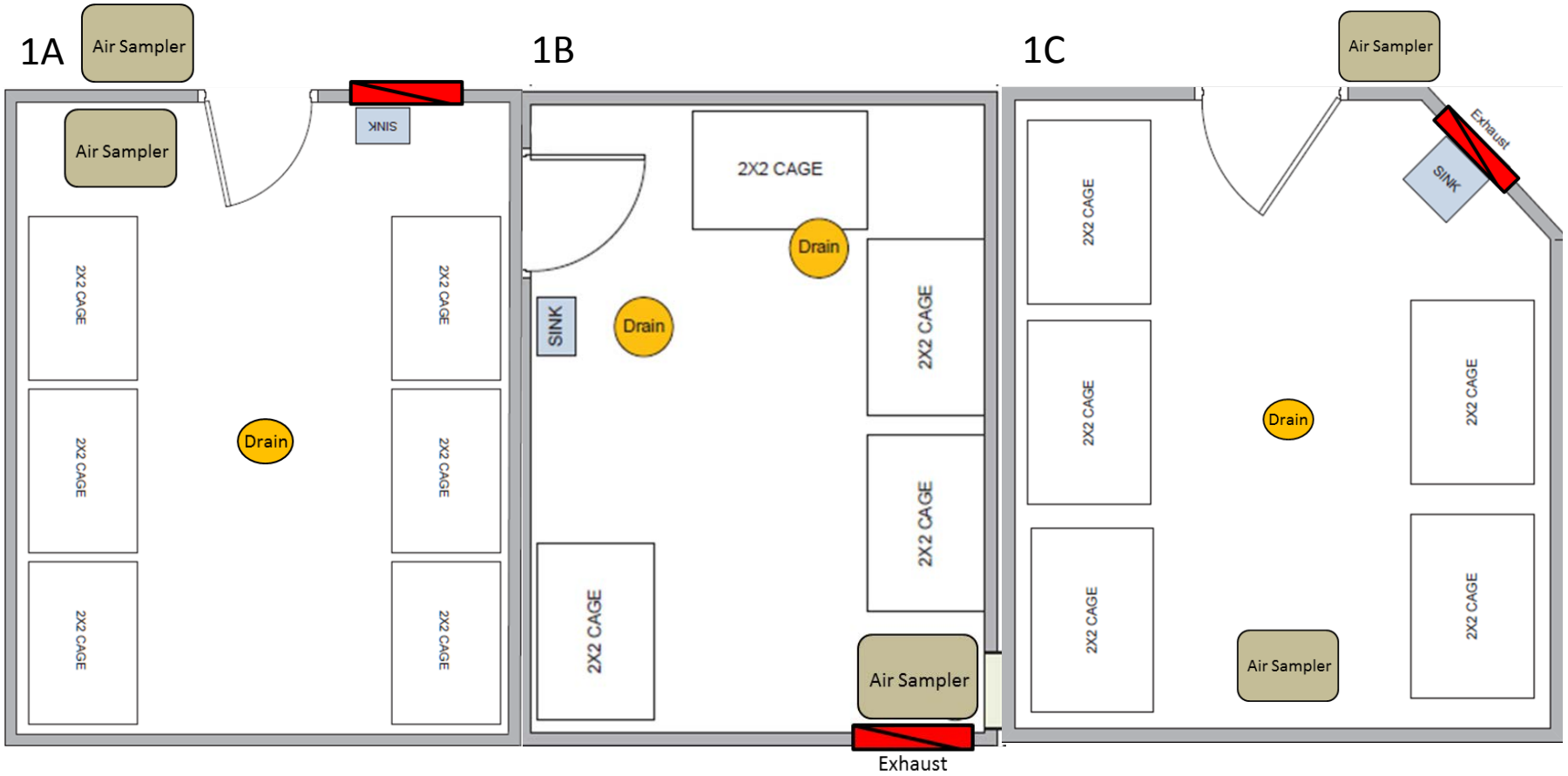

Study I

Study II

Study III