

Dead or alive: animal sampling during Ebola hemorrhagic fever outbreaks in humans

Sarah H. Olson^{1,2}, Patricia Reed³, Kenneth N. Cameron³,
Benard J. Ssebide⁴, Christine K. Johnson⁵,
Stephen S. Morse^{5,6}, William B. Karesh⁷, Jonna A. K. Mazet⁵
and Damien O. Joly^{1*}

¹Wildlife Health Program, Wildlife Conservation Society, Nanaimo, BC, Canada; ²Center for Sustainability and the Global Environment, University of Wisconsin, Madison, WI, USA; ³Wildlife Health Program, Wildlife Conservation Society, Brazzaville, Republic of Congo; ⁴Mountain Gorilla Veterinary Project, Kampala, Uganda; ⁵School of Veterinary Medicine, University of California, Davis, CA, USA; ⁶Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA; ⁷EcoHealth Alliance, New York, NY, USA

There are currently no widely accepted animal surveillance guidelines for human Ebola hemorrhagic fever (EHF) outbreak investigations to identify potential sources of *Ebolavirus* (EBOV) spillover into humans and other animals. Animal field surveillance during and following an outbreak has several purposes, from helping identify the specific animal source of a human case to guiding control activities by describing the spatial and temporal distribution of wild circulating EBOV, informing public health efforts, and contributing to broader EHF research questions. Since 1976, researchers have sampled over 10,000 individual vertebrates from areas associated with human EHF outbreaks and tested for EBOV or antibodies. Using field surveillance data associated with EHF outbreaks, this review provides guidance on animal sampling for resource-limited outbreak situations, target species, and in some cases which diagnostics should be prioritized to rapidly assess the presence of EBOV in animal reservoirs. In brief, EBOV detection was 32.7% (18/55) for carcasses (animals found dead) and 0.2% (13/5309) for live captured animals. Our review indicates that for the purposes of identifying potential sources of transmission from animals to humans and isolating suspected virus in an animal in outbreak situations, (1) surveillance of free-ranging non-human primate mortality and morbidity should be a priority, (2) any wildlife morbidity or mortality events should be investigated and may hold the most promise for locating virus or viral genome sequences, (3) surveillance of some bat species is worthwhile to isolate and detect evidence of exposure, and (4) morbidity, mortality, and serology studies of domestic animals should prioritize dogs and pigs and include testing for virus and previous exposure.

Keywords: *ebolavirus*; *disease outbreaks*; *veterinarians*; *ecology*; *vertebrates*; *epidemiology*

To access the supplementary material to this article: Appendix A, B and C, please see Supplementary files under Article Tools online.

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On May 13, 2011, a 12 year-old girl from Zirowbe, Uganda, was diagnosed with a strain of EBOV most similar to *Sudan ebolavirus* (SEBOV) (1). The Ugandan government quickly convened a national task force with a Ministry of Health-led outbreak response team, which included the World Health Organization's Regional Office for Africa (WHO/AFRO), the

United States Centers for Disease Control and Prevention (CDC), and the United States Agency for International Development (USAID) as partners. As part of the response, wildlife disease experts were asked to sample both bats and non-human primates near the outbreak site. This request prompted a discussion of historical animal sampling results related to human EHF outbreaks

by the authors, the findings of which we present in this review. Our purpose is to help field teams during an EHF outbreak identify animal species and sampling strategies that will most likely yield a positive finding by targeting species already implicated as potential reservoirs, vectors, or hosts (dead-end or otherwise) as supported by diagnostic data. This strategy carries the best chance of elucidating the possible source of the human infection, encountering a range of susceptible species within that particular context, identifying potential reservoirs, and effectively informing public health efforts.

