The temporal RNA virome patterns of a lesser dawn bat (Eonycteris spelaea) colony revealed by deep sequencing

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Abstract

The virosphere is largely unexplored and the majority of viruses are yet to be represented in public sequence databases. Bats are rich reservoirs of viruses, including several zoonoses. In this study, high throughput sequencing (HTS) of viral RNA extracted from swabs of four body sites per bat per timepoint is used to characterize the virome through a longitudinal study of a captive colony of fruit nectar bats, species Eonycteris spelaea in Singapore. Through unbiased shotgun and target enrichment sequencing, we identify both known and previously unknown viruses of zoonotic relevance and define the population persistence and temporal patterns of viruses from families that have the capacity to jump the species barrier. To our knowledge, this is the first study that combines probe-based viral enrichment with HTS to create a viral profile from multiple swab sites on individual bats and their cohort. This work demonstrates temporal patterns of the lesser dawn bat virome, including several novel viruses. Given the known risk for bat–human zoonoses, a more complete understanding of the viral dynamics in South-eastern Asian bats has significant implications for disease prevention and control. The findings of this study will be of interest to U.S. Department of Defense personnel stationed in the Asia-Pacific region and regional public health laboratories engaged in emerging infectious disease surveillance efforts.

Key words: Eonycteris; virome; bat; metagenomic; sequencing; viral genomics.

Introduction

One health, or the concept that humans, animals, and the environmental health are intrinsically linked, has provided a lens to study possible cross-species transmission of viruses from bats to humans or other amplifying hosts. The majority of viruses is previously unknown or not represented in public sequence databases.
databases, making virome characterization a particularly challenging task. Approximately 263 viruses from 25 families are known to infect humans (Geoghegan et al. 2016; Carroll et al. 2018), but viruses of 40,000 species are estimated to infect mammals. Of those viruses, approximately 10,000 are estimated to have zoonotic potential (Carlson et al. 2019). Innovative advancements in unbiased high throughput sequencing (HTS), coupled with increased computational power, have broadened the capacity for viral discovery in recent years (Kuhn et al. 2019). Describing and classifying previously unknown viruses and sharing them in public sequence databases not only helps the scientific community to better understand basic biology, but can ultimately improve detection and facilitate the prediction of viral emergence, and hence helping prevention or mitigating future disease outbreaks (Kuhn et al. 2019).

More than half of all human infectious diseases result from zoonotic pathogens, and of those, 75 per cent have emerged from wildlife reservoirs (Woolhouse and Gowtage-Sequeria 2005). Bats are the most widely distributed land animals (Calisher et al. 2006), represent the second-most speciose mammalian order at 1,300 species, and harbor a significantly higher number of viruses than any other host species, with the latter two being agriculturally important (Li et al. 2005; Li et al. 2017). As the number of bats that roost in urban areas continues to increase due to anthropogenic land changes bringing bats closer into contact with livestock and humans, spillover events from bats globally, especially in South-eastern Asia, has gained recognition as a potential source of pandemic infections (Phelps et al. 2019). In particular, phylogenetic data suggest that bats host the progenitor strains of alpha- and beta-coronaviruses that infect humans or other incidental hosts, such as severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus, porcine epidemic diarrhea virus, and swine acute diarrhea syndrome coronavirus, with the latter two being agriculturally important (Li et al. 2005; de Groot et al. 2013; Zhou et al. 2018). Bats are also the natural reservoir for other medically important viruses, including henipaviruses, lyssaviruses, and filoviruses (Drexler et al. 2012; Banyard et al. 2014; Yang et al. 2019).

Bats are ecosystem service providers, acting as pollinators, seed dispersers, and insect consumers as well as producing guano that is used for fertilizer (Kunz et al. 2011). Unfortunately, the displacement of bats through agricultural land conversion and urbanization increases the probability of human–bat interactions and increases the risk of zoonotic spillover via amplifying hosts (Plowright et al. 2015; Lacroix et al. 2017). The recognition of bats as a reservoir of infectious diseases, initially driven by the SARS-CoV outbreak, has led to an increase in bat-borne virus surveillance (Wang and Cowled 2015; Han et al. 2016). A comprehensive understanding of the viral dynamics in South-eastern Asian bats would have significant implications by informing pathogen surveillance, prevention, and intervention.

While the majority of viral surveillance has focused on the detection of known emerging threats or their near-neighbors, there is a recent history of broadly characterizing the bat virome with HTS. The bat virome can be defined as all of the viruses that exist within a single bat or a population of bats. Surveillance often focuses on viruses of zoonotic potential. However, a significant proportion of previously reported viruses detected in virome studies of bats are not known to infect humans. For example, it has been reported that a high proportion of viruses detected in the bat virome reflect diet-associated viruses (Salmier et al. 2017; Bennett et al. 2019). Phage-related sequences have also been explored in the literature (Yinda et al. 2018) but are excluded from this work. Additionally, family-specific PCRs often focus on conserved internal genes (such as the RNA-directed-RNA polymerase) and are unable to provide information on surface proteins which are responsible for cellular entry and can be used to predict receptor usage (Plowright et al. 2016).

