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RESEARCH ARTICLE

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Viruses associated with ill health in wild chimpanzees

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Abstract

Viral infection is a major cause of ill health in wild chimpanzees (*Pan troglodytes*), but most evidence to date has come from conspicuous disease outbreaks with high morbidity and mortality. To examine the relationship between viral infection and ill health during periods not associated with disease outbreaks, we conducted a longitudinal study of wild eastern chimpanzees (*P. t. schweinfurthii*) in the Kanyawara and Ngogo communities of Kibale National Park, Uganda. We collected standardized, observational health data for 4 years and then used metagenomics to characterize gastrointestinal viromes (i.e., all viruses recovered from fecal samples) in individual chimpanzees before and during episodes of clinical disease. We restricted our analyses to viruses thought to infect mammals or primarily associated with mammals, discarding viruses associated with nonmammalian hosts. We found 18 viruses (nine of which were previously identified in this population) from at least five viral families. Viral richness (number of viruses per sample) did not vary by health status. By contrast, total viral load (normalized proportion of sequences mapping to viruses) was significantly higher in ill individuals compared with healthy individuals. Furthermore, when ill, Kanyawara chimpanzees exhibited higher viral loads than Ngogo chimpanzees, and males, but not females, exhibited higher infection rates with certain viruses and higher total viral loads as they aged. Post-hoc analyses, including the use of a machine-learning classification method, indicated that one virus, salivirus (*Picornaviridae*), was the main contributor to health-related and community-level variation in viral loads. Another virus, chimpanzee stool-associated virus (chisavirus; unclassified Picornavirales), was associated with ill health at Ngogo but not at Kanyawara. Chisavirus, chimpanzee adenovirus (*Adenoviridae*), and bufavirus (*Parvoviridae*) were also associated with increased age in males. Associations with sex and age are consistent with the hypothesis that nonlethal viral infections cumulatively reflect or contribute to senescence in long-lived species such as chimpanzees.

KEYWORDS
chimpanzee, conservation, disease, health, metagenomics, virus

Abbreviations: LMM, linear mixed model; RLMM, robust linear mixed model; vRPM/kb, viral reads per million per kilobase of target sequence.

1 | INTRODUCTION

Viruses are among the most important causes of ill health and mortality in wild chimpanzees and represent a grave threat to conservation (Dunay et al., 2018; Glasser et al., 2021; Köndgen et al., 2008; Leendertz et al., 2006; Melin et al., 2020; Williams et al., 2008). Respiratory diseases caused by viral infections, including those of coronaviruses, metapneumoviruses, and rhinoviruses, have been reported in chimpanzee populations across equatorial Africa (Kaur et al., 2008; Köndgen et al., 2008, 2010; Negrey et al., 2019; Patrono et al., 2018; Scully et al., 2018). However, most information about viral infection in wild chimpanzees comes from conspicuous disease outbreaks with high morbidity and mortality (Formenty et al., 1999; Köndgen et al., 2008; Negrey et al., 2019; Scully et al., 2018). Although virulence may, in some cases, augment viral transmission (Alizon & Michalakis, 2015), avirulent or minimally virulent pathogens can persist in populations due to greater long-term transmission success (Méthot, 2012). Therefore, many less virulent pathogens may have important but protracted health implications, perhaps acting cumulatively over time to affect health, as suggested for humans (Pawelec et al., 2010). Alternatively, viruses may respond to hosts' ill health by proliferating, offering a potentially useful biomarker of declining physiological or immunological function.

Viruses and other microparasites of low virulence can have important effects at both the individual and population levels (Anderson & May, 1979). Effects can be both direct (e.g., cellular damage) and indirect (e.g., limiting energy available for nonimmunological processes). Furthermore, viruses can either cause ill health directly, or viral infection and shedding can result from ill health. In humans, several inflammatory bowel diseases are associated with expansion of the gastrointestinal virome (Norman et al., 2015), and in rhesus macaques (*Macaca mulatta*), pathogenic progression of simian immunodeficiency virus (SIV) infection entails expansion of the gut virome, including coinfection with potentially pathogenic agents such as adenovirus (Handley et al., 2012, 2016). A study of wild gorillas (*Gorilla gorilla*) reported that the gut virome differed between individuals with and without SIV infection; SIV-infected individuals not only shed greater quantities of viruses in the families *Herpesviridae* and *Reoviridae* but were the only individuals in the sample to shed viruses in the family *Adenoviridae* (D'arc et al., 2018).

In the present study, we used metagenomics to characterize gastrointestinal viromes (i.e., viral communities characterized in fecal samples) of wild eastern chimpanzees (*P. t. schweinfurthii*) from the Kanyawara and Ngogo communities of Kibale National Park, Uganda. The communities are separated by approximately 10 km within an estimated population of 1500–2000 individuals (Plumptre & Cox, 2006). For the last 27 to 34 years, both communities have grown in size and exhibit high individual survival, with life expectancy higher at Ngogo than at Kanyawara (Muller & Wrangham, 2014; Wood et al., 2017). Using methods previously applied to this chimpanzee population (Negrey et al., 2020), we investigated links between gastrointestinal viruses and general health. Our goal was to identify which viruses, if any, were associated with observed episodes of ill health in this population and may act as biomarkers of illness. We sampled individuals both before and during episodes of illness to assess within-individual changes in viral infection associated with ill health.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The noninvasive procedures used in this study were approved by the Uganda Wildlife Authority, the Uganda National Council for Science and Technology, and by the Institutional Animal Care and Use Committees (IACUCs) of Harvard University (protocol number 96-03), Tufts University (protocol number M2019-83), and the University of New Mexico (protocol number 18-200739-MC). The study was formally exempt from review by the IACUCs of the University of Michigan and Boston University. All procedures complied with the American Society of Primatologists Ethical Principles for the Treatment of Non-Human Primates.