Materials and methods

We began our review using ‘animal’, ‘wildlife’, and ‘Ebola’ as ISI Web of Knowledge search terms for reports of animal sampling efforts associated with outbreaks. Sampling efforts published in a peer-reviewed publication comprised live capture or carcass sampling efforts, during which vertebrate animals were collected from an area associated with a human EHF outbreak and tested for EBOV or antibodies. Live capture efforts often involved trapping and euthanizing live animals, whereas carcass-sampling efforts involved sampling animals that were found dead. Sampling event locations and years were recorded. Data from multiple publications were merged when it was apparent that they reported on the same sampling effort. Samples were sorted by diagnostic assay type, and if the assay type was not disclosed the study was excluded from further analysis. Diagnostic tests that directly detected the EBOV infection were often conducted with histopathologic examination of tissues or involved virus isolation in Vero cell culture, antigen capture assays, and virus-specific PCR (2, 3). Antibody tests used to detect previous exposure to EBOV included enzyme-linked immunosorbent assays (ELISA) targeting virus-specific host immunoglobulin G (IgG) antibodies (4). We classified EBOV detection assays into the two broad detection categories, virus or antibody, without adjusting for their specific reliability or validity, which has changed dramatically over the past 35 years. We considered an animal EBOV-positive after a single positive test. Further documentation of EBOV diagnostics is provided in the Appendix and the original publications. Each diagnostic test result was counted independently and in some cases both antibody and virus detection tests were carried out on one animal and therefore counted as two test samples. Species counts were estimated within each order. Sets of animals that were reported as unclassified were grouped and considered to come from one species. Scientific names were standardized according to Mammal Species of the World, 3rd edition, taxonomy (5).

Our analysis focused on animal sampling strategies associated with human EHF outbreak investigations to identify potential sources of EBOV spillover into humans

and other animals, which may further propagate transmission cycles. This objective is distinct from prospective surveillance of natural EBOV reservoir species and sylvatic cycles, which would ideally be carried out prior to outbreaks.

Results

Live animal and carcass sampling

We evaluated 14 reports of outbreak related sampling efforts, nine targeting live animals and five targeting carcasses, with test results available in peer reviewed journals, covering the period 1976–2011. Sampling efforts had a basic structure in common with adaptations for each specific outbreak. During an outbreak event, national authorities permitted teams of zoologists and veterinarians to trap, and in at least three instances, hunt individual animals for testing (2, 6, 7). The length of time between a reported human case and the first sampling ranged from 2 months prior to 22 months post outbreak (Table 1). Small game animals were also collected from bushmeat markets and researchers occasionally used payment schemes to target collection of specific species in villages (2, 6). Rodents, if sampled, were collected with Sherman and pitfall traps on trap lines and point sites, dispersed near the outbreak and hospital treatment areas (6). Bats were captured with mist nets (which are more effective in capturing some species of bats than others) at sampling sites that were not always located adjacent to an outbreak event (8–10). Unfortunately data were not always available to differentiate which bats were caught in which location, and therefore our analysis uses the pooled data from these sampling efforts (9, 10). Most live captured animals were euthanized in the field (2, 6, 7). General baseline data included location of sample (latitude and longitude), sample type (blood, tissue etc.), genus, species, identification number, sex, age, weight, morphometric measurements, and body condition. Researchers also detected carcasses often with the help of local people (11–13). For diagnostics, there was a lack of consistency in gene targets (e.g., polymerase, glycoprotein, and nucleoprotein) and procedures used for reverse transcription-PCR (RT-PCR) and molecular characterization. Publication of outbreak related animal sampling data from the 1970s through the present was delayed on average 3.4 years (Table 1). Six papers reported complementary information on two separate sets of animal sampling efforts (7, 11–15).

The average sample size of a live capture sampling effort was 1,214 individual animals and comprised 1–84 species, whereas the mean sample size of a carcass sampling effort was 19 individual carcasses comprised of 1–10 species. Together, researchers using both live and carcass sampling methods collected and tested 13,404 samples, representing roughly 158 species. In total, EBOV

Table 1. Vertebrate sampling efforts associated with EHF outbreaks

Years of sampling(s)	Location	Method	Assay	Subtype(s) assayed ^a	Reference	Year published	Sampling delay (months)
1976	DRC ^b , Yambuku	Live	Virus	Z	7	1978	2
1976	DRC, Yambuku	Live	Virus	Z	15	1978	2
1979–1980	DRC ^c	Live	Both	Z	2	1999	22
1995	DRC, Kikwit	Live	Both	Z,S,R	6	1999	7
1996	Gabon, Booué	Carcass	Virus	NA	27	1997	NA
1985–2000	Gabon & RoC ^d	Carcass	Virus	Z	14	2004	NA
2001	Gabon & RoC ^e	Carcass	Virus	Z	11	2004	1
2001–2002	Gabon ^f	Live	Antibody	Z	34 ^g	2005	NA
2002, 2003	Gabon & RoC ^h	Live	Both	Z	8	2005	4
2001–2003	Gabon & RoC	Carcass	Virus	Z	12	2005	–2
2003–2006	Gabon & RoC	Live	Antibody	Z	9	2007	20
2001–2006	Gabon & RoC	Carcass	Virus	Z	13	2007	0
2003, 2005, 2006, 2008	Gabon & RoC	Live	Antibody	Z	10	2009	0
2008–2009	Philippines	Live	Antibody	R	16	2011	>6