Viral persistence and shedding in bats are, in part, driven by birth pulses, social contact, roost size, flight, and migration (Plowright et al. 2016). As the only truly flying mammals, bats possess a suite of characteristics that includes unique immunological factors to accommodate for the physiologically taxing nature of flight. These mechanisms may have evolved to minimize inflammation from the production of oxygen free radicals during flight, which in turn reduces damage to DNA (Thomas and Suthers 1972; Chionh et al. 2019). Bats tolerate most viral infections without displaying symptoms due to a tighter control of innate responses that have a higher basal expression of certain defense genes (such as interferon and interferon-stimulated genes), and at the same time have multiple mechanisms to control for over-induction of inflammatory genes (Zhou et al. 2016; Subudhi, Rapin, and Misra 2019). It is herefore unknown whether bats serve as viral reservoirs, maintaining persistent infection at the population level by repeated viral infection of naive individuals (juveniles with waning maternal antibodies) or through long-term persistence of virus within individual bats (Subudhi, Rapin, and Misra 2019). In response to conflicting reports in the literature with regard to the persistence of viruses, longitudinal studies on captive colonies provide a controlled environment to fill the knowledge gap (Plowright et al. 2015).

This longitudinal study of a captive bat colony presents a unique opportunity to study the viral genomes that persist or circulate within a closed community. Herein, we present a comprehensive RNA virome analysis and longitudinal evaluation of viral population persistence in a captive colony of lesser dawn bats where we characterized the virome by addressing population-level viral dynamics over time. We collected head, body, oral, and rectal swabs from each bat (excluding pregnant and newborn bats) at 3- or 4-month intervals over the course of 18 months. RNA was extracted from each swab to perform shotgun and target enrichment sequencing from six time points from April 2016 to September 2017. These datasets were analyzed to ascertain the RNA virome diversity and how it changed over the study course in both individuals and cohorts. The aim of this study was to characterize the RNA virome by addressing population-level viral dynamics. Herein, we present a comprehensive RNA virome analysis and longitudinal evaluation of viral population persistence in a captive colony of lesser dawn bats.

Materials and Methods

Bat Colony Structure and Sampling Strategy

To establish a breeding colony of lesser dawn bats, wild-caught bats were brought into captivity in April 2016. Sixteen bats resided in the colony throughout the study, two were euthanized and four were born or added to the colony after April 2016 (Fig. 1). New bats were introduced to the colony in July 2017 and housed in separate cages situated approximately 1-m apart. Head, body, oral, and rectal swabs were collected from each individual bat every 3 or 4 months over the course of 18 months while the bats were kept in captivity.
Bats were housed in stainless steel mesh cages with ample room for roosting and flight. Each set of cages housed a maximum of 25 bats. The top of the cage consisted of wire meshing, allowing the bats to hang without obstruction. Burlap was strategically placed at the corner of the cage to provide seclusion. Bats were fed a liquid diet mixture of water, apple juice, glucose powder, low fat milk powder, powdered pollen, Wombaroo, and tegically placed at the corner of the cage to provide seclusion. Burlap was stra-
mum of 25 bats. The top of the cage consisted of wire meshing,
room for roosting and flight. Each set of cages housed a maxi-

Figure 1. Total RNA from head, body, oral, and rectal swabs was sequenced using two different, complementary methods: unbiased shotgun sequencing for broad detection of all organisms including potentially very divergent viruses, and target enrichment sequencing for more sensitive detection of 83 viruses of concern to human health and/or biosurveillance as well as their near neighbors (Paskey et al. 2019). The green bar represents the original resident bat colony, the blue bar represents births and additions to the bat colony, and the red bar represents 14 new bats that were introduced to the colony in July 2017. Samples from the 14 new bats are not included in this study. Solid circles represent samples that were sequenced by shotgun and/or target enrichment. Open circles represent samples that were not sequenced due to RNA input constraints.

Nucleic Acid Extraction and Sequencing

Total RNA was extracted from head, body, oral, and rectal swabs of each bat using a QIAGEN RNasy Kit with on-column DNase digestion (Qiagen; Valencia, CA, USA). RNA was eluted twice with RNase-free water. RNA was extracted with the aim to sequence genomic RNA and transcripts from RNA viruses, as well as transcripts from nonencapsidated DNA viruses. A maximum of 18 µl of the extract was used as input to prepare RNA TruSeq libraries. Illumina’s recommendations for the RNA TruSeq protocol were followed with a modification of fragmentation time to 4 min as described by Blackley et al. to account for potentially degraded RNA samples (Illumina; San Diego, CA, USA) (Blackley et al. 2016). Conventional HTS (shotgun) libraries were multiplexed in pools of 24 for sequencing on the NextSeq 500 platform using v2 chemistry with 2 × 150 bp read lengths. Post-library enrichment probe targets and preparation methods were previously described by Paskey et al.; samples were probed in pools of 12 and multiplexed for sequencing on the MiSeq platform using v3 chemistry with 2 × 300 bp read lengths (Paskey et al. 2019).

Bioinformatic Analyses

Each sample was processed both by using VirusSeeker (virus discovery pipeline, Zhao et al. 2017) and MetaSPAdes assembler v3.11.1 (Nurk et al. 2017). The Eonycteris spelaea genome was removed from each sample by read mapping to assembly GCA_003508835 (Wen et al. 2018) prior to MetaSPAdes assembly or by VirusSeeker (Zhao et al. 2017). VirusSeeker is a virus discovery pipeline that stitches paired reads together into a single read in addition to performing assembly using Newbler. Paired reads and contigs are then classified as potentially viral or discarded after being compared with viral databases using BLASTX and BLASTN algorithms (cutoff < e–5). False positives are then removed by comparing the candidate viral sequences to the complete nucleotide and non-redundant protein databases (cutoff < e–10). Viral reads as determined by VirusSeeker were normalized by number of reads per sample (formula below) and taxonomic assignments were filtered to exclude the possibility of sample carryover and only include assignments based on more than one read to semi-quantitatively evaluate abundance among samples.

