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RESEARCH ARTICLE

Detection of Viruses Using Discarded Plants from Wild Mountain Gorillas and Golden Monkeys

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Infectious diseases pose one of the most significant threats to the survival of great apes in the wild. The critically endangered mountain gorilla (*Gorilla beringei beringei*) is at high risk for contracting human pathogens because approximately 60% of the population is habituated to humans to support a thriving ecotourism program. Disease surveillance for human and non-human primate pathogens is important for population health and management of protected primate species. Here, we evaluate discarded plants from mountain gorillas and sympatric golden monkeys (*Cercopithecus mitis kandti*), as a novel biological sample to detect viruses that are shed orally. Discarded plant samples were tested for the presence of mammalian-specific genetic material and two ubiquitous DNA and RNA primate viruses, herpesviruses, and simian foamy virus. We collected discarded plant samples from 383 wild human-habituated mountain gorillas and from 18 habituated golden monkeys. Mammalian-specific genetic material was recovered from all plant species and portions of plant bitten or chewed by gorillas and golden monkeys. Gorilla herpesviral DNA was most consistently recovered from plants in which leafy portions were eaten by gorillas. Simian foamy virus nucleic acid was recovered from plants discarded by golden monkeys, indicating that it is also possible to detect RNA viruses from bitten or chewed plants. Our findings show that discarded plants are a useful non-invasive sampling method for detection of viruses that are shed orally in mountain gorillas, sympatric golden monkeys, and potentially other species. This method of collecting specimens from discarded plants is a new non-invasive sampling protocol that can be combined with collection of feces and urine to evaluate the most common routes of viral shedding in wild primates. Am. J. Primatol. 78:1222–1234, 2016.

Key words: mountain gorilla; golden monkey; pathogen; non-invasive; sampling

INTRODUCTION

Infectious diseases pose one of the greatest threats to the survival of great apes in the wild [Kondgen et al., 2008; Leendertz et al., 2006; Pedersen et al., 2007]. Pathogens of human origin are suspected to be major contributors to infectious diseases in great apes, particularly those that have close proximity contact with humans through ecotourism and research activities [Kondgen et al., 2008]. Epidemics, such as ebolavirus in western chimpanzees (*Pan troglodytes verus*) and western lowland gorillas (*Gorilla gorilla gorilla*), respiratory disease due to human metapneumovirus in mountain gorillas and chimpanzees, and human paramyxovirus in chimpanzees, have led to widespread morbidity and mortality [Bermejo et al., 2006; Formenty et al., 1999; Kaur et al., 2008; Kondgen et al., 2008; Palacios et al., 2011].

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The estimated 880 remaining critically endangered mountain gorillas (*Gorilla beringei beringei*) inhabit two isolated forest regions, the Virunga Volcanoes Conservation Range spanning Rwanda, Uganda and the Democratic Republic of Congo (DRC) and the Bwindi Impenetrable Forest in Uganda [Gray et al., 2011; Robbins et al., 2011]. These gorillas are at high risk for contracting human pathogens because approximately 60% of their population is habituated to humans making it the most habituated of any great ape species in the wild [Gray et al., 2011; Robbins et al., 2011]. Habituation involves making animals more accessible to researchers and tourists by gradually familiarizing selected groups to the close presence of humans [Woodford et al., 2002]. Inadvertently, this conservation strategy can expose great apes to human pathogens [Spelman et al., 2013]. Mountain gorillas are exposed to people from local communities and around the world through active research projects and tourism programs and because the conservation areas in which they live are surrounded by the densest human population in continental Africa [Sandbrook & Semple, 2006; Williamson & Fawcett, 2008]. Given this intense human contact, monitoring pathogens of human and gorilla origin is critical to ensuring proper mountain gorilla management practices [Travis et al., 2008]. Preventing the transmission of pathogens from people is of utmost importance and only through population level monitoring can authorities be confident that their practices are effective.

Collection of whole blood samples, oral swabs, and rectal swabs are often considered optimal specimen types for detection of viruses in non-human primates (hereafter referred to as primates). However, in order to collect these samples, individuals are usually anesthetized. Given the inherent risk of field anesthesia and the endangered status of mountain gorillas, interventions requiring anesthesia are generally only permitted if a gorilla is sick or injured due to a human-induced cause or the injury or illness is life threatening. Population-wide health monitoring in mountain gorillas and other great apes, therefore, often relies upon non-invasively collected samples, such as feces and urine [Gillespie et al., 2008; Leendertz et al., 2006]. Feces and urine, however, can be logistically challenging to collect and do not cover all biological routes of pathogen shedding [Cranfield & Minnis, 2007; Nizeyi et al., 2001; Sleeman & Mudakikwa, 1998] (Table I). Fecal collection for population wide studies of mountain gorillas typically requires identifying night nests where gorillas may no longer be present. Directly observing mountain gorillas defecating happens less frequently. Urine collection also requires directly observing a gorilla producing a urine sample, which can limit the number of individuals to be sampled and increase time and effort.