^aZ, S, and R indicate an assay for EBOV subtype Zaïre, Sudan, or Reston respectively. NA indicates an observation was not available.

^bDemocratic Republic of Congo (formerly Zaïre).

^cYalosemba, Tandala (DRC).

^dRepublic of Congo.

^eZaïre region (Gabon) and Lossi Sanctuary (RoC).

^fMkeambo, Ekata, and Mazingo.

^gData from human cases and animal source locations.

^hEkata (Gabon) and Mbomo (RoC).

or antibodies were detected in 1.6% (211/13,404) of animal samples and 12 species. All sampling efforts were tested for the *Zaïre ebolavirus* (ZEBOV) subtype, with the exceptions of those collected by Taniguchi et al., which reported for *Reston ebolavirus* (REBOV) subtype (16). The paucity of sampling and testing for SEBOV versus ZEBOV reflects the smaller overall number of EHF outbreaks in humans attributed to SEBOV.

Live capture samples were dominated by species from the Chiroptera (bat) and Rodentia (rodent) orders, followed by non-human primates and birds. The live capture prevalence of EBOV was 2.2% (180/8,050) for antibody tests and 0.2% (13/5,309) for virus detection (Table 2).

The prevalence of EBOV in animal carcasses found and opportunistically sampled in association with human outbreaks was 32.7% (18/55; Table 2). Overall carcass samples represented just 0.4% (55/13,404) of the collection but 58.1% (18/31) of positive virus detection samples. Two thirds of the carcasses collected were non-human primates, including gorillas, chimpanzees, and *Cercopithecus cephus* (Moustached Guenon). Fifty-nine percent (13/22) of gorilla and 50.0% (4/8) of chimpanzee carcasses tested positive for EBOV (Table 3). While antigen detection, immunohistochemical staining, and molecular tests identified EBOV, tests of degraded muscle tissue from these gorilla and chimpanzee carcasses have

yet to detect EBOV specific IgG antibodies, an indication that animals may not have mounted an IgG immune response to the infection (11, 12, 14). EBOV was detected in 16.7% (1/6) of duiker (*Cephalophus sp.*) carcasses and there are no published accounts of Chiroptera (bat) or Rodentia (rodent) carcass sampling (Table 3). The rate of carcass decay in the tropics is 4–7 days, with only bones present beyond 21 days (12,17). RT-PCR successfully detected EBOV genetic material in bone marrow from carcasses (skulls and long bones) 1 week to 1 year post mortem (12).

The yield by order, assay, and sampling method for species with a minimum of one positive EBOV test is shown in Table 3. EBOV antibody tests were used on 60.0% (8,040/13,404) of samples and virus detection tests on 40.0% (5,364/13,404; Table 2). Notably, 26% (3,474/13,349) of all live capture samples were not tested with the newer ELISA or PCR methods used to test carcass samples. If newer methods have fewer false negatives, our results could underestimate the true prevalence in live capture samples.

Domestic animal sampling

Efforts targeting domestic animals (cow, goat, sheep, pig, and dog) represented 0.9% (114/13,404) of all samples in the collection (Appendix C). With the exception of

Table 2. Number of animals sampled from 1976 through 2011 in association with EHF outbreaks