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\text{Normalized reads} = \frac{\text{# reads for one viral taxa in one sample}}{\frac{\text{virus genome length (nt)}}{1000} \times \text{total # QC reads in that sample}}.
\]

Quality controlled (QC) reads are defined as trimmed, deduplicated reads that did not map to bacterial, and eukaryotic reference sequences with greater than 70 per cent nucleotide identity using the tools described as follows. Quality control and removal of non-viral reads were performed using fastqc v0.11.5, bmap, and bbduk v37.78 (Andrews 2010; Bushnell 2014). De novo assembly was performed using MetaSPAdes (average contig length 198.65 bp); reads were mapped back to contigs for validation and sequenced relatives were determined by DIAMOND using the BLASTX algorithm against RefSeq viral protein sequences from NCBI as of December 4, 2018 (Altschul et al. 1990; Buchfink, Xie, and Huson 2015; Nurk et al. 2017). Results were visualized using MEGAN 6 (Huson et al. 2016). Phage-related sequences are excluded from this work. Detailed analysis of contigs and reads was performed with CLC Genomics Workbench V11 (QIAGEN Bioinformatics; Redwood City, CA, USA).

The International Committee for the Taxonomy of Viruses (ICTV) defines viral species as “a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria” and identity cutoffs vary by genus and are determined by natural and experimental host range, cell and tissue tropism, pathogenicity, vector specificity,
antigenicity, and the degree of relatedness of genomes (ICTV 2018). In this study, cutoffs of 90 per cent or higher at the amino acid level and 95 per cent or higher at the nucleotide level for the definition of a virus were used in viral classification. Sequences that were not sufficiently related to known species were classified using the following naming convention: “novel [name of viral family] virus.”

Taxonomic assignments were grouped as zoonotic-related, dietary-associated, or “other” based on the literature. Zoonotic-related viruses were defined as having a near-neighbor that was previously known to cause disease in vertebrates. Dietary-associated viruses were defined as plant-associated taxa that were associated with components of the bats’ diet. “Other” viruses included species that could have been detected in the environment were not previously associated with human infection or could have possibly existed as misclassified host material due to the possibility of integration (i.e., retroviruses) or similarity to mammalian genomes.

The evolutionary history of a novel filovirus-like nucleotide sequence detected in the sample was inferred by using the maximum likelihood method based on the general time reversible model (Nei and Kumar 2000) with 100 bootstrap replications. The tree with the highest log likelihood (−39,135.14) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. The analysis involved ten full-length filovirus genome sequences (NC_014373, NC_014372, NC_002549, NC_004161, NC_006432, NC_016144, KX371887, NC_024781, NC_001608, NC_03945) and one filovirus consensus sequence from Swab 340. There were a total of 3,588 nucleotide positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Principal Component Analysis

Data for twenty-nine zoonotic-related viruses that persisted for more than three collection dates were evaluated by principal component analysis. Evaluated elements included the number of collection dates for which the virus was detected, the total number of times the virus was detected, the length of the virus genome and binary code for the following parameters: human infectivity, cytoplasmic replication, segmented vs. non-segmented genome, vector-borne, single-stranded genome, RNA vs. DNA genome, and enveloped vs. non-enveloped virion. The data were clustered using kmeans, k = 4. The number of clusters was determined by using factoextra to evaluate the optimal k for the first three components by the Silhouette and Elbow methods (Kassambara and Mundt 2017). The first three principal components represented 65 per cent of the variance, and the first two principal components represented 48 per cent of the variance. This analysis was performed using built-in statistical packages in R v3.4.1 (R Core Team 2018).

Rarefaction Curves

Rarefaction curves were produced using zoonotic-related reads as classified by VirusSeeker (Zhao et al. 2017) to evaluate the extent at which mammalian viral diversity was recovered by using zoonotic-related species detected in each sample. The vegan package was utilized in R v3.4.1 for this analysis (Dixon 2003).

Statistical Evaluation of Potential Confounders

To evaluate the potential for confounding influences on this analysis, we evaluated analysis of variance (ANOVA) for viral abundance. Features of concern include NextSeq 500 batch grouping, bat ID number, length of sample storage time, and virus genome length. No statistically significant difference was found among groups (P > 0.1). Taken together, these analyses suggest that taxonomic classifications of viruses were not biased by potentially confounding features of the study. We performed a two-way ANOVA to evaluate the impact of changing the collection month or number of samples sequenced on the number of taxa detected. The P-value for collection month was 0.06 and 0.018 for the number of samples sequenced per time point. There was no significant synergistic effect for collection month and number of samples sequenced (P > 0.1). We interpret these results to indicate that a change in the number of sequencable samples would impact the number of detected viral taxa.

This analysis was performed using built-in statistical packages in R v3.4.1 (R Core Team 2018).

Results

Colony Structure and Sampling Strategy

To establish a breeding colony of lesser dawn bats, wild-caught bats were brought into captivity in April 2016. Head, body, oral, and rectal swabs were collected from each individual bat every 3 or 4 months over the course of 18 months while the bats were kept in captivity. Total RNA was extracted from each swab and 210 swabs were successfully sequenced by shotgun sequencing. Among them, 134 swabs were also sequenced using target enrichment sequencing, resulting in 0.027 per cent of QC target-enriched reads to be classified as viral (Fig. 1, Table 1).