2.2 | Study sites, subjects, and sample collection

We conducted health monitoring and collected corresponding fecal samples in the Kanyawara and Ngogo chimpanzee communities of Kibale National Park, Uganda, from July 2015 to May 2019. On January 1, 2016, Kanyawara and Ngogo contained 49 and 194 individual chimpanzees, respectively. Research personnel collected daily health observations from all individually identified chimpanzees in the study communities. Observers noted the presence or absence of clinical signs and provided detailed descriptions when signs were severe. We converted these data into a categorical health scoring system: (1) no signs, (2) coughing or sneezing, (3) diarrhea or otherwise abnormal feces, (4) an unhealed wound, (5) skin abnormality or swelling, and (6) mobility deficits (e.g. reduced mobility/limping). Whenever a chimpanzee experienced one or more signs (categories 2–6), that individual was classified as ill.

As previously described (Negrey et al., 2020), we collected fecal samples on a quarterly basis and homogenized them at a 1:1 ratio with RNAlater buffer (Thermo Fisher Scientific). We stored samples in an ice-filled thermos until returning to camp, at which point we transferred samples to freezers at −20°C. We then transported samples on ice to the United States. To examine changes in viral infection corresponding to periods of ill health, our analyses only included chimpanzees that were classified as ill during the study period. For each episode of illness, we selected one pair of samples from the individual in the quarter before the illness event, and a second sample from the individual during the quarter in which they were ill. In three cases, fecal samples were not collected in the quarters immediately preceding observed illness, so we therefore used samples collected two or three quarters before observed illness (Table S1).

2.3 | Viral identification and sequencing

We analyzed 40 samples from 19 individuals ranging in age from 2 to an estimated 54 years at the time of illness (Kanyawara Males = 4; Kanyawara Females = 3; Ngogo Males = 9; Ngogo Females = 3), collected during quarters when they exhibited clinical signs of illness outside of disease outbreaks (Table S1). We sampled one individual male at Ngogo for two different episodes of illness that occurred 2.1 years apart. Following previously described protocols (Goldberg et al., 2017, 2018, 2019; Negrey et al., 2020; Sibley et al., 2016; Toohey-Kurth et al., 2017), we used metagenomic methods to identify viruses in chimpanzee feces and to estimate their loads. In brief, we added 200 µl of feces (preserved in RNAlater) to 800 µl of Hanks' Balanced Salt Solution and then homogenized the solution by bead beating. We treated the homogenate with nucleases to reduce unencapsidated nucleic acids (Allander et al., 2001) and performed subsequent extractions with the Qiagen QIamp MinElute Virus Spin Kit (Qiagen). We synthesized cDNA from the extracted RNA, purified cDNA using Agencourt AmpureXP beads (Beckman Coulter), and prepared libraries using the Illumina Nextera XT kit (Illumina). We sequenced libraries on an Illumina MiSeq instrument using 2 × 150 cycle paired-end chemistry. As previously described (Bennett et al., 2020; Toohey-Kurth et al., 2017), we mitigated "index hopping" or "bleed over" between samples by performing additional library cleanups to remove free adapters, storing libraries at −20°C, and sequencing libraries on an MiSeq instrument, which minimizes index hopping by using a nonpatterned flow cell and bridge amplification clustering (Bentley et al., 2008). We also ran blank samples and examined all data post-sequencing for evidence of cross-contamination with unique sequences.

To complement the methods above, we also analyzed samples by multiplex RT-PCR (NxTAG Respiratory Pathogen Panel, Luminex). The panel detects 20 human respiratory pathogens, including several, such as human respiratory syncytial virus, human metapneumovirus, and rhinovirus, that have caused respiratory disease in wild apes (Grützmacher et al., 2016; Negrey et al., 2019; Scully et al., 2018). Our prior studies of respiratory disease in these chimpanzee communities benefitted from this highly sensitive and specific assay (Negrey et al., 2019; Scully et al., 2018); see Scully et al. (2018) for further description of this method.

2.4 | Bioinformatics

To identify and reconstruct viral sequences, we used CLC Genomics Workbench (CLC Bio). We trimmed short and low-quality sequences, removed reads that mapped to chimpanzee DNA and known contaminants, and assembled the remaining reads de novo. We used BLAST algorithms to compare the resulting contiguous sequences (contigs) with viral sequences in the GenBank sequence database at both the nucleotide and amino acid level (Altschul et al., 1990; Gish & States, 1993). For the purposes of this analysis, we retained only contigs that matched viruses with mammalian hosts or were primarily associated with mammals, under the assumption that contigs matching viruses of plants, insects, and similar taxa were associated with dietary items. For all downstream analyses, we used replicase genes when possible; exceptions included viruses with genomes consisting of single open reading frames and those for which only the capsid-encoding gene was available. Because we used comparable genomic segments for each virus, we avoided classifying separate segments of the same genome as different viruses.

Following common methods for metagenomics analyses of viruses from fecal and sewage samples (Hjelmsø et al., 2017; Siqueira et al., 2018; Tisza & Buck, 2021), we measured viral loads by mapping reads from each sample to viral contigs and calculating the proportion of total reads that mapped to each identified virus, from which we also calculated the total load of all viruses detected in the sample. We subsequently normalized this proportion to 1 million reads and adjusted for target sequence length (kilobases). The resulting measure of viral load, viral reads per million per kilobase of target (vRPM/kb), has been validated by real-time quantitative polymerase chain reaction (Huang et al., 2019; Toohey-Kurth et al., 2017) and has proven useful for quantifying viral loads in wild chimpanzees (Negrey et al., 2020).