Order/totals	Live capture						Carcass		
	Antibody			Virus			Virus		
	No.	+	Yield	No.	+	Yield	No.	+	Yield
Total									
No. species	126	9		129	3		10	3	
No. samples	8040	180	2.2%	5309	13	0.2%	55	18	32.7%
Positive findings									
Artiodactyla	23	0	0.0%	58	0	0.0%	13	1	7.7%
Carnivora	87	21	24.1%	49	0	0.0%	6	0	0.0%
Chiroptera	4883	158	3.2%	1418	13	0.9%			
Non-human primates	275	0	0.0%	285	0	0.0%	33	17	51.5%
Rodentia	2431	1	0.04%	2540	0	0.0%			
Null findings									
Afrosoricida				5	0	0.0%			
Hyracoidea	7	0	0.0%	7	0	0.0%			
Macroscelidae	28	0	0.0%	29	0	0.0%			
Pholidota	66	0	0.0%	95	0	0.0%			
Proboscidea							2	0	0.0%
Soricomorpha	105	0	0.0%	123	0	0.0%			
Mammal unspecified	20	0	0.0%	124	0	0.0%			
Non-mammals									
Class Aves	85	0	0.0%	421	0	0.0%			
Class Reptilia	30	0	0.0%	155	0	0.0%			

one goat carcass, all were live samples, and all samples tested negative for EBOV (Appendix A, B, and C). Only dog samples were tested for EBOV antibody, which was detected at 26.3% (21/80) prevalence. Conversely, the overall antibody detection prevalence was 2.0% (159/7,960) for wild species (Appendix A). The 80 dog (*Canis lupus familiaris*) samples were collected during two sampling efforts associated with human outbreaks in Democratic Republic of Congo (DRC) 1979–1980 and Gabon 2001–2002 (Appendix A). The 12 pig (*Sus scrofa*) samples tested were collected during the DRC Yambuku 1976 and DRC Kikwit 1995 human outbreaks (Appendix B).

Trends in EHF outbreak animal sampling

Some of the first EHF outbreak animal sampling efforts targeted a wide range of vertebrates and arthropods, but analysis of the samples consistently failed to detect EBOV. Scientists were performing sampling efforts to search for the EBOV reservoir, or ‘one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population’ (18). Following the first medically recognized outbreak in 1976, up through the 1990s, tests for EBOV

in both domestic and wild animals were uniformly negative. One study sampled nearly 28,000 arthropods, and another study sampled over 3,000 vertebrates without identifying a single positive sample (6, 19).

There are several possible explanations as to why early sampling efforts were unable to detect evidence of EBOV in animal reservoirs. First, the early animal tests relied solely on Vero cell cultures to isolate the virus, because antigen and molecular detection assays were not yet developed. These latter methods have proven to be less dependent on sample quality and may be more sensitive. Second, the total sample size of these studies was large, but the samples were spread across numerous species that, in hindsight, were unlikely reservoirs (2). Lastly, when the sampling effort was conducted months after a presumed index transmission event from animals to humans, seasonal shifts in habitat between the wet and dry season and natural patterns of animal migration meant the reservoir species may have left the outbreak site or high levels of enzootic transmission in reservoirs may have ceased (20, 21).

Non-human primate sampling

Non-human primate susceptibility to EBOV was evident in the scientific literature. EBOV belongs to the same

Table 3. Summary of species with at least one positive test collected in areas associated with EHF outbreaks

Method (Assay)	Live capture (Antibody)			Live capture (Virus)			Carcass sampling (Virus)			References
	No.	+	Yield	No.	+	Yield	No.	+	Yield	
Order/subtotals										
Total	2161	111	5.1%	355	8	2.3%	36	18	50.0%	
Artiodactyla (n = 12) ^a										
<i>Cephalophus sp.</i>							6	1	16.7%	11, 12, 13
<i>Philantomba monticola</i>	17	0	0.0%	28	0	0.0%				6, 7, 15
<i>Sylvicapra grimmia</i>				4	0	0.0%				6
Subtotal	17	0	0.0%	32	0	0.0%	6	1	16.7%	
Carnivora (n = 10)										
<i>Canis lupus familiaris</i>	80	21	26.3%	14	0	0.0%				2, 34
Chiroptera (n = 47)										
<i>Epomops franqueti</i>	1670	69	4.1%	140	5	3.6%				8, 9, 10
<i>Hypsignathus monstrosus</i>	253	18	7.1%	22	4	18.2%				8, 9, 10
<i>Micropteropus pusillus</i>	275	4	1.5%	78	0	0.0%				10
<i>Mops (Mops) condylurus</i>				64	0	0.0%				10
<i>Mops (Mops) condylurus & Hipposideros gigas</i> ^b	24	3	12.5%							10
<i>Myonycteris (Myonycteris) torquata</i>	1185	33	2.8%	141	4	2.8%				8, 9, 10
<i>Roussetus aegyptiacus</i>	307	24	7.8%							10
<i>Roussetus (Roussetus) amplexicaudatus</i>	16	7	43.8%							16
Subtotal	2060	89	4.3%	305	8	2.6%				
Non-human primates (n = 15)										
<i>Gorilla gorilla</i>							22	13	59.1%	11, 12, 13
<i>Pan troglodytes</i>							8	4	50.0%	11, 12, 13, 27
Subtotal							30	17	56.7%	
Rodentia (n = 47)										
<i>Anomalurus derbianus</i>	4	1	25.0%	4	0	0%				2