Results Figure 2 provides an overview of whole-colony level data, including all swab sites (head, body, oral, and rectal). The most frequently detected zoonotic agent-related viruses belong to Orthomyxoviridae, Coronaviridae, Astroviridae, Reoviridae, Picornaviridae, and Paramyxoviridae. The most abundant and consistently detected zoonotic-related viruses were unclassified betacoronaviruses, Rousettus bat coronavirus GCCDC1, and influenza A virus. Ubiquitous viruses that are native to bats but not of concern to humans such as unclassified herpesvirus, bat retroviruses and anelloviruses were classified as other or host-specific, and were frequently detected (Gentile and Micozzi 2016; Parker 2016; Rascovan, Duraisamy, and Desnues 2016; Freer et al. 2018). Dietary-associated viruses such as watermelon silver mottle tospovirus, an unclassified totpivirus, unclassified crinivirus, and potyvirus were frequently detected.

There was no significant difference in total number of viruses detected at each time point between zoonotic-related, other or dietary viruses (Supplementary Fig. S1, Table S1). Normalized abundance varied among time points. Among individual bats, there was variation in both abundance and distribution. Few complete or nearly complete viral genomes were recovered through this study due to the complexity and microbial “noise” of metagenomic samples. The complete genome for Rousettus bat coronavirus GCCDC1 was recovered, as well as a nearly complete genome for a novel bat mumps virus and partially complete novel bat paramyxovirus. All other zoonotic-related taxonomic assignments were made using partial gene coverage. Overall, data obtained from these 210 swabs represent massive viral diversity and broad variability in abundance among swab types and taxa. Analysis was limited to viruses
cent nucleotide identity for Zika virus strains MR766 and R11265 containing human adenovirus strains 4 and 51, as well as 89 percent nucleotide identity in a contrived, complex sample (Krishnamurthy and Wang 2017). "dark matter" that did not match any sequence in GenBank potential viral sequence, including unclassifiable viral reads or total of 293 giga base pairs (Gbp) of sequence were identified as known sequences. For reference, approximately 5.3 million base number of taxonomic assignments was limited to previ- sion dynamics such as contact rates, food abundance and served despite the condition of captivity, where drivers of infec- We considered whether trends in detected viruses could be ob- determined by abundance and significance with a known se- quenced relative, therefore excluding unmatched reads.

Comparison of Two Different Sequencing Strategies

Target enrichment for known viruses of biosurveillance concern resulted in a simplified dataset consisting of primarily zoonotic-related taxonomic classifications. Data from target enrichment sequencing were used to guide bioinformatic analysis of shotgun sequencing data. The number of zoonotic-related taxonomic assignments was compared between matched swabs sequenced both by shotgun and by target enrichment sequencing. Of 317 taxa assigned by VirusSeeker to shotgun data, 33.1 percent of assignments were zoonotic-related. Conversely, 72.1 percent of 44 target-enriched assignments were zoonotic-related, a result which is expected as the probe panel used here for target enrichment intentionally biases our dataset toward known viruses of biosurveillance concern (Fig. 3). It is important to note that the number of taxonomic assignments was limited to previously known, classifiable references and cannot account for unknown sequences. For reference, approximately 5.3 million base pairs (Mb) of sequence were unequivocally classified as viral. A total of 293 giga base pairs (Gbp) of sequence were identified as potential viral sequence, including unclassifiable viral reads or “dark matter” that did not match any sequence in GenBank (Krishnamurthy and Wang 2017).

Previously, we demonstrated binding tolerance as low as 79 percent nucleotide identity in a contrived, sample containing human adenovirus strains 4 and 51, as well as 89 percent nucleotide identity for Zika virus strains MR766 and R11265 in contrived samples (Paskey et al. 2019). Therefore, the capacity for target enrichment probes to bind slightly off-target but related (near-neighbor) sequences in the current samples was evaluated. In a subset of enriched reads classified by VirusSeeker as belonging to Filoviridae, Orthomyxoviridae, Paramyxoviridae, Picornaviridae, and Reoviridae, the lowest percent identity at the nucleotide level between probe and captured sequence was 88 percent. Taken together, these data demonstrate that target enrichment probes exhibit a binding tolerance that could permit the enrichment from environmental samples of previously unknown near-neighbors that have yet to be represented in public sequence databases.

Evaluation of a Dynamic Virome in a Captive Colony of Bats

We considered whether trends in detected viruses could be ob- served despite the condition of captivity, where drivers of infec- tion dynamics such as contact rates, food abundance and quality, environmental perturbations, reproduction, and popu- lation density were partially controlled or minimized (Flowlri- ght et al. 2016). While numerous zoonotic-related viruses such as bat orthoreovirus, mumps-like bat paramyxovirus, unclassified polyomavirus, unclassified sapovirus, and unclassified bat rotavirus did not persist over time, several viruses were detectible at five or six out of six collection dates (Supplementary Fig. S2). Persistence within the colony could only be measured to the fifth collection date because new, wild-caught bats were intro- duced to the colony in July 2017, prior to the final swab collection date. Thus, new viruses could have been introduced or reintroduced to the original colony. Notable zoonotic-related vi- ruses that were detectable at multiple collection dates belonged to the families Astroviridae, Orthomyxoviridae, Picornaviridae, Paramyxoviridae, Reoviridae, and Coronaviridae. Other and dietary- associated viruses were detected more consistently, as expected, due to frequent reintroduction from food and environ- mental sources, with the exception of notable diet changes in the first year of the study.