Many viruses are shed or present in oropharyngeal secretions, including respiratory viruses and herpesviruses. Oral samples have been used for the detection of primate viruses including some with frequent cross-species transmission, such as Ebola, herpes B, and simian immunodeficiency virus [Huff et al., 2003; Thomas et al., 2010; Weingartl et al., 2012]. Oral samples have also been used for the detection of viruses in humans and could be applied to primate samples, such as dengue fever, Ebola, hepatitis A, Marburg, and measles [Formenty et al., 2006; Grolla et al., 2011; Hutse et al., 2010; Mackiewicz et al., 2004; Poloni et al., 2010]. Viruses that are shed orally or in the respiratory tract are likely better detected through a sampling method targeting saliva and oropharyngeal cells then other non-invasive methods [Robinson et al., 2008; Streckfus & Gibler, 2002; Van Velzen et al., 2013]. Oral samples have been collected non-invasively using ropes thrown out to habituated primates, and samples have been used for the detection of both DNA and RNA viruses [Smiley Evans et al., 2015]. It has also been suggested that wadged plant material dropped by chimpanzees could be used as a sample for respiratory pathogens [Kaur et al., 2008; Kondgen et al., 2010]. To the authors' knowledge, there have been no published reports of viruses detected using wadge samples from chimpanzees or other primate species. Detecting viruses in wadge-like samples has been documented for Nipah virus in partially chewed fruit samples from flying foxes [Chua et al., 2002]. Chimpanzee wadge samples have been used to successfully detect *Staphylococcus aureus* [Schaumburg et al., 2012, 2013] and chimpanzee fruit wedges have been used to successfully detect *Streptococcus sp*.; however, these bacterium can remain intact in the environment for prolonged periods of time [Eells et al., 2014; Kramer et al., 2006]. The detection of RNA viruses is particularly important for primate pathogen surveillance because RNA viruses have higher mutation rates and are more likely to shift hosts resulting in zoonotic or anthropozoonotic disease transmission [Woolhouse et al., 2005]. However, RNA viruses are especially labile and therefore pose a particular challenge for disease investigations, especially in remote settings.

Mountain gorillas spend a great portion of their day foraging and manipulating a variety of plant species with their mouths [Watts, 1988]. They discard large quantities of bitten or partially chewed plants providing an easily accessible sample type at a much greater frequency than feces or urine [Smiley et al., 2010]. Oral samples have been recovered from bitten plants discarded by mountain gorillas and chimpanzees and used for the detection of primate genetic material [Shimada et al., 2004; Smiley et al., 2010]. However, it is unknown whether viruses can

TABLE I. Comparison of Non-Invasive Sample Collection Techniques for Pathogen Detection Used with Non-Human Primates

Sample type	Field collection techniques	Analyses performed	Example pathogens	Example citations
Feces	Collection upon observation of defecation; collection from night nests/sleeping locations	Extraction of viral DNA/RNA; extraction of bacterial DNA; extraction of parasite DNA; extraction of antibodies; bacterial culture; identification of parasites	HIV-1, HBV, hMPV, RSV, SFV, SIV, *P. falciparum*, *Mycobacterium tuberculosis*, *Salmonella, Shigella, Camphylobacter, Yersinia, Enterococcus*, nematode sp., protozoan sp., trematode sp.	Santiago et al. [2002]; Makuwa et al. [2003]; Gillespie [2006]; Keele et al. [2006]; Van Heuverswyn et al. [2006]; Liu et al. [2008]; Rwego et al. [2008]; Kondgen et al. [2010]; Prugnolle et al. [2010]; Howells et al. [2011]; Goffe et al. [2012]; Blasse et al. [2013]; Wolf et al. [2015]
Urine	Collection upon observation of urination and pipetting from leaves; collection using tarps underneath night nests	Extraction of antibodies; extraction of parasite DNA	STLV, *T. b. gambiense*, SIV	Ling et al. [2003]; Leendertz et al. [2004]; Ngotho et al. [2015]
Saliva/ oropharyngeal cells	Collection from chewed vegetation; collection using distributed ropes	Extraction of viral DNA/RNA; bacterial culture	*S. aureus*, *Streptococcus sp.*, RhCMV, SFV	Schaumburg et al. [2012]; Schaumburg et al. [2013]; Smiley Evans et al. [2015]; Denapaite et al. [2016]

HIV-1 = human immunodeficiency virus-1; HBV = hepatitis B virus; SIV = simian immunodeficiency virus; SFV = simian foamy virus; hMPV = human metapneumovirus; RSV = human respiratory syncytial virus; P. falciparum = plasmodium falciparum; S. aureus = *Staphylococcus aureus; STLV = simian T-cell leukemia virus; T. b. gambiense = Trypanosoma brucei gambiense; RhCMV = rhesus cytomegalovirus.*

be recovered from these discarded plant samples and if so, which plants and collection methods are best for viral detection.

Here, we evaluate discarded plants from mountain gorillas and sympatric golden monkeys (*Cercopithecus mitis kandti*) as potential diagnostic samples for virus detection. The objectives of this study were to evaluate: (1) feasibility of collecting discarded plant samples from wild human-habituated mountain gorillas for a population wide study; (2) detection of primate DNA and RNA viruses from discarded plant samples from gorillas and golden monkeys; and (3) variability in quality of specimens recovered from various plant types. To validate this method for application in field sampling of wild primates, we tested discarded plant samples for the presence of mammalian beta-actin and two ubiquitous DNA and RNA primate viruses, herpesviruses, and simian foamy virus [Voevodin & Marx, 2009a, b]. In addition, because mountain gorillas suffer seasonal respiratory illness outbreaks, mountain gorilla samples were screened for viral families / genera that could play a role in respiratory disease: *Coronaviridae*, *Enteroviridae*, *Paramyxoviridae*, and *Influenza virus*. By optimizing this non-invasive sampling technique for the recovery of oral specimens from discarded plants and the detection of viruses, we provide an important novel method for sampling wild great apes and other primate species. These methods could be applied to detection of viruses in wild species where health monitoring is critical for conservation management.