^aTotal number of species sampled within an order.^bStudy counted both *Mops condylurus* and *Hipposideros gigas*.

virus family as *Marburgvirus*, and a Marburg hemorrhagic fever outbreak had been linked to green monkeys (*Chlorocebus aethiops*) 9 years prior to the first recognized EHF outbreak in Yambuku, DRC (22). However, it was not until the early nineties that evidence again hinted that non-human primates provided a transmission link between the sylvatic cycle of the virus and human outbreaks. Late in 1989 outbreaks of REBOV, at that time a new strain of EBOV, occurred in non-human primate quarantine centers in the United States, putatively killing monkeys (*Macaca fascicularis*) imported from the Philippines and causing seroconversion but no disease in humans who handled the monkeys (23–25). In November 1994 a natural outbreak of EHF occurred in chimpanzees (*Pan troglodytes*) in Taï National Park, Côte d'Ivoire, and a researcher who necropsied an ape carcass became infected with another new subtype of EBOV, Côte d'Ivoire ebolavirus (CIEBOV) (26). Around that same time in Gabon a set of three human EHF outbreaks began that coincided with deaths of non-human primates. The index human cases of the last outbreak in Gabon, in the spring of 1996, had a history of butchering chimpanzees. A chimpanzee carcass found near the hunting grounds of an index case tested positive for EBOV by an immunohistochemical skin biopsy (27).

The importance of the human-great ape interface was further supported during another series of outbreaks in Gabon from 2001 through 2003. Leroy and colleagues were able to trace 'epidemic chains' back to 10 index cases, all hunters who had handled a gorilla, chimpanzee, or duiker carcass (11). While the carcasses at the head of the epidemic chains could not be sampled, in sum the researchers were able to confirm 64 carcasses and sample 36 within a 2-hour walking distance of villages: 22 gorillas (13 positive), eight chimpanzees (four positive), and six duikers (one positive) (11–14).

Epidemiological evidence shows that hunting of great apes is an important transmission interface, yet their role as a natural reservoir remains unlikely. One study between 1985 and 2000 showed the IgG ELISA based seroprevalence of EBOV in captive-born non-human primates was 0% (0/165) compared to 6.3% (39/618) in wild-caught captive non-human primates, with chimpanzees representing three quarters of the positives in Cameroon, Gabon, and the Republic of Congo (14). The wild-caught captive non-human primate species that tested positive for EBOV specific IgG antibodies were *Cercopithecus neglectus* (De Brazza's Monkey), *Gorilla gorilla* (Western Gorilla), *Mandrillus leucophaeus* (Drill), *Mandrillus sphinx* (Mandrill), *Pan troglodytes* (Chimpanzee), and *Papio anubis* (Olive Baboon). The study's authors concluded that non-lethal EBOV naturally circulated in the area prior to human exposure (14).

Within non-human primates to date just gorilla, chimpanzee, and *Cercopithecus cephus* (Mustached

Guenon) carcasses have been sampled, and only gorilla and chimpanzees have tested positive for EBOV by RT-PCR. In this light, and in addition to the high mortality rates of gorillas and chimpanzees, some researchers concluded that non-human primates are not the natural EBOV reservoir (12, 13, 28, 29). Explanations for the presence of EBOV antibody in some populations and outright mortality in other populations may include individual differences in protective immunological responses (30), differential virulence of emerging EBOV strains, or differences in transmission pathways.