Table 2 summarizes biosurveillance-related viruses detected in this study and reported in the literature, listing viral family, sampling country, references, virus, and total reads if the result is from this study (Chen et al. 2014).

Variation of Viral Abundance at Both the Colony and Individual Level

Data from oral and rectal swabs were used as an indicator of potential replication in and shedding from particular bats, while data from head and body swabs (presumably derived from contact with other, shedding, bats but not necessarily in- dicating shedding from the particular bat on whose head or body it is identified) were used to characterize the overall di- versity of viruses present within the colony. Bat coronavirus was previously found in the small intestine of experimentally infected Leschenault’s rousettes (Rousetts leschenaultia; Watanabe et al. 2010); therefore, we hypothesized that corona- viruses would be frequently detected from rectal swabs. In fact, rectal swabs contained the greatest proportion of all coronaviruses detected (30.3 percent), followed by body swabs (28.3 percent), head swabs (23.7 percent), and oral swabs (17.8 percent). We hypothesize that the generally high abun- dance of viruses on the skin of bat bodies is due to the gregar- iousness of lesser dawn bats.

Fractional abundance was used to evaluate detection of vi- ruses on the colony level and is graphed in Fig. 4. The zoonotic agent-related viruses detected within the colony are consistent with the literature (Fig. 4A) and many dietary-associated viruses have been associated with elements of the colony’s diet (Fig. 4B).
(Mendenhall et al. 2017a, b, 2019; Yinda et al. 2018). Results are biased toward RNA viruses due to our technical approach, and it is likely that DNA virus diversity is not fully represented in these analyses. This dataset does, however, represent RNA from multiple related viruses that were detected at various time points throughout the study. Supplementary Fig. S3 displays the sporadic detection of even the most frequently detected zoonotic-related viruses. Notably, this inconsistency is in contrast to the more consistent detection of other and dietary viruses over time. One should note that bat 763D86 was born...
into the colony and first sampled in 2017. Additionally, bat 763576 F was euthanized between the October 2016 and January 2017 time points.

Persistence of Certain Viral Populations at the Colony Level

Figure 5 highlights four zoonotic-related and two dietary viruses, which were “more persistent” than other viruses detected. Individual bats rarely shed specific viruses during consecutive time points. Overall, viruses were not restricted to individual bats, but rather were detected in multiple bats at numerous time points. Dietary virus shedding was more consistent and detected at the overall highest normalized abundance. We observed that bat astrovirus was not detected beyond the October 2016 time point. Influenza A virus was detected sporadically, and never in the same shedder swab type from the same bat at subsequent time points. This pattern could reflect intermittent shedding or cycles of infection, transmission, and reinfection or persistent virus replication at levels near or slightly below the limit of detection for our assay. In this study, we observed read support in 24 swabs for an influenza A-like virus by both shotgun and enrichment sequencing. No full sequences for all eight segments were detected, preventing unequivocal characterization of all known viruses. Despite the improvement of viruses of biosurveillance concern in our samples, how- ever, unbiased shotgun sequencing, were utilized to evaluate the extent of viruses of biosurveillance concern. In so doing, we were able to evaluate the strengths and limitations of target enrichment, while also obtaining informative data with regard to the colony-level virome in a captive colony of bats. Our results showed that there is sufficient binding tolerance to enrich for near-neighbors of probe targets. This flexibility enabled for the detection of viruses of biosurveillance concern in our samples, however, unbiased shotgun sequencing was necessary for characterization of all known viruses. Despite the improvement in detection of classifiable reads gained by the use of target enrichment, less than 1 per cent of target-enriched or shotgun reads that were possibly viral could be unequivocally classified. This is consistent with reports that viral dark matter can indicating a propensity for spillover (Olival et al. 2017). In fact, cluster one had a higher known average capacity to infect humans (0.92) and also included the most frequently detected viruses as compared to clusters two through four (0.5, 0.44, 0.2, respectively). The hypothesis was supported by unsupervised clustering, and we further analyzed the clustering results to make several observations (Fig. 6). Cluster one (thirteen members; gold points) was composed of single-stranded RNA viruses. Significantly more of these viruses are known to infect humans, and none of these viruses is vector-borne. Cluster two (two members; red points) was composed of the enveloped, double-stranded DNA viruses: asfivirus and unclassified bat poxvirus. This group had the largest genomes. Cluster three (nine members; gray points) is represented by segmented RNA viruses. Cluster four (five members; green points) was composed of non-enveloped DNA viruses. Overall, the viral members of the largest cluster, Cluster one, also included the largest number of human-infecting viruses that are associated with multivariate characteristics that are prone to zoonotic spillover such as those possessed by orthomyxoviruses, paramyxovi- ruses, and coronaviruses (Wang and Anderson 2019).

In addition to the vast complexity of each swab type, we observed a surprisingly even distribution of detection among swab types. As shown in Fig. 7, many viruses are detected in all four swab types. As previously discussed, there is a consistent pattern of dietary and other viruses that contrasts with the sporadic detection of zoonotic-related viruses. Some individual bats appear to shed or carry a larger number of zoonotic-related taxa as compared to other individuals. We hypothesize that this is influenced by both the immunological predispositions and behavior of each bat.