To infer viral phylogenetic relationships, we first used TranslatorX (Abascal et al., 2010) to align newly generated viral sequences with those of related viruses retrieved from GenBank, removing poorly aligned regions via the Gblocks algorithm (Castresana, 2000). We then used PhyML v3.0 (Lefort et al., 2017) to generate maximum likelihood phylogenetic trees with 1000 bootstrap replicates and used FigTree v1.4.4 (Rambaut, 2018) to display the resulting trees.

2.5 | Inferential statistics

We used the modified Wald method (Agresti & Coull, 1998) to calculate viral prevalence by health status, sex, and study community. We then conducted linear mixed model (LMM) analyses of the association of clinical status and covariates with viral richness (the number of distinct viruses per sample) and total viral load (vRPM/kb for all viruses) in R v4.0.3 (R Core Team, 2021). We used the "lmer" function in package lmerTest v3.1.3 (Kuznetsova et al., 2017) as modified from lme4 (Bates et al., 2015) to run LMMs with Gaussian error structures and fitted with restricted maximum likelihood. We ran two LMMs in which the response variables were viral richness and total viral load, respectively. The fixed effects included the individual's health status, study community, sex, and age at the time of sample collection. Chimpanzee age was determined as previously described (Negrey et al., 2020) and was included as a continuous variable. We also included interactions among health status with age, sex, and study community, as well as the interaction between age and sex, based on prior findings from this population (Negrey et al., 2020). To mitigate type 1 and type 2 error, we removed interaction terms for which the *p*-value was greater than or equal to alpha (0.05). Finally, we included subject ID as a random variable to control for multiple sampling of individuals. We calculated *p*-values using the

Satterthwaite method (Luke, 2017). After detecting that model residuals deviated moderately from normality (see Supporting Information), we ran both models again as robust linear mixed models (RLMMs) using the "rlmer" function in package robustlmm (Koller, 2016). We provide results for both the original LMMs and the accompanying RLMMs.

To determine which individual viruses contributed to patterns identified in the LMMs, we applied random forest classification using the "randomForest" function in R package randomForest (Liaw & Wiener, 2002). The random forest algorithm is a machine learning tool that generates a large number (i.e., from dozens to thousands) of decision trees via random, independent sampling of a data set, and that can be used to predict or classify a given set of values (Breiman, 2001). Each decision tree is trained on a subset of the original data set. We generated a series of random forests in which we analyzed response variables that were significant by mixed modeling (e.g., total viral load, community, age). For all random forests, predictor variables were viral loads (vRPM/kb) for each individual virus. Importance values of each virus for predicting the given response variable were calculated as the mean decrease in node impurity. As node impurity decreases, the forest becomes more effective at predicting and/or classifying samples (Segal & Xiao, 2011). For continuous and categorical response variables, node impurity was calculated using the residual sum of squares and Gini index, respectively (Liaw & Wiener, 2002).

R script and data are available on Figshare (Negrey et al., 2021).

3 | RESULTS

3.1 | Virus detection

We selected and analyzed 20 episodes of ill health in 19 chimpanzees, all but one of whom were sexually mature (Table S1). Clinical signs included coughing and/or sneezing (19 episodes) and diarrhea (two episodes). One individual exhibited respiratory signs and diarrhea concurrently. No cases of skin abnormalities, wounding, or limping were included in our analyses. Episodes of ill health were acute: None of the observed episodes resulted in mortality, and at other times, each chimpanzee was apparently healthy.

In fecal samples collected before and proximate to episodes of ill health, we found 18 viruses that are either thought to infect mammals or are primarily associated with mammals (Table 1), including nine viruses previously identified in fecal samples from Kibale chimpanzees. Contigs derived from metagenomic analyses mostly corresponded to replication-associated genes; only contigs for bufavirus, chisavirus, and salivirus represented most of the genome. The similarity of amino acid sequences to known viruses ranged from 46.3% to 99.8%. We did not detect any viruses in 2 of the 40 fecal samples. Viral prevalence varied considerably (Table S2), ranging from 2.5% for unclassified chimpanzee ssDNA virus 1 (95% CI: 0.0%, 14.0%) to 52.5% for eastern chimpanzee-associated porprismacovirus 9 (95% CI: 37.5%, 67.1%). Eleven of the 18 viruses were present at both Ngogo and Kanyawara. Chimpanzee stool-associated RNA virus (chisavirus), three porprismacoviruses, and an unclassified single-stranded DNA (ssDNA) virus were detected only in Ngogo samples, whereas an enterovirus and a second unclassified ssDNA virus were found only in samples from Kanyawara. We found no evidence of "index hopping: or "bleed over" in read data from blanks or chimpanzee fecal samples.

Targeted multiplex RT-PCR assays identified no viruses other than those identified using metagenomics. This assay identified adenovirus in 19 samples from 13 chimpanzees, compared with 10 samples identified using metagenomics. Concordance between the metagenomic and RT-PCR assay results identified 31 agreements (77.50%) and 9 disagreements (22.5%), yielding a Cohen's kappa value of 0.538 ± SE 0.120 (95% CI: 0.303, 0.774).