Chiroptera (bat) sampling

Strong circumstantial evidence implicates bats as a natural EBOV reservoir. A bat reservoir was suspected early on when bat roosts were observed in a cotton warehouse at the center of EHF outbreaks in Nzara, Sudan (31). Swanepoel et al. experimentally inoculated 19 vertebrate species with EBOV and demonstrated that only bats became infected and shed the virus in feces, strongly suggesting them as a potential reservoir (32). In 2004 researchers in South East Asia and Australia established that fruit bats could transmit *Nipahvirus* and *Hendravirus* (33). Researchers have since found 151 individual bats from six species were seropositive for EBOV antibodies: *Epopomops franqueti* (Franquet's Epauletted Fruit Bat), *Hypsignathus monstrosus* (Hammer-headed Fruit Bat), *Micropteropus pusillus* (Peters's Lesser Epauletted Fruit Bat), *Mops (Mops) condylurus* (Angolan Free-tailed Bat), *Myonycteris (Myonycteris) torquata* (Little Collared Fruit Bat), and *Rousettus (Rousettus) aegyptiacus* (Egyptian Rousette or Fruit Bat) (see Appendix A, 8, 9, 10). In 2009, following an outbreak linked to the Kitaka Cave, Uganda, comprehensive evidence from epidemiological observations, serological and genetic testing, and virus isolation identified *Rousettus (Rousettus) aegyptiacus* as a natural *Marburg virus* reservoir (34). The close genetic relationship between *Marburg virus* and EBOV suggests that bat species may also be reservoirs for EBOV, but other potential reservoir species have not been ruled out. Very recently REBOV antibodies were found in *Rousettus (Rousettus) amplexicaudatus* in the Philippines following a disease outbreak in pigs (16). While mounting serologic and PCR evidence points towards bats as natural EBOV reservoirs, researchers have not isolated any strain of EBOV from naturally infected bats.

Discussion

We describe the cumulative history of published EHF outbreak animal sampling data assayed for ZEBOV and exposure to ZEBOV. The data were collected from 14 peer-reviewed publications and analyzed for general trends over time, sampling method, assay type, and domesticated status. Over time the list of target wildlife species narrowed and sampling of domesticated animals

declined as clues implicated dead non-human primates and live bats as important primary routes of exposure to humans and bats as putative EBOV reservoirs (35).

In outbreak situations, field response teams and researchers can use the recommendations presented here to confirm exposure to EBOV in animals, potentially detect the source of immediate spillover, and improve our knowledge of implicated animal species by targeting high yield vertebrate species with optimized sampling approaches that reduce destructive animal sampling, especially for wild species whose distributions are poorly documented and may be rare. When trying to identify natural cycles of EBOV in free-ranging wildlife during non-outbreak situations, broader surveillance strategies may still be appropriate. In either situation, the tables and appendices provide quick reference for sample strategies and we report prevalence for sampling efforts by species, EBOV assay, and sampling method. These values should be used as starting points, as they may over- or underestimate the true value depending on the reliability and validity of each assay as performed by different labs.

Our analysis, combined with new insights, suggests some species have not been sampled sufficiently and should be targeted in the future. Allela and colleagues present a strong gradient of distance and antibody prevalence in dogs, yet no follow up study has sampled dogs for ZEBOV (36). More recently REBOV was detected in domestic pigs in the Philippines, and laboratory studies confirmed domestic pigs are susceptible to ZEBOV infection, shed the virus in large quantities, and can infect co-housed pigs (37, 38). No trace of EBOV was found in the 12 domestic swine (*Sus scrofa domesticus*) that were sampled in 1976 and 1995 in the DRC. Future sampling efforts should include wild and domestic pigs in greater numbers. While it seems unlikely that domestic animals, or their wild suid or canid relatives, are the natural reservoir of EBOV, they may become sources of new infections when infected by spillover from a reservoir species or from human cases.