METHODS

Study Population

We surveyed Virunga mountain gorillas living in the 447 km^2 Virunga Volcanoes conservation area, which is comprised of the Volcanoes, Virunga, and Mgahinga Gorilla National Parks spanning the borders of Rwanda, Democratic Republic of Congo and Uganda and Bwindi mountain gorillas living in the 331 km^2 Bwindi Impenetrable National Park in Southwestern Uganda. We surveyed golden monkeys living on the lower slopes of Mount Sabinyo in the Volcanoes National Park.

Mountain Gorilla Sample and Data Collection

This study protocol was approved by the Uganda Wildlife Authority, the Rwanda Development Board, and the Institutional Animal Care and Use Committee of the University of California, Davis. All research conducted adhered to the American Society of Primatologists principles for the ethical treatment of non-human primates. To determine if discarded plant samples could be collected for a population-wide survey of wild human-habituated mountain gorillas, all accessible habituated gorilla groups in the Volcanoes National Park, Bwindi Impenetrable Forest, and Mgahinga Gorilla Park were visited a minimum of three times. Discarded plant samples ($n = 383$) were collected from 294 individual mountain gorillas from 26 family groups between the months of November 2012 and June 2013. When possible, plants discarded by every individual within a family group were collected. Gorillas were observed from a minimum of 7 m while they ate and discarded pieces of plants (Figs. 1 and 2). Gorillas were identified using facial features with the help of trackers and guides that were familiar with each individual in the group. Infants were defined as gorillas <3 years of age, juveniles as 3–5, sub-adults as 6 to 7, adult females as 8 years old and greater, black-backs as males 8–11, and silverbacks as males 12 years old and greater [Robbins et al., 2009; Williamson & Gerald-Steklis, 2001]. Samples of dropped plants were collected when a gorilla moved a minimum of 7 m to a new foraging area. Using gloved hands, the most bitten or chewed portion of a discarded plant, as determined by teeth marks and visible deposited saliva, was cut using disposable sterile scalpel blades and plant pieces

Fig. 1. Infant mountain gorilla playing with *Urera* species vine in Volcanoes National Park, Rwanda (Photo T. Smiley Evans).

Fig. 2. Female mountain gorilla eating wild celery (*Peucedanum linderi*) in Volcanoes National Park, Rwanda (Photo T. Smiley Evans).

Fig. 3. Golden monkey discarding chewed plants in the Volcanoes National Park, Rwanda (Photo T. Smiley Evans).

placed in 2 ml cryovial tubes containing 1 ml of viral transport media (Remel M6^{TM}, Thermo Scientific, Lenexa, KS). Sufficient sample material was cut from multiple locations on the bitten or chewed plant to fill one 2 ml tube. Samples were immediately frozen in liquid nitrogen, the optimal technique for long-term viral storage, when liquid nitrogen dewars could be transported to study locations. When this transport was not possible, samples were immediately placed on ice packs and transported outside the park to liquid nitrogen dewars within 4 hr.

Golden Monkey Sample and Data Collection

To determine if discarded plants could be collected from a different primate species living in close proximity to the mountain gorillas, one habituated golden monkey group in the Volcanoes National Park, consisting of approximately 80 members, was visited on three separate sampling dates. Discarded plant samples were collected from 18 individuals between March and June 2013. Golden monkeys were observed eating and discarding plants, and samples were collected as described for mountain gorillas (Fig. 3).

Classification of Plants

Discarded plants were identified by species using literature on local plants and the East African plant photo database (available online at: http://www.eastafricanplants.senckenberg.de; [Alphonse et al., 2010; Ganas et al., 2004, 2008]. The portion of the plant consumed and eaten by gorillas was also observed and recorded. Data were categorized into plant types: fruit, herb, grass, shrub, tree, mushroom, and parasite categories based on previous literature on mountain gorilla feeding behaviors [Ganas et al., 2004, 2008]. Portions of the plant eaten were categorized into fruit, whole stalk, leaves, inner pith, grass blades, bark, root, mushroom, peel, and whole parasitic plant based on the portion of the plant the gorilla was observed eating.

Nucleic Acid Extraction and PCR

Total nucleic acid was extracted from discarded plant samples to purify primate genetic material and viral nucleic acids that were present in oral specimens deposited on the plants. Discarded plant samples stored in viral transport media were thawed on ice, vortexed, and centrifuged for 10 min at 3,000 rpm. Total nucleic acid was extracted from