3.2 | Virus shedding and health status

Virus prevalence varied by health status, sex, and study community (Table 2). Mixed models of viral richness showed no statistically significant variation by health status, age, sex, or community (Table S3). However, mixed models of viral load revealed substantial variation by these same predictors (Table 2; Figure 1). Notably, overall viral loads were 2.4 times higher in chimpanzees exhibiting clinical illness signs than in healthy chimpanzees (Table 2; Figure 2a). Healthy and ill chimpanzees exhibited mean total viral loads of 0.314 (SD ± 0.538) and 0.758 (SD ± 0.783) Log_{10}(vRPM/kb), respectively. Random forest classification identified three viruses as most important for predicting total viral load in ill chimpanzees regardless of subject age, sex, or community, and these were (in descending order of importance): salivirus, porprismacovirus 9, and chisavirus (Table S4; Figure 3a). Post-hoc modeling indicated the associations of salivirus and porprismacovirus 9 with total viral load were not driven by differences in prevalence: ill health did not predict overall prevalence of salivirus ($\beta = -0.077$, SE = 0.994, $p = 0.938$; Table S5a) or porprismacovirus 9 ($\beta = 0.272$, SE = 0.733, $p = 0.710$; Table S5b). However, the overall prevalence of chisavirus was positively associated with ill health ($\beta = 27.4$, SE = 11.4 $p = 0.002$; Table S5c).

The interaction of viral load and community was significant (Table 2; Figure 2b), with higher total viral load in ill chimpanzees in Kanyawara than in Ngogo. Ill chimpanzees at Kanyawara and Ngogo exhibited mean total viral loads of 1.138 and 0.554 Log_{10}(vRPM/kb), respectively. To determine if this relationship was affected by the time between observed illness and sample collection, we ran a robust multiple linear model ("rlm" function in R package "MASS") on samples collected during quarters with observed clinical signs, with study community and days from health observation to sample collection as the two predictor variables. The relationship between community and total viral load remained significant ($\beta = -0.937$, SE = 0.321, $F = 8.42$, $p = 0.010$); however, there was no relationship between the number of days between sample collection and total viral load ($\beta = -0.187$, SE = 0.157, $F = 1.53$, $p = 0.232$). Random forest classification indicated that salivirus most strongly differentiated between ill chimpanzees at Kanyawara and Ngogo (Table S4). Post-hoc generalized mixed models controlling for subject sex indicated that, among ill chimpanzees, salivirus was more

TABLE 1 Viruses detected in 40 fecal samples from wild chimpanzees in the Kanyawara and Ngogo communities of Kibale National Park, Uganda

	Virus	Closest match (accession number[a])	Family	Host (country, Year)	Genome	Length (nt)[b]	% identity[c]	Accession number[d]
1	Chimpanzee adenovirus	Chimpanzee adenovirus Y25 (YP_006272954)	*Adenoviridae*	Chimpanzee (—, 1969)	dsDNA	2403	99.75	MW876510
2	Chimpanzee bufavirus	Human bufavirus (AOR39545)	*Parvoviridae*	Human (Tunisia, 2013)	ssDNA	1500	81.40	MT076200
3	Chimpanzee circovirus 1	Dromedary stool-associated circular ssDNA virus (AIY31253)	*Circoviridae*	Camel (United Arab Emirates, 2013)	ssDNA	1134	60.65	MW876514
4	Chimpanzee circovirus 2	Circovirus sp. (QBA83760)	*Circoviridae*	Pig (China, 2017)	ssDNA	1179	46.31	MW876515
5	Chimpanzee enterovirus	Enterovirus B112 (AJA74399)	*Picornaviridae*	Chimpanzee (Gabon, 2009)	ssRNA	6150	92.78	MW876511
6	Chimpanzee picobirna-like virus	Kumba picobirna-like virus (QAA77647)	Unclassified Picornavirales	Human (Cameroon, 2014)	RNA	1188	93.43	MT076202
7	Chimpanzee salivirus	Salivirus FHB (YP_009067077)	*Picornaviridae*	Human (China, 2011)	ssRNA	7125	98.27	MW876512
8	Chisavirus (chimpanzee stool-associated RNA virus)	Husavirus (AWU65954)	Unclassified Picornavirales	Human (Venezuela, 2015)	ssRNA	8379	62.95	MW876513
9	Eastern chimpanzee- associated porprismacovirus 1	Macaca mulatta feces-associated virus 4 (APG55823)	*Smacoviridae*	Rhesus macaque (USA, 2014)	ssDNA	777	64.00	MT076205
10	Eastern chimpanzee-associated porprismacovirus 2	Porcine-associated porprismacovirus (QBP37091)	*Smacoviridae*	Pig (Vietnam, 2013)	ssDNA	735	71.97	MT076206
11	Eastern chimpanzee-associated porprismacovirus 3	Porcine-associated porprismacovirus 8 (YP_009054991)	*Smacoviridae*	Pig (USA, 2011)	ssDNA	786	55.91	MT076207
12	Eastern chimpanzee- associated porprismacovirus 4	Chimpanzee stool-associated circular ssDNA virus (ADB24816)	*Smacoviridae*	Chimpanzee (Tanzania, 2004)	ssDNA	816	99.26	MT076208
13	Eastern chimpanzee- associated porprismacovirus 6	Macaca mulatta feces-associated virus 7 (APG55812)	*Smacoviridae*	Rhesus macaque (USA, 2014)	ssDNA	780	68.34	MT076210
14	Eastern chimpanzee-associated porprismacovirus 7	Chimpanzee associated- porprismacovirus 2 (YP_009508863)	*Smacoviridae*	Chimpanzee (Tanzania, 2004)	ssDNA	586	92.27	MW876516
15	Eastern chimpanzee-associated porprismacovirus 8	Chicken smacovirus mg4_964 (QIR82267)	*Smacoviridae*	Chicken (USA, 2017)	ssDNA	786	77.27	MW876517
16	Eastern chimpanzee- associated porprismacovirus 9	Chicken smacovirus mg4_964 (QIR82267)	*Smacoviridae*	Chicken (USA, 2017)	ssDNA	771	68.98	MW876518
17	Unclassified chimpanzee ssDNA virus 1	Unidentified circular ssDNA virus (APG55818)	Unclassified	Macaque (USA, 2014)	ssDNA	639	96.70	MW876519
18	Unclassified chimpanzee ssDNA virus 2	Uncultured virus (AUM61717)	Unclassified	Wastewater (USA, 2015)	ssDNA	1020	58.31	MW876520