Our results show that testing of dead wild animals for EBOV should not be neglected and may improve our knowledge regarding the full range of dead end hosts. Opportunistic sampling of carcasses may be a low hanging fruit to investigate alternative transmission pathways as carcass sampling has so far yielded the highest prevalence of virus detection, over 150 times more than live capture. Across all the sampling efforts we show the virus was present in 1% of live capture bat samples and 52% of gorilla and chimpanzee carcass samples collected in association with a human EHF outbreak. Animals acting sick or debilitated should also be targeted for sampling as not all hosts have succumbed to EBOV infection (14). In the face of human outbreaks, quickly determining if the virus is present in animals (wild or domestic) will help investigators understand the

transmission dynamics in these populations and possible infection sources during a human outbreak, focus on factors associated with spillover risk (e.g., seasonality, geography), and inform public health efforts.

We recognize that it is possible that a natural reservoir species for EBOV, such as bats are considered, may not exhibit high mortality levels with infection, and therefore live sampling is an important component for determining their role in the spillover event in question. In addition, even if the natural reservoir species exhibits high mortality levels with infection their carcasses may be difficult to find in some field conditions due to their small size and rapid decomposition.

Briefly reflecting on the quality of the data provided in the published literature, there are easy steps researchers can take to improve our collective knowledge of zoonotic disease ecology. Presently data are scattered, unorganized, incomplete, and static. Published tables are truncated for space and often lack individual sample information, such as the specific date, location, or assays performed. These samples are extremely valuable, but there is no centralized tracking of what tests have been run at which labs or where they are currently located. New techniques and assays are emerging rapidly and revisiting the freezer of 20 years ago may tell us something important that was missed the first time. Researchers should consider collecting and reporting a proxy of field sampling effort, such as the number of sampling days, or the GPS track length. This information is particularly useful to statistical modelers to allow adjustment for sampling biases. Standardizing RT-PCR genetic targets will also improve downstream genetic analysis, but at minimum samples should be taken and preserved with standard protocols to maximize the possibilities for future analysis.

Another observation was that overall reporting progress has improved, delay in publication of EBOV testing results dropped from 20 to 2 years, and a model of standardized wildlife disease data collection terminology was recently developed by the USAID PREDICT project (39). On the front end, surveillance teams are on the ground shortly after outbreak events. On the back end quickly circulating all surveillance findings to the scientific community can help promote adaptive surveillance strategies.

All surveillance includes both positive and negative findings. One caveat to our analysis is that when negative results are left unpublished the conclusions can be 'dangerously misleading' (40). Although the track record of publishing null results in EHF research is impressive (31,000 arthropods and vertebrates negative for EBOV and counting) it is possible that field investigators, authors, or editors failed to publish results because they were fatigued by response activities and negative findings. Similarly only carcasses of a few species have been tested.

Table 4. Animal surveillance recommendations for human EHF outbreaks

- Prioritize free-ranging non-human primate mortality (antigen testing) and morbidity (antigen or antibody testing)
- Investigate and report any wildlife morbidity or mortality
- Survey dogs and suids for EBOV and EBOV antibodies
- Most likely to recover virus RNA from carcasses of free-ranging species
- Carcass sampling yields higher likelihood of EBOV recovery than live capture
- Bat surveillance is indicated but requires large sample sizes ($n > 100$)

Our analysis raises questions and observations for future studies. Why are animal carcass sampling efforts limited to a handful of species? Why were duikers involved and did other animals die as a result of EBOV infections? Are carcasses of different species detected at the same rate? Does bat mortality occur? Have dogs or pigs been sources of human infection?

The challenge of identifying EBOV spillover species is real, but this extensive literature review offers some guidance towards top priorities (Table 4). First, our review indicates that the field team should not overlook the importance of sampling and testing dogs and pigs for EBOV and previous exposure as part of an overall response strategy. Second, all wildlife morbidity or mortality events in wildlife, and especially (although by no means exclusively) non-human primates should be reported and investigated. Our analysis shows EBOV is most likely to be recovered from free-ranging species by opportunistically sampling dead animals. Carcass sampling yields much higher recovery rates of EBOV than live capture sampling. Lastly, our findings also support the importance of bat surveillance in the hunt for the definitive reservoir, but encourage careful sample size planning, as the likelihood of finding evidence of EBOV in bats is very low.

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***Damien O. Joly**

Wildlife Health Program
 Wildlife Conservation Society
 900 Fifth Street, Building 373
 Nanaimo, BC V9R 5S5
 Canada
 Tel: +1 917 512 2890
 Fax: +1 250 483 6475
 Email: djoly@wcs.org