600 μl of sample supernatant using the NucliSENS® MiniMAG® system (bioMérieux, Inc., Durham, NC) according to manufacturers instructions. To determine the overall quality of the specimen recovered from discarded plants, samples were tested for mammalian beta-actin, a component of host mammalian cells that should be present in the sample. Beta-actin has been used in other studies as an indicator of overall specimen sample quality [Lin et al., 2009] and has been commonly used as an indicator of sample quality in wildlife diagnostic studies [Smiley Evans et al., 2015]. RNA was reverse transcribed and cDNA synthesized using SuperscriptIII First Strand Synthesis cDNA kits (Invitrogen, Carlsbad, CA) according to manufacturer instructions. Complementary DNA was analyzed by polymerase chain reaction (PCR) amplifying a 544 bp fragment of the mammalian beta-actin gene [Hammond et al., 2005]. To determine if DNA viruses could be detected in specimens recovered from discarded plants, DNA was analyzed by PCR to detect herpesviral DNA utilizing degenerative primers amplifying a 450 bp region of the terminase (TERM) gene and 225 bp region of the DNA polymerase (DPOL) gene [Chmielewicz et al., 2001; Ehlers et al., 1999]. These primers are designed to amplify all herpesviruses within the alpha, beta, and gamma herpesvirinae sub-families. To determine if RNA viruses could be detected in specimens from discarded plant samples, cDNA was analyzed by PCR to detect simian foamy virus, an RNA retrovirus, amplifying a 357 bp region of the long terminal repeat (LTR) gene and 632 bp region of the polymerase (POL) gene [Goldberg et al., 2009]. In order to opportunistically detect viral families contributing to respiratory disease, cDNA was also analyzed by PCR to detect *Coronaviridae*, amplifying a 328 bp region of the RNA polymerase gene [Quan et al., 2010]; *Enteroviridae*, amplifying a 350–450 bp region of the VP1 gene [Nix et al., 2006]; *Paramyxoviridae*, amplifying a 561 bp region of the polymerase gene [Tong et al., 2008] and *Influenza virus*, amplifying a 243 bp region of the M gene [Anthony et al., 2012]. PCR products of appropriate size were cloned using TOPO® TA cloning kits (Invitrogen), and sequencing was performed using Sanger dideoxy sequencing at the University of California, Davis DNA sequencing laboratory. Sequences were compared to other published herpesviral DNA sequences in the GenBank Database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD).

Statistical Methods

Associations between detection of beta-actin, herpesviruses, and various individual, demographic, and plant characteristic factors were evaluated by the one-sided χ_2 test and the odds ratio (OR). Confounding and effect modification were evaluated for significant associations by stratification on putative risk variables and comparing the OR for individual strata. If confounding on the stratified variables was determined to be substantial (>10% of the OR), the adjusted Mantel–Haenszel test OR was reported.

Logistic regression was used to assess the association between independent factors (specific plant species, category of plant eaten, and portion of the plant eaten) and overall sample quality (as determined by the presence of beta-actin). Mixed-effects logistic regression was used to assess the association of factors (specific plant species, category of plant eaten, and portion of plant eaten) and combined results for all herpesvirus sub-families (alpha, beta, and gamma). Mixed-effects models implement a likelihood-based estimation method that allows for all available data to be used in the analysis while accounting for correlation among individuals with similar risk. Gorillas within the same family group were likely to be similar to each other with respect to herpesvirus shedding due to similar stresses imposed on an individual group at a certain sampling time and shared viruses transmitted by close contact. Lymphocryptoviruses have been shown to be reactivated during periods of stress [Yamamoto et al., 2010]. We, therefore, incorporated family group as a random effect variable in the model. An unstructured covariance matrix was chosen for the random effects. Plant species, plant category, portion of the plant eaten, and gorilla age were evaluated as fixed effect variables. Age was incorporated as a fixed variable because in other primate species, such as rhesus macaques, certain age groups are more or less likely to be shedding ubiquitous herpesviruses such as herpes B and CMV [Huff et al., 2003]. The decision whether to include a variable in the model was evaluated using the likelihood-ratio test to determine whether each significantly improved model fit ($P < 0.1$), compared to a model without that variable. Variables were retained in the model if they improved fit, while minimizing AIC, or were determined to be important confounders based on bivariate analyses [Hosmer et al., 2013]. All statistical analyses were performed using STATA version 13.1 [StataCorp, 2013].

RESULTS

Mountain gorillas were observed eating and discarded plant samples were successfully collected during every visit to a gorilla group. Samples were collected from all age groups, including infants, and discarded plants representing 35 different plant species were collected (Table II). Gorillas were most frequently eating and discarding *Urera* plants during the sampling period. Adult and juvenile gorillas would most commonly push the vines

TABLE II. Discarded Plant Species Collected from Mountain Gorillas and the Detection of Gorilla Beta-Actin, Herpes, and Simian Foamy Virus Nucleic Acid