[a]Accession number of closest match in GenBank.

[b]Length of the nucleotide sequence used to analyze phylogenetic relationships and viral load.

[c]% amino acid similarity of the new virus to its closest match in GenBank.

[d]GenBank accession number of viral sequence from this study.

TABLE 2 Results of (a) a linear mixed model and (b) a robust linear mixed model assessing variation in total viral load

(a) Linear mixed model						
Predictor	***β***	**SE**	**95% CI**	**DF**	***t***	***p***
Intercept	0.405	0.283				
Health status[a]	**0.857**	**0.224**	**[0.406, 1.327]**	**19.521**	**3.821**	**0.001**
Age	−0.295	0.248	[−0.793, 0.192]	14.856	−1.191	0.252
Sex[b]	−0.024	0.265	[−515,0.475]	14.698	−0.090	0.930
Community[c]	−0.052	0.345	[−0.773,0.621]	20.313	−0.151	0.882
Age × Sex	**0.681**	**0.263**	**[0.161, 1.214]**	**14.699**	**2.591**	**0.021**
Health status × Community	**−0.643**	**0.278**	**[−1.232, −0.086]**	**19.513**	**−2.310**	**0.032**
(b) Robust linear mixed model						
Predictor	***β***	**SE**	**—**	**—**	***t***	***p***
Intercept	0.359	0.178	—	—		
Health status	**0.803**	**0.214**	—	—	**3.756**	**0.001**
Age	**−0.327**	**0.136**	—	—	**−2.404**	**0.030**
Sex	−0.132	0.144	—	—	−0.916	0.374
Community	0.002	0.218	—	—	0.010	0.992
Age × Sex	**0.539**	**0.143**	—	—	**3.769**	**0.002**
Health status × Community	**−0.619**	**0.265**	—	—	**−2.335**	**0.030**

Notes: The marginal and conditional R^2 for the linear mixed model were 0.37 and 0.69, respectively.

Bold font denotes predictors for which $p < 0.05$.

[a]The reference category is "healthy".

[b]The reference category is "female".

[c]The reference category is "Kanyawara".

prevalent at Kanyawara than Ngogo ($\beta = -0.705$, SE = 0.159, $p < 0.001$; Table S5d), and that among Kanyawara chimpanzees, salivirus was more prevalent among ill individuals than healthy ones ($\beta = 15.2$, SE = 8.39, $p = 0.011$; Table S5e). Similarly, chisavirus was only found in males at Ngogo (Table S2) and was more prevalent among ill males than healthy males ($\beta = 27.4$, SE = 11.2, $p = 0.002$; Table S5f).

The age by sex interaction was also significant (Table 2), indicating that males, but not females, exhibited higher total viral loads as they aged. Random forest classification indicated that chisavirus, adenovirus, and bufavirus were most important for differentiating males by age (Table S4; Figure 3b). Post-hoc generalized linear modeling indicated that male age was positively related to the prevalence of chisavirus ($\beta = 3.95$, SE = 2.85, $p = 0.007$; Table S5g) and adenovirus ($\beta = 1.24$, SE = 0.565, $p = 0.012$; Table S5h). Conversely, bufavirus prevalence decreased with age, but not significantly ($\beta = -0.130$, SE = 0.470, $p = 0.781$; Table S5i).

4 | DISCUSSION

We observed changes in the fecal viromes of clinically ill chimpanzees compared with when they were previously healthy. Specifically, we observed a 2.4-fold increase in total viral load when chimpanzees became ill. Of the viruses identified, salivirus was the strongest contributor to this trend. Salivirus was first described in humans suffering from gastroenteritis (Li et al., 2009) and has since been detected in sewage and water samples around the world (Adineh et al., 2019; Badru et al., 2018). Although members of family *Picornaviridae* cause a wide variety of illnesses in humans (Tuthill et al., 2010), the relationship between salivirus and gastroenteritis has been inconsistent, with some studies reporting a causal relationship (Itta et al., 2016; Shan et al., 2010) and others not (Aldabbagh et al., 2015; Yu et al., 2015). Interestingly, salivirus was also detected in a postmortem nasopharyngeal swab from a child suffering from a severe adenovirus coinfection (Pei et al., 2016), suggesting that salivirus infection may not be limited to the gastrointestinal tract. Moreover, in a previous study of this chimpanzee population, we identified salivirus in a higher proportion of aged males than in younger males (Negrey et al., 2020). In Kibale chimpanzees, salivirus may therefore be a cause of ill health, a biomarker of diminished immune function, or both.