Discussion

To our knowledge, this is the first comprehensive, longitudinal study to evaluate virome dynamics in a colony of old world bats. Beyond the challenge of limited known viral sequences, it can be difficult to detect viral genomes amidst the “noise” of bacterial commensals in a complex environmental sample (Frey and Bishop-Lilly 2015). Computational methods for virome characterization rely on database-independent analyses such as de novo assembly to obtain scaffolded or complete viral genomes that share little homology but can be compared to distantly related, published references. In particular, Meta-SPAdes is an effective tool for de novo assembly of virome data (Nurk et al. 2017; Sutton et al. 2019). With limited tools to classify viruses from complex samples, in addition to the diversity of previously unknown viral genomes, the full characterization of a virome requires extensive manual analysis.

Two sequencing methods, unbiased shotgun sequencing and viral target enrichment, were utilized to evaluate the extent of viruses of biosurveillance concern. In so doing, we were able to evaluate the strengths and limitations of target enrichment, while also obtaining informative data with regard to the colony- level virome in a captive colony of bats. Our results showed that there is sufficient binding tolerance to enrich for near-neighbors of probe targets. This flexibility enabled for the detection of viruses of biosurveillance concern in our samples, however, unbiased shotgun sequencing was necessary for characterization of all known viruses. Despite the improvement in detection of classifiable reads gained by the use of target enrichment, less than 1 per cent of target-enriched or shotgun reads that were possibly viral could be unequivocally classified. This is consistent with reports that viral dark matter can
| Viral family      | Sampling country | References                                                | Virus                                                                 | Total reads (this study) |
|------------------|-----------------|-----------------------------------------------------------|----------------------------------------------------------------------|--------------------------|
| Adenoviridae     | Singapore       | Viruses 2019, 11(3):pii: E250                             | Adenoviridae sp. DNUS-Bat-Polymerase-Esp                            | NA                       |
| Arenaviridae     | Singapore       | This study                                                | Mamarenavirus                                                        | 2                        |
| Asfarviridae     | Singapore       | This study                                                | Asfivirus                                                            | 6                        |
| Astroviridae     | Singapore       | This study                                                | Bat astrovirus                                                       | 294                      |
| Astroviridae     | Laos            | Infect Genet Evol 2016, 47:41–50                          | Mamastrovirus sp. PREDICT MAstV-13 strains                         | NA                       |
| Caliciviridae    | Singapore       | This study                                                | unclassified saposivirus                                             | 42                       |
| Circoviridae     | Singapore       | One Health, 2017, 4:27–33                                 | unclassified circovirus                                              | 8                        |
| Coronaviridae    | Singapore       | This study                                                | Roussetus bat coronavirus GCCDC1                                    | 2,765                    |
| Coronaviridae    | Singapore       | This study                                                | unclassified Betacoronavirus                                         | 84                       |
| Coronaviridae    | Singapore       | This study                                                | unclassified Alphacoronavirus                                        | 6                        |
| Coronaviridae    | Singapore       | Transbound Emerg Dis 2017, 64(6):1790–1800                | Bat betacoronavirus Es/Singapore/2014                                | NA                       |
| Coronaviridae    | Cambodia        | Infect Genet Evol 2016, 48:10–18                          | Bat coronavirus RK strains                                           | NA                       |
| Coronaviridae    | China           | Virol Sin, 2018, 33(1):87–95                              | Bat coronavirus GCCDC1 BatCoV Eonycteris spelae/Mengla/2016          | NA                       |
| Coronaviridae    | Laos            | Infect Genet Evol 2016, 48:10–18                          | Coronavirus PREDICT CoV-22 PREDICT_CoV-22/LAP11-D0063              | NA                       |
| Filoviridae      | Singapore       | This study                                                | unclassified filovirus                                               | 22                       |
| Filoviridae      | China           | Emerg Infect Dis, 23(3):482–486                           | Bat filovirus Eonycteris spelae/China/2009, 2015 strains            | NA                       |
| Flaviviridae     | Singapore       | Viruses 2019, 11(3):pii: E250                             | Flaviviridae sp. DNUS-Bat-E-Esp                                     | NA                       |
| Herpesviridae    | Singapore       | This study                                                | unclassified Hepevirida                                              | 609                      |
| Herpesviridae    | Singapore       | This study                                                | unclassified Herpesvirida                                            |                          |
| Herpesviridae    | Singapore       | This study                                                | Percivirus                                                           | 30                       |
| Herpesviridae    | Singapore       | This study                                                | Cytomegalovirus                                                      | 4                        |
| Herpesviridae    | Malaysia        | Unpublished                                              | unidentifed herpesvirus acc_AB125970                                 | NA                       |
| Nodaviridae      | Singapore       | This study                                                | Nodamura virus                                                       | 43                       |
| Orthomyxoviridae | Singapore       | This study                                                | Influenza A virus                                                    | 36                       |
| Orthomyxoviridae | Singapore       | This study                                                | Influenza B virus                                                    | 6                        |
| Papillomaviridae | Singapore       | This study                                                | Betapapillomavirus 1                                                 | 4                        |
| Papillomaviridae | Singapore       | Viruses 2019, 11(3):pii: E250                             | Papillomaviridae sp. DNUS-Bat-E1-Exp                                 | NA                       |
| Paramyxoviridae  | Singapore       | This study                                                | Mumps                                                                | 248                      |
| Paramyxoviridae  | Singapore       | This study                                                | novel bat paramyxovirus                                              | 112                      |
| Paramyxoviridae  | Singapore       | This study                                                | Respirovirus                                                         | 2                        |
| Paramyxoviridae  | China           | Viruses 2014, 6(5):2138–2154                              | Henipavirus YN12069/CHN/2012                                         | NA                       |
| Paramyxoviridae  | Singapore       | Viruses 2019, 11(3):pii: E250                             | Paramyxoviridae s. DNUS-Bat-L-9, N                                   | NA                       |
| Paroviridae      | Singapore       | This study                                                | Bocaparvovirus                                                      | 16                       |
| Paroviridae      | Singapore       | Viruses 2019, 11(3):pii: E250                             | Permomyxoviridae sp. DNUS-Bat-VP1, VP2                               | NA                       |
| Picornaviridae   | Singapore       | This study                                                | unclassified Picornavirus                                            | 222                      |
| Picornaviridae   | Singapore       | Viruses 2019, 11(3):pii: E250                             | Picornaviridae sp. DNUS-Bat-3D-Esp, Polyprotein-Esp                 | NA                       |
| Pneumoviridae    | Singapore       | This study                                                | unclassified pneumovirus                                             | 2                        |
| Polyomaviridae   | Singapore       | This study                                                | unclassified polyomavirus                                            | 166                      |
| Polyomaviridae   | Singapore       | Viruses 2019, 11(3):pii: E250                             | Polyomaviridae sp. DNUS-Bat-V7-Esp                                  | NA                       |
| Reoviridae       | Singapore       | This study                                                | Seadornavirus                                                        | 40                       |
| Reoviridae       | Singapore       | This study                                                | unclassified bat rotavirus                                           | 333                      |
| Reoviridae       | Singapore       | This study                                                | unclassified bat orthoreovirus                                      | 112                      |
| Reoviridae       | Singapore       | Viruses 2019, 11(3):pii: E250                             | Orthoreovirus sp. DNUS-Bat-L2, M2-Esp                               | NA                       |
| Reoviridae       | Philippines     | Arch Virol, 2017, 162(6):1529–1539                        | Pteropine orthoreovirus Samal-24                                     | NA                       |
| Reoviridae       | Philippines     | Arch Virol, 2017, 162(6):1529–1539                        | Pteropine orthoreovirus Talikud strains 73, 74, 81, 83              | NA                       |
| Reoviridae       | Singapore       | Viruses 2019, 11(3):pii: E250                             | Rotavirus sp. DNUS-Bat-Vp1, Vp7-Esp                                 | NA                       |
| Retroviridae     | Singapore       | This study                                                | Bat retrovirus                                                       | 1,807                    |
| Retroviridae     | China           | Viruses 2014, 6(5):2138–2154                              | Bat gammaretrovirus comp48905_c0                                     | NA                       |
represent as much as 90 per cent of sequences (Krishnamurthy and Wang 2017). Nevertheless, approximately 5.3 Mb of 293 Gbp of viral sequence were unequivocally classified. Coverage of detectable taxonomic groups varied in breadth and depth, as is typical in sequencing of metagenomic samples (Frey and Bishop-Lilly 2015). To address the challenge of low coverage depth in data including viruses with varied genome lengths, a normalization approach that takes into account the nucleotide length of the virus genome was used and with a requirement of both forward and reverse read coverage.