Plant species	Part eaten[a]	Plant category	Beta-actin (%)	Herpes viruses (%)	Simian foamy virus (%)
Aframomum sp.	Fruit	Fruit	1/1 (100)	1/1 (100)	0/1 (0)
Bambusa vulgaris	Stalk	Shrub	20/22 (91)	14/22 (64)	0/22 (0)
Basella alba	Leaves	Herb	6/7 (86)	6/7 (86)	0/7 (0)
Brillantaisia sp.	Pith	Herb	17/19 (89)	13/19 (68)	0/19 (0)
Cardus sp.	Stalk	Herb	36/45 (82)	18/45 (40)	0/45 (0)
Chrysophyllum sp.	Fruit	Fruit	16/18 (89)	3/18 (17)	0/18 (0)
Cyathea manniana	Stalk	Shrub	6/8 (75)	1/8 (13)	0/8 (0)
Cyperus latifolius	Grass	Grass	0/1 (0)	0/1 (0)	0/1 (0)
Dendrosenecio johnstonii	Pith	Shrub	1/1 (100)	0/1 (0)	0/1 (0)
Discopodium penninervium	Pith	Tree	5/5 (100)	3/5 (60)	0/5 (0)
Eucalyptus sp.	Bark	Tree	4/4 (100)	2/4 (50)	0/4 (0)
Ficus sp.	Bark	Tree	7/7 (100)	4/7 (47)	0/7 (0)
Galiniera coffeeoides	Pith	Tree	1/1 (100)	0/1 (0)	0/1 (0)
Galium sp.	Leaves	Herb	1/1 (100)	1/1 (100)	0/1 (0)
Impatiens niamniamensis	Leaves	Shrub	2/3 (67)	1/3 (33)	0/3 (0)
Ipomea sp.	Leaves	Herb	1/1 (100)	1/1 (100)	0/1 (0)
Kniphofia acraea	Stalk	Herb	3/4 (75)	2/4 (50)	0/4 (0)
Laportea sp.	Peel	Herb	13/15 (87)	4/15 (27)	0/15 (0)
Mimulopsis arborescens	Pith	Herb	3/5 (60)	2/5 (40)	0/5 (0)
Mimulopsis solmsii	Peel	Herb	9/10 (90)	5/10 (50)	0/10 (0)
Momordica sp.	Leaves	Herb	1/2 (50)	2/2 (100)	0/2 (0)
Mushroom sp.	Mushroom	Mushroom	1/1 (100)	0/1 (0)	0/1 (0)
Myrianthus holstii	Bark	Tree	7/8 (88)	4/8 (50)	0/8 (0)
Pennisetum parpureum	Grass	Grass	5/6 (83)	5/5 (100)	0/5 (0)
Peucedanum linderi	Pith	Herb	41/44 (93)	27/44 (61)	0/44 (0)
Peucedanum linderi root	Root	Herb	2/3 (67)	0/3 (0)	0/3 (0)
Piper capense	Pith	Herb	1/1 (100)	1/1 (100)	0/1 (0)
Pycnostachys goetzenii	Peel	Shrub	1/3 (33)	0/3 (0)	0/3 (0)
Teclea noblis	Fruit	Tree	2/2 (100)	1/2 (50)	0/2 (0)
Triumfetta sp.	Leaves	Herb	3/4 (75)	2/4 (50)	0/4 (0)
Unidentified berries	Berries	Fruit	8/8 (100)	6/8 (75)	0/8 (0)
Unidentified tree parasite	Parasite	Parasite	1/2 (50)	1/2 (50)	0/2 (0)
Urera sp.	Peel	Herb	40/54 (74)	11/54 (20)	0/54 (0)
Vernonia calongensis	Pith	Shrub	3/5 (60)	2/5 (40)	0/5 (0)
Xymalos monospora	–	Tree	1/1 (100)	0/1 (0)	0/1 (0)

[a]Fruit = gorilla consumed seed bearing portion of plant/tree leaving portions or stems of fruit; stalk = gorilla consumed entire stalk of plant leaving a chewed end; leaves = gorilla consumed leaves of plant leaving stems or parts of leaves; pith = gorilla consumed inner pith of stalk of plant leaving outer portion of stalk; grass = gorilla consumed grass blades leaving ends of blades; bark = gorilla consumed tree/shrub bark leaving skinned portion of tree/shrub; mushroom = gorilla consumed fungus leaving partially bitten portion of fungus; peel = gorilla consumed outer peel of plant stalk leaving inner portion of stalk; parasite = gorilla consumed plant parasite growing on a tree/bush leaving partially bitten portion of parasite.

through their mouths to remove the peel and discard large portions of the remaining inner pith of the plant. Infants, although still predominantly nursing, were frequently observed playing with *Urera* vines. While not consuming the plant, infant teeth marks and oral specimens were left on the vines, and adequate samples were collected (Fig. 1). The second most frequently eaten and discarded plant species was wild celery (*Peucedanum linderi*). Gorillas would most commonly scrape the inner pith of the celery stalk with their teeth leaving large portions of the fibrous exterior of the plant (Fig. 2). These plant species were easily collected after being discarded by gorillas and teeth marks and saliva could be clearly visualized on the discarded bitten or chewed portions.

Golden monkeys were also observed eating and discarded plant samples were successfully collected during every visit to the habituated group (Fig. 3). Golden monkeys were predominantly eating bamboo shoots (*Bambusa vulgaris*) during the sampling period. They were observed consuming the inner portion of the shoot, leaving the more fibrous outer portions behind. Sample collection from golden monkeys was more challenging, compared to gorillas, given their arboreal nature. Discarded plants were often dropped onto tree branches after being carried some distance through the canopy.

Gorilla beta-actin was detected in oral specimens recovered from 34/35 (97%) plant species and 321/383 (83.8%) total discarded plant samples (Tables II and III). On bivariate analyses of plant

species, beta-actin was 4.8 times as likely to be detected in specimens recovered from wild celery (95%CI: 1.28–17.92, $P = 0.01$) compared to other plant species. On bivariate analyses of portions of the plants eaten by gorillas, beta-actin was 2.27 times as likely to be detected in plants in which gorillas ate the inner pith (95%CI: 1.09–7.15, $P = 0.002$) and 0.11 times as likely to be detected from plants in which gorillas ate the root (95%CI: 0.03–0.53, $P = 0.00$) compared to other portions of plants eaten. Wild celery, plant species in which gorillas consumed the inner pith and plant species in which they ate the roots were evaluated for associations with detection of beta-actin by multivariable logistic regression modeling. Of all variables evaluated, only the root portion of the plant was significantly associated with a lower likelihood of detecting beta-actin (OR: 0.11, 95%CI: 0.22–0.49, $P = 0.01$).