Porprismacovirus 9 was also a strong contributor to total viral load, although prevalence and load did not differ greatly between healthy and ill chimpanzees. Porprismacoviruses (family: *Smacoviridae*) are small, circular, single-stranded DNA viruses found in the feces of primates and pigs (Varsani & Krupovic, 2018). Relatively little is

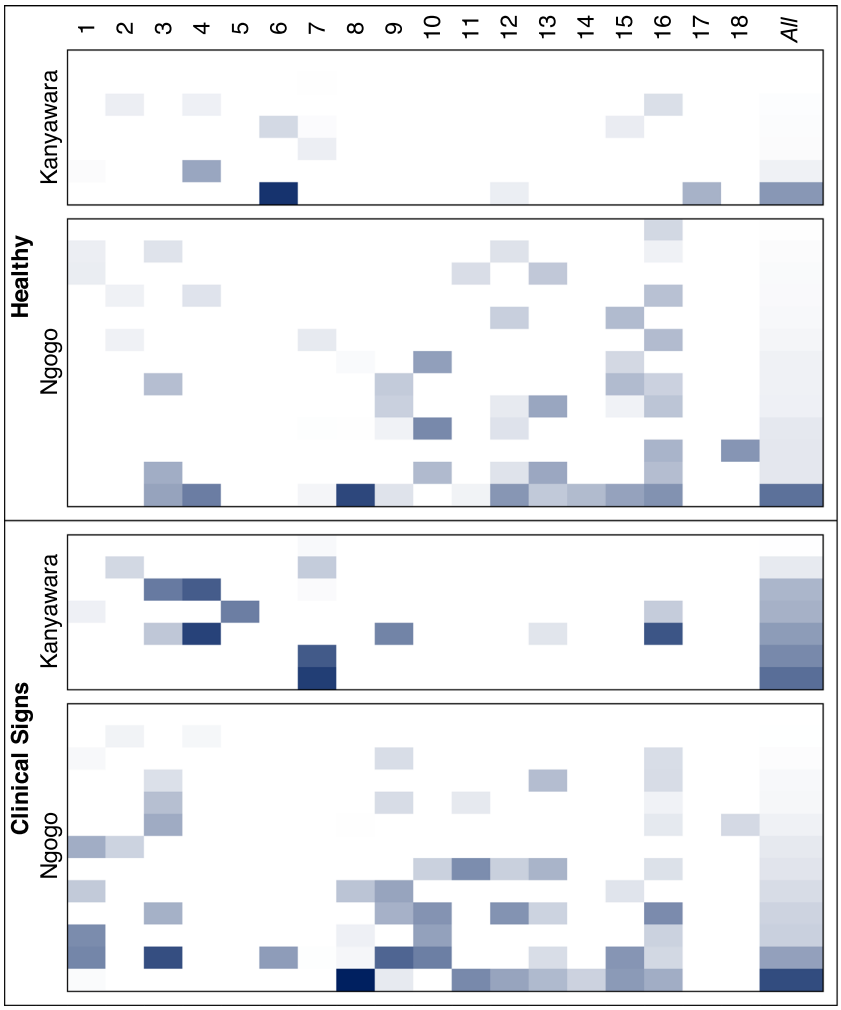

FIGURE 1 Heatmap of viral loads for all 19 chimpanzees, grouped by community and health status. Values range from 0 (lightest) to 3.3 Log_{10}(vRPM/kb) (darkest). Viruses are numbered as per Table 1

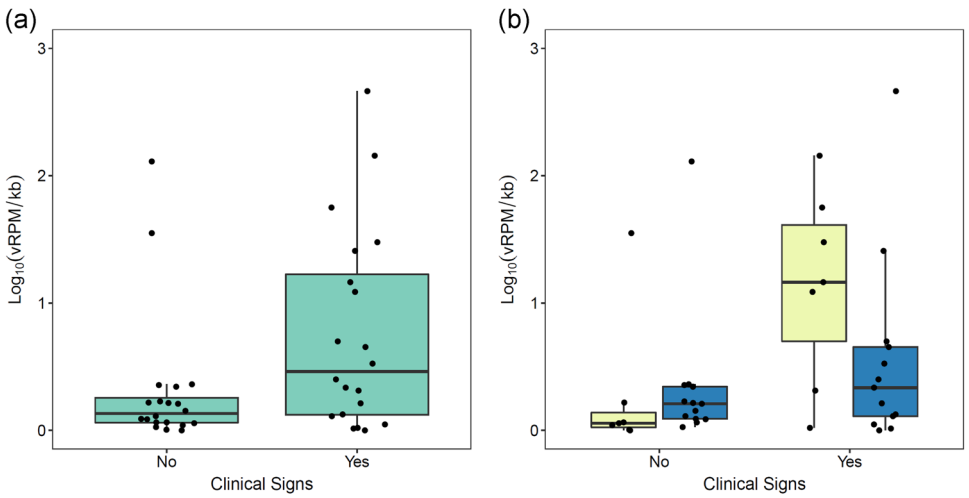

FIGURE 2 Chimpanzee gastrointestinal total viral load as a function of clinical signs (a) in all individuals and (b) by study community. In panel (b), light and dark boxes represent Kanyawara and Ngogo, respectively. Thick horizontal bars represent medians, and the upper and lower bounds of each box represent the 75th and 25th percentiles, respectively

known about porprimsmacoviruses, and any relationships between smacoviruses and vertebrate disease remain unclear. For instance, smacoviruses were detected in samples from a relatively large proportion of unsolved outbreaks of diarrhea, including 28% of outbreaks in the United States and 67% in France (Ng et al., 2015), and from as many as 19% of diarrheal samples from Peruvian children (Phan et al., 2016). However, smacoviruses have also been routinely detected in the absence of illness or immune suppression in a number of