Virus detection rates vary with several factors, including specimen type, cold chain logistics, date of sampling, host age, and taxonomy. Common surveillance methodologies include culture/isolation, serological assays, conventional and quantitative polymerase chain reaction (PCR) assays, HTS, and target enrichment sequencing (Young and Olival 2016). Each of the aforementioned methods has advantages and disadvantages with regard to portability and necessary prior knowledge about the viruses present within a given sample. Rarefaction curves were produced using VirusSeeker data to evaluate the extent at

Figure 4. The proportion of reads from unbiased shotgun RNA sequencing data representing viral families in each virus source are graphed. (A) zoonotic agent-related viruses and (B) dietary-associated-associated viruses.
which mammalian viral diversity was recovered by using zoonotic-related species detected in each sample (Supplementary Fig. S4). The rarefaction curves illustrate that some samples were more completely sampled by comparison and that there is variation in the detection of zoonotic-related species per sample. One caveat to this study is that there is a correlation between the number of detected taxa and the number of samples successfully prepared for sequencing. Multivariate factors may contribute to the failure of samples that were unable to be sequenced, including low concentration or quality RNA, decreased shedding of certain viral taxa, behavior changes in the bat, or technical error in preparation.

Consistently detected dietary-associated viruses such as watermelon silver mottle tospovirus could be attributed to the bat’s diet being supplemented with watermelon as part of their enrichment regime. For this reason, dietary-associated viruses that were consistently reintroduced by the diet or environment were used as a comparison alongside zoonotic-related viruses that are not found in dietary sources. Interestingly, several relevant zoonotic agent-related viruses that were detected at four or more time points have been associated with prior viral spillover (Coronaviridae, Reoviridae, Paramyxoviridae) from bats to humans. Our findings are consistent with the existing literature (Mendenhall et al. 2017a, b, 2019), in that Pteropodidae family bats have been previously demonstrated to be associated with viruses of the following: Paramyxoviridae, Adenoviridae, Herpesviridae, Astroviridae, Coronaviridae, Rhabdoviridae, Polyomaviridae, Flaviviridae, Iflaviridae, Hepadnaviridae, Bunyavirales, Togaviridae, Caliciviridae, Orthomyxoviridae, Papillomaviridae, Reoviridae, Retroviridae, Filoviridae, Paroviridae, and Circoviridae (Young and Olival 2016).