Gorilla herpesviral DNA was detected in oral specimens recovered from 28/35 (80%) plant species and 161/383 (42%) total discarded plant samples (Tables II and III). Herpesvirus strains detected included a cytomegalovirus (KU578072) with 95.7% nucleotide similarity to Panine herpesvirus 2 (AF480884) across a fragment of the TERM gene, a rhadinovirus (KU578070, KU578071) with 97% nucleotide similarity to Gorilla rhadinovirus-1 (AY177144) across a fragment of the DPOL gene and 83% nucleotide similarity to human herpesvirus 8 (KT271468) across a fragment of the TERM gene, and a lymphocryptovirus (KU578068, KU578069) with 96.1% nucleotide similarity to Gorilla gorilla LCV-1 (AF250885) across a fragment of the DPOL gene and 90.6% nucleotide similarity to *Cercopithecus* herpesvirus 15 (AY037858) across a fragment of the TERM gene. On bivariate analyses of plant species, herpesviral DNA was 2.4 times as likely to be detected in oral specimens recovered from wild celery (Mantel–Haenszel OR 95%CI: 1.24–4.64), 4.02 times as likely from *Brillantaisia* (Mantel–Haenszel OR 95%CI: 1.28–12.62), 0.25 times as likely from *Chrysophyllum* (Mantel–Haenszel OR 95%CI: 0.07–0.86), and 0.24 times as likely from *Urera* (Mantel–Haenszel OR 95%CI: 0.12–0.51) compared to all other plant species. On bivariate analyses of portion of the plant eaten by gorillas, herpesviral DNA was 2.43 times as likely to be detected in oral specimens recovered from plants in which the inner pith was eaten (Mantel–Haenszel OR 95%CI: 1.42–4.16) and 3.77 times as likely to be detected from plants in which the leaves were eaten (Mantel–Haenszel OR 95%CI: 1.34–10.62) compared to other portions of plants eaten. Age was a confounder of both plant species and portion of the plant bitten or chewed on stratified analysis.

Wild celery, *brillantaisia*, *Urera*, *and Chrysophyllum*, plant species in which gorillas consumed the inner pith, plant species in which gorillas consumed the leaves, age and family group were evaluated for associations with detection of herpesviral DNA by mixed-effects logistic regression modeling. Although gorilla age did not significantly predict detection of herpesviral DNA, the stratified bivariate analyses were consistent with age confounding the detection of herpesviruses from specimens collected from different plant species and portion of the plant bitten or chewed. Therefore, gorilla age was included in the mixed effect logistic model. Of all variables evaluated, the only significant associations detected were that herpesviral DNA was 0.23 times as likely be detected in specimens recovered from *Chrysophyllum* plants (95%CI: 0.06–0.89, $P = 0.03$) and 3.84 times as likely to be detected from plants in which the leafy portion of the plant was eaten (95%CI: 1.27–11.65, $P = 0.02$).

Simian foamy virus and viruses causing potential respiratory diseases (*Coronaviridae*, *Enteroviridae*, *Paramyxoviridae*, and *Influenza virus*) were not detected in specimens recovered from any plant collected from mountain gorillas. Golden monkey beta-actin was recovered from 12/15 (80%) and beta and gamma herpesviral DNA from 12/15 (80%) discarded bamboo plant samples. Herpesvirus strains detected included a lymphocryptovirus (KU578073) with 96.9% nucleotide similarity to *Macaca fascicularis* lymphocryptovirus (JQ062969) across a fragment of the DPOL gene, a cytomegalovirus (KU578074) with 86.5% nucleotide similarity to Cynomologus macaque cytomegalovirus (JN227533) across a fragment of the TERM gene, and a rhadinovirus (KU578075) with 95.8% nucleotide similarity to chlorocebus rhadinovirus 2 (AJ251574) across a fragment of the DPOL gene. In golden monkeys, simian foamy virus nucleic acid was detected in specimens recovered from 2/15 (13.3%) discarded bamboo plant samples. Simian foamy virus strains detected (KU578076) had 90.9% nucleotide similarity to a simian foamy virus isolated from a human in the Democratic Republic of Congo

TABLE III. Detection of Mountain Gorilla Beta-Actin and Herpesviral Nucleic Acid in Specimens Recovered from Discarded Plants by Portion of Plant Eaten

Portion of plant eaten	Beta-actin (%)	Herpesviruses (%)
Fruit	29/31 (93.6)	12/31 (38.7)
Pith	72/77 (93.5)	47/76 (61.8)
Stalk	69/82 (84.2)	37/80 (46.3)
Leaves	16/20 (80.0)	15/20 (75.0)
Root	4/8 (50.0)	0/8 (0.0)
Grass	5/6 (83.3)	6/6 (100.0)
Parasite/fungus	4/5 (80.0)	3/5 (60.0)
Bark/peel	84/102 (82.4)	30/102 (29.4)
Wood	9/9 (100.0)	4/9 (44.4)

(JX157540). Golden monkey data were not modeled given the small sample size and because only one species of plant was collected.

DISCUSSION

Discarded plant samples were successfully collected from wild mountain gorillas and golden monkeys for detection of DNA and RNA viruses. This is the first report of the successful use of discarded food items for viral detection in primates. Discarded plant samples were collected from all 26 habituated mountain gorilla family groups tracked in Uganda and Rwanda. Discarded plant samples were readily available, collection resulted in minimal behavioral disruption of the gorilla groups and, in most cases, allowed for specimen collection from every individual within a group during a feeding session, including infants. Compared to traditional sample collection methods for which primates are darted and anesthetized, discarded plant specimen collection resulted in less behavioral disruption and minimal risk while allowing for a much greater number of individuals to be sampled.