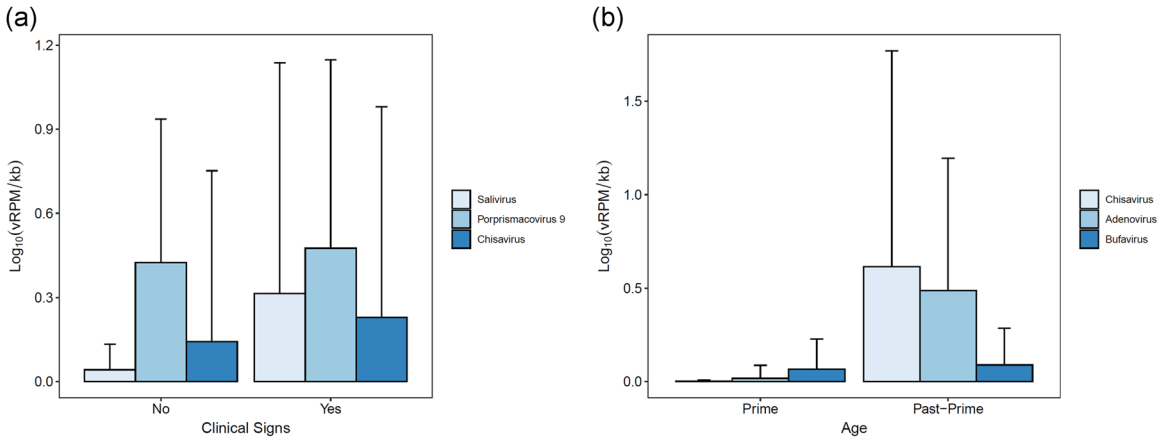

FIGURE 3 Mean viral loads for (a) all chimpanzees by health status and (b) male chimpanzees by age. "Prime" and "past-prime" refer to individuals aged <30 years and ≥30 years, respectively. Vertical bars indicate standard deviations

vertebrates (Varsani & Krupovic, 2018). Recent evidence even suggests that archaea, rather than animals, act as hosts for smacoviruses (Díez-Villaseñor & Rodriguez-Valera, 2019). Given their ubiquity, uncertainty surrounding their life cycles, and comparable shedding by healthy and ill chimpanzees, porprismacoviruses are unlikely to cause ill health in chimpanzees. However, we cannot discount the possibility that porprismacoviruses have long-term and subtle clinical consequences.

Chimpanzee stool-associated RNA virus (chisavirus) was the third most important component of total viral load in ill chimpanzees, and its prevalence was predictive of ill health among Ngogo chimpanzees. Chisavirus is closely related to a cluster of unclassified members of *Picornavirales* infecting a taxonomically broad range of hosts; these viruses include husaviruses in humans, posaviruses in pigs, and fisaviruses in fish (Aoki et al., 2019; Oude Munnink et al., 2017). Often observed at low prevalence, husavirus has not displayed any consistent relationship to ill health in humans (Mohammad et al., 2020; Oude Munnink et al., 2017). However, genomic (Shan et al., 2011) and epidemiological (Siqueira et al., 2018) evidence suggests that husaviruses and their relatives could be shed by gastrointestinal nematodes. Chisavirus presence and load may therefore indirectly reflect burdens of nematode infection.

We also detected chimpanzee adenoviruses at high prevalence (14.3% and 35.0% in healthy and ill individuals, respectively). A prior study suggested that adenovirus was associated with a respiratory disease outbreak in chimpanzees in the Mahale Mountains, Tanzania (Tong et al., 2010). However, other studies indicate that adenoviruses are widespread and largely apathogenic in nonhuman primates (Hoppe et al., 2015; Negrey et al., 2019; Scully et al., 2018; Seimon et al., 2015; Wevers et al., 2011). In our study, adenovirus prevalence and load were not associated with ill health. However, adenovirus prevalence and load were associated with increasing age in male chimpanzees. These findings mirror a pattern we previously observed for salivirus, chisavirus, and a porprismacovirus, and suggest an association with immunosenescence (Negrey et al., 2020). In humans, some adenoviruses cause disease in elderly individuals (Kandel et al., 2015; Loeb, 2019), although less commonly than other frank pathogens such as rhinoviruses and influenza viruses (Chasqueira et al., 2018).

Notably, ill chimpanzees at Kanyawara exhibited greater total viral loads than did those at Ngogo. This accords with a recent study of female chimpanzees in the same population showing that Kanyawara chimpanzees shed more gastrointestinal parasites than those at Ngogo (Phillips et al., 2020). The Ngogo and Kanyawara territories exhibit differences in resource quality that may drive health differences. Notably, dietary quality impacts immune function (Scrimshaw & SanGiovanni, 1997), such that diminished or compromised nutrition may reduce resistance to viral infection or shedding. A 2009 study indicated that Ngogo chimpanzees exhibit higher noninvasive measures of energy balance than do Kanyawara chimpanzees (Emery Thompson et al., 2009), reflecting the greater availability of ripe fruit at Ngogo (Potts et al., 2011). Differences in energetic status may therefore manifest as differences in physiological function and tolerance to viruses at Ngogo than at Kanyawara through such processes as immune responsiveness. Community-level differences may also arise from other factors, such as ecological or behavioral differences in exposure to viruses. For example, the Kanyawara territory abuts the park boundary but the Ngogo territory does not, such that the Kanyawara chimpanzees interact more frequently with anthropogenically altered environments than do the Ngogo chimpanzees (Mackenzie et al., 2011). Differences in exposure to humans and their environments may also affect exposure to viruses of humans and domestic animals. For instance, the prevalence of salivirus was highest in samples from ill chimpanzees at Kanyawara, where excursions into surrounding villages occur more frequently than at Ngogo. Resolving the transmission dynamics of poorly known viruses such as salivirus would require more detailed epidemiological studies and accurate estimates of viral evolutionary rates. Regardless of sources and origins, however, viral systems may be particularly suited to examining host physiological/immunological function, including the influence of external factors such as ecology and sociality, given that viruses are obligate intracellular molecular parasites (Rivers, 1927).

In wild apes, certain anthroponoses are highly virulent and sometimes lethal (Grützmacher et al., 2016; Köndgen et al., 2008; Leendertz et al., 2006; Negrey et al., 2019; Palacios et al., 2011; Patrono et al., 2018; Scully et al., 2018). Our results show that wild chimpanzees also experience nonfatal infections of presumably low virulence, some of which increase in occurrence or load with ill health. In Kanyawara, respiratory disease was the most important cause of mortality between 1987 and 2017 (Emery Thompson et al., 2018). For example, a single epidemic of human rhinovirus C killed 8.9% of the community in 2013 (Scully et al., 2018). However, 53.2% of mortality during the same period remained unexplained (Emery Thompson et al., 2018). At Ngogo, respiratory disease caused by metapneumovirus killed 12.2% of the community in 2017 (Negrey et al., 2019). However, approximately 60% of deaths from 1995 to 2016 were from unknown causes (Wood et al., 2017). Infections of mild virulence during nonepidemic periods may account for some of the unexplained mortality in Kanyawara, Ngogo, and other wild chimpanzee communities, especially given that mortality rates are particularly high among younger and older individuals (Muller & Wrangham, 2014; Wood et al., 2017). If, as our data show, low-virulence viral infections are endemic and recurring within communities and affect individuals more as they senesce—especially males, as suggested in our present results and previously published work (Negrey et al., 2020)—such infections could cumulatively impact the chimpanzee aging process (i.e. "wear and tear"). Indeed, this idea has underlain key evolutionary theories of senescence. Even though the precise mechanisms whereby low-virulence infections cause cumulative health effects remain poorly understood (McHugh & Gil, 2018), aging seems to result from the accumulation of senescent cells (Childs et al., 2015). Cellular senescence, in turn, is caused by such factors as oxidative stress (Liguori et al., 2018) and telomere shortening (Oeseburg et al., 2010). Viral infections are known to contribute to such processes (Beck et al., 2000; Camini et al., 2017; Dowd et al., 2017; Gong et al., 2001; van de Berg et al., 2010).

We acknowledge that our inferences are based on small sample sizes. This limitation is inherent to studies of long-lived primates such as chimpanzees. We also note that the metagenomic methods used, although shown to be as sensitive as quantitative RT-PCR for detecting viruses in serum (Toohey-Kurth et al., 2017), may behave differently for other sample types such as feces. Such differences may account for lower detection rates of adenovirus using metagenomic methods than using RT-PCR. Because of these limitations, however, we suspect that including more chimpanzee communities over longer time periods (ideally, over the entire life course) and employing specific PCR assays for viruses of interest would likely increase the strength of the trends we have documented, and perhaps bring other trends to light.

We emphasize that the viruses associated with bouts of ill health we have identified here may not be the causes of disease themselves but rather reflective of declines in health caused by other factors. Such patterns have been observed in humans. For example, anelloviruses, which are generally benign in humans (Kaczorowska & van der Hoek, 2020), are associated with compromised immune function and ill health. Anelloviruses flourish in transplant recipients (Young et al., 2015), AIDS patients (Shibayama et al., 2001), and children with acute encephalitis or meningoencephalitis (Eibach et al., 2019). We also note that, despite strong trends with viral load, we did not detect associations between viral richness and ill health or any demographic variables. This finding reinforces the notion that viruses within populations respond differently (and perhaps independently) to host-related factors (e.g., Kapusinszky et al., 2017).

5 | CONCLUSIONS

In summary, we observed that fecal viromes of wild chimpanzees change predictably as chimpanzees experience episodes of ill health. We found that viral loads increase during episodes of ill health and that the magnitude of this change varied by study community, suggesting ecological effects on host responses to viral exposure and infection. Furthermore, given that the best predictors of ill health were viruses not previously linked to disease outcomes (e.g., salivirus), our results emphasize the importance of broad-spectrum diagnostics for identifying causes or biomarkers of ill health. Identification and investigation of such little-known viruses may prove valuable, with implications for both the study of virology and health monitoring of free-ranging animals.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Tony L. Goldberg conceived the study; Tony L. Goldberg and Jacob D. Negrey drafted the manuscript; Jacob D. Negrey performed metagenomics lab work, bioinformatics, and statistical analyses; James E. Gern, Tressa E. Pappas, and Kristine A. Grindle performed viral diagnostics; Jacob D. Negrey, John C. Mitani, Richard W. Wrangham, Emily Otali, Rachna B. Reddy, Zarin P. Machanda, Martin N. Muller, Kevin E. Langergraber, and Melissa Emery Thompson coordinated

data collection; all authors made significant intellectual contributions and revised the manuscript.

DATA AVAILABILITY STATEMENT

Viral nucleotide sequences are available on GenBank under accession numbers MT076199 to MT076210 and MW876510 to MW876520. Additional data and R code are available on Figshare (DOI:10.6084/m9.figshare.14879934).

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