Compared to traditional non-invasive collection methods, such as the collection of feces and urine, discarded plant collection also allowed for a greater number of samples to be collected during a single sampling session, along with accurate identification of the age of the sample and the individual gorilla associated with the sample. Collection of fecal samples from night nests is generally the preferred method for larger studies because it is efficient for collecting samples from multiple individuals within a group. Mountain gorillas make a new nest every evening and defecate in a designated portion of the nest at some time during the night or early morning. Directly observing mountain gorillas defecating happens far less frequently. Therefore, the identification of individuals usually requires genetic analysis of the sample. The age of the sample is also often difficult to ascertain when a nest is found and a sample is collected. Urine samples can be collected opportunistically from mountain gorillas when they urinate onto vegetation. This process requires observing the gorilla urinate and immediately pipetting the sample from leaves. While it is possible to collect a sample from every individual, urine sample numbers are limited given the infrequency of observed urination. Surveillance that relies on fecal and urine samples is also limited to the pathogens shed by these routes. Discarded plants allowed sampling for the oropharyngeal route of viral shedding and potentially viruses not detectable through fecal or urinary samples.

Discarded plants were also successfully collected from sympatric golden monkeys living in close proximity to the mountain gorillas in Rwanda. This primate species, however, proved more challenging for sampling, given their arboreal nature and the manner in which they consumed plant forage. Golden monkeys handled plants with their mouths less, thus generally depositing less saliva and oropharyngeal cells on plant material. Whereas gorillas tended to use their mouths to strip outer peels and bark from plants, golden monkeys more commonly used their hands. Golden monkeys also consumed a larger portion of the plant they were eating, therefore, leaving less material behind for potential sampling. This sampling method was useful, in any case, and samples could be collected with the technique, albeit on a smaller scale. For golden monkeys and other primate species with similar arboreal nature and eating behaviors, additional sampling visits should be planned in order to reach target sample volume.

Discarded plant sample collection proved to be feasible across a variety of different habitats when gorillas and golden monkeys were eating different species of plant foliage. Both beta-actin and herpesviral DNA were detected from a variety of discarded plant species where gorillas and golden monkeys were eating different portions of the plants. These findings are consistent with previous studies, which have shown that multiple discarded plant species can be used to collect genetic material from gorilla oral specimens [Smiley et al., 2010]. Here, however, we show for the first time that primate viruses can also be detected using these methods.

Simian foamy virus was detected in oral specimens recovered from discarded plants from golden monkeys but not mountain gorillas. This finding demonstrates that it is possible to detect RNA viruses from primate oral specimens recovered from discarded plants. However, it is unclear whether this sample collection method is less effective for the collection of RNA viruses in mountain gorillas or whether mountain gorillas shed simian foamy virus less frequently than other primates. Simian foamy virus is considered the most likely RNA virus to be present in primates and has been detected non-invasively in the wild [Voevodin & Marx, 2009b]. Simian foamy virus RNA has been detected in fecal samples of wild chimpanzees and simian foamy virus DNA in blood samples of western lowland gorillas however, this virus has not yet been detected in wild ape saliva samples nor in mountain gorillas by any sampling technique [Blasse et al., 2013; Calattini et al., 2004; Goffe et al., 2012; Liu et al., 2008]. While low levels of proviral simian foamy virus DNA or endogenous retroviral DNA can be detected in tissues and blood, viral RNA, indicative of viral replication, is abundant in differentiated superficial oral mucosal cells that are shed into the oral cavity [Falcone et al., 1999; Murray et al., 2008]. The PCR protocols used in this study targeted a conserved region of the simian foamy virus POL and LTR genes and should have detected all simian foamy viruses potentially present in a sample. Based

on these facts, the assumption was made that mountain gorillas would have detectable simian foamy virus RNA in saliva recovered from discarded chewed plant samples. Concurrent antibody screening of serum samples from a small sample of the same population of mountain gorilla ($n = 77$), however, did not detected simian foamy virus [Gilardi et al., 2011]. In addition, a pilot study conducted by these authors with a small group of western lowland gorillas ($N = 5$) at the San Francisco Zoo did not find simian foamy virus by PCR in paired oral swab and discarded plant samples. Thus, it is likely that simian foamy virus was not detected in discarded plant samples from mountain gorillas in this study because they were either not infected or not shedding the virus during this sampling period.

Similarly, no respiratory viruses were detected in specimens recovered from discarded plants from mountain gorillas. This was not unexpected, as all samples were collected from healthy individuals; we did not encounter gorillas with respiratory signs during our study. Unlike herpesviruses and simian foamy viruses, which are often shed in healthy individuals, we did not expect that respiratory viruses would be shed and therefore detected in healthy individuals, unless a gorilla was by chance sampled during a prodromal phase of viral shedding just before becoming ill. Identifying respiratory viruses circulating in this population has been challenging to date because samples have only been acquired from gorillas undergoing anesthesia for treatment of their respiratory illness. Even if a respiratory outbreak cannot be recognized, population-wide screening using non-invasive techniques should be utilized, in the chance that a respiratory virus could be identified because opportunities for anesthetizing gorillas are so rare. Further efforts to collect both oral and nasal specimens and discarded plant samples from gorillas exhibiting signs of respiratory illness will help evaluate the utility of discarded plants as a diagnostic sample during respiratory disease outbreaks. In addition, the collection of fecal samples during respiratory outbreaks is recommended as human metapneumovirus and human respiratory syncytial virus have been detected in chimpanzee fecal samples during respiratory outbreaks [Kondgen et al., 2010].

Evaluation of sensitivity or specificity of the discarded plant sampling technique was beyond the scope of this study. In order to do so, we would have needed to collect paired non-invasive discarded plant and invasive oral swab samples from the same individual. This was not possible given the associated risk of field anesthesia in wild mountain gorillas. Evaluation of sensitivity and specificity of a similar sampling technique was possible in another study by which non-invasive oral samples were collected using a distributed rope, along with paired oral swabs [Smiley Evans et al., 2015]. In this study, the non-invasive sampling technique had a lower sensitivity compared to oral swabs for RNA viruses and no significant difference in specificity. The non-invasive sampling technique did not have a significant difference in sensitivity or specificity compared to oral swabs for DNA viruses. Similar results would be likely with this discarded plant sampling method and we would expect sensitivity, particularly for RNA viruses, to be lower for discarded plants compared to oral swabs. When plants are dropped from the primate's mouth, they are exposed to the environment, which includes plant enzymes, and UV light, as well as different moisture levels and temperature, all of which rapidly degrade RNA, and to a lesser extent, DNA. Sensitivity and specificity of this technique should be evaluated for viruses targeted for surveillance and lower sensitivity of this technique should be considered when interpreting a negative discarded plant sample. In addition, the best available cold chain should be implemented. In this study, we stored samples in viral transport media because it was considered optimal for long-term storage of viruses and we wanted to maintain the ability to culture virus. Alternative storage medias such as RNAlater (Ambion, Inc., Foster City, CA), which is commonly used to store fecal samples, can likely also be used to store discarded plants and should be further evaluated.

Field studies investigating viruses in primates are laborious and expensive in both sample collection and laboratory diagnostics. It is, therefore, important to optimize the types of samples collected in order to maximize the viral data collected and to save precious conservation resources. For future studies involving great apes and other primate species employing this sampling method, we recommend collecting a plant that is frequently consumed by the primate species of interest in order to create greater comparability among sample data. In this study, the optimal plant species to collect from mountain gorillas due to ease of sample collection, as well as a strong detection of beta-actin and herpesviruses, was wild celery. Gorillas frequently consumed wild celery during the sampling period across parks, elevations, and gorilla family groups. Gorillas left the largest quantities of discarded plant material when eating wild celery as they scraped out the inner pith with their mouths and discarded the outer fibrous stalk. This feeding behavior allowed researchers to find the highest quality sample by visually inspecting for deposited saliva and teeth marks from a discarded pile with many pieces of bitten or chewed celery.

In addition and when possible, we recommend collecting discarded plants in which the leafy portion of the plant is consumed. In this study, herpesviral DNA was most likely to be detected in specimens recovered from discarded plants in which the leafy portion of the plant was eaten. Gorillas run the leaves

through their mouths stripping off the desired portion of the leaves and discarding the stems and less desirable portions of the leaves. This consumption method produces discarded plant samples that have had maximum contact between the plant and the gorilla's mouth, and it is, therefore, logical that greater amounts of oral specimen could be recovered from plants eaten using this method.

We recommend avoiding collection of plants in which the root portion of the plant is eaten; eating the root portion of the plant was negatively associated with the detection of beta-actin, likely due to a lack of deposited quality oral specimen using this eating method. Gorillas did not push the root through their mouths when eating as with other plants and, therefore, deposited less oral specimen. We also recommend avoiding collection of *Chrysophyllum* plant species. *Chrysophyllum* plant species were negatively associated with the detection of herpesviruses, likely due to the viscous inner pith of the fruit, which may inhibit recovery of viruses. In laboratory settings some plant materials, including cotton, have proven to have negative effects on herpesvirus recovery due to viruses becoming lodged in the fibrous material [Smiley Evans et al., 2015]. *Chrysophyllum* plant species may have similar effects on recovery of herpesviruses and other viruses of interest because viruses could become lodged in the viscous inner pith material. Seven other plant species did not allow for detection of herpesviral DNA. These plants, however, had small sample sizes, and it cannot be determined whether the few gorillas eating these plants were simply not shedding herpesviruses at the time they were eating the plant or whether these plants inhibited virus detection. For application of this technique to other primate species, we recommend evaluating the feeding behavior of the species of interest and selecting the best plant for collection based on frequency of consumption, and the eating behavior that promotes depositing the greatest amount of specimen on discarded plant material, along with consideration of likely plant based PCR inhibitors.

In the remote tropical locations where great apes and other primate species live, many populations are not consistently monitored for pathogens that could be of conservation or public health concern. This lack of health monitoring hinders the understanding of disease dynamics within great ape populations and the other species with which they interact and also promotes a largely reactionary approach in which steps are taken only after an outbreak of disease has occurred. Here, we suggest the collection of discarded plants as a non-invasive sampling method that can capture the oral route of viral shedding and improve viral detection in difficult to monitor primate species. Together with other available non-invasive sample types, such as feces and urine, discarded plants can be used to capture the majority of routes for viral oral shedding. We have demonstrated that discarded plants are readily available for evaluation and that quality oral specimens can be effectively recovered for viral detection in wild habituated mountain gorillas, golden monkeys, and potentially other wildlife species. Discarded plants are relatively easy to collect compared to other non-invasive biological samples, the individual that produced the sample can be identified and the specimens can be used to investigate known as well as novel viruses at a population wide scale. Implementing discarded plant collection as part of a comprehensive non-invasive sampling effort for routine viral detection activities and as a complementary technique for outbreak response efforts could greatly enhance our understanding of human and primate viruses circulating among great apes and sympatric primate species.

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