We observed population persistence of the following viral families: Astroviridae, Orthomyxoviridae, Picornaviridae, Paramyxoviridae, Reoviridae, and Coronaviridae. Of these families, Paramyxoviridae, Reoviridae, and Coronaviridae are each known to be associated with spillover from bats to humans (Drexler et al. 2012; Anthony et al. 2017; Wang and Anderson 2019). This knowledge should inform future directions for biosurveillance of viruses within each of the aforementioned families that have the potential to cross the species barrier from bats to humans or amplifying hosts.

The circulation of orthomyxoviruses and filoviruses in wild reservoirs is relevant to public health and these viruses were sporadically detected in the colony. Although influenza A virus has previously been detected in South American bats (Tong et al. 2012, 2013), the capacity for old world bats to serve as a reservoir for influenza A-like viruses was only recently discovered (Kandeil et al. 2019). Questions have been raised with regard to the geographic range of bat-borne influenza viruses and the capacity for reassortment of bat-borne orthomyxoviruses with known human influenza A viruses (Wu et al. 2014). Serologic evidence of filoviruses has been reported in lesser dawn bats (Laing et al. 2018) but, to our knowledge, sequence data have not been previously reported. It is apparent from both target enrichment and shotgun sequence data that a novel filovirus was present in one rectal swab and two body swabs, but there was not significant breadth of coverage as compared to any previously known filovirus reference genomes. The closest sequenced relative is

Figure 5. Abundance of select taxa detected from unbiased shotgun sequencing data derived from shedding sites is displayed in a heat map. Individual bat identification numbers are listed along the y-axis, parsed by oral and rectal swab types. Sample collection date is listed along the x-axis, divided by specific viral taxa and virus source. (A) Zoonotic-related viruses bat astrovirus, influenza A, Rousettus bat coronavirus GCCDC1, and unclassified betacoronavirus. (B) Dietary viruses potyvirus and watermelon silver mottle tospovirus. Reads were normalized to virus size and total number of reads in each sample.
Mengla virus, which was recently isolated from Rousettus bats in China (Yang et al. 2019; Supplementary Fig. S5).

Zoonotic-related viruses were intermittently detected in samples from individual bats. One caveat of this study lies within the variability of nucleic acids that could be sequenced. Results with regard to individual bats should be interpreted with caution because it is unknown whether this phenomenon was observed due to intermittent shedding below the limit of detection or cycles of recurrent infection (susceptible-infectious-recovered-susceptible [SIRS] or susceptible-infectious-latent-infectious [SILI] models of infection (Plowright et al. 2016)). However, one compelling finding of this study was that multiple viruses exhibited population persistence despite the isolation from contact with new, wild bats for 20 months. We interpret these results to indicate that persistent viruses possess a propensity for spillover due to their comparatively higher recurrence in shedder sites at high enough abundance for detection. These viruses share multivariate qualities with viruses known to infect humans (Fig. 6) and could represent viruses that are most likely to cross the species barrier to humans or intermediate hosts in the manner that henipaviruses and SARS-CoV caused outbreaks in the past. The persistence of Paramyxoviridae, Reoviridae and Coronaviridae family viruses without external reintroduction to this captive community indicates that lesser dawn bats may serve as a maintenance host. As changing human factors such as urbanization impact the potential for disease interface by increasing urban-adapted (synanthropic) animal populations (Hassell et al. 2017), targeted assays, such as PCR, for these persisting viruses should continue to be conducted by regional public health laboratories engaged in emerging infectious disease surveillance efforts.

Furthermore, viruses that were detected less frequently may be of a lesser concern for biosurveillance as they did not exhibit robust, colony-level persistence. In particular, this study utilized swabs of the exterior of the bat (head and body) to evaluate the virus population. This could be a useful sample site for future surveillance to extrapolate population-level infection and recapitulates the observation that host-microbiome dynamics of gregarious species such as bats should be observed on the colony rather than an individual level (Kolodny et al. 2019). Taken together, we conclude that noninvasive surveillance methods that target the body of bats not only detect viruses shed within the colony, but can also represent viral populations dispersed throughout the entire colony. As shown in Fig. 7, the results across internal (oral and rectal) and external (head and body) swabs are homogeneous and reflect the viral populations for the entire colony. External swabs could be informative targets for sample collection. In conclusion, we have provided novel insight to the virome of South-eastern Asian bats that should be used to inform future surveillance methods in the region.

Figure 6. Principal component analysis of frequently detected, zoonotic-related viruses (k = 4) from unbiased shotgun sequencing data. Data are graphed in PC1 and PC2 space, representing 48 per cent of the total variance. Cluster number (color): 1 (gold), 2 (red), 3 (grey), and 4 (green).

Vertebrate Animal Care and Safety

All bats were housed and handled by Duke-National University of Singapore Medical School animal facilities. Trained laboratory personnel provided daily care for the animals according to the guidelines agreed upon by Duke-NUS Institutional Animal Care and Use Committee (2015/SHS/1088) and the Agri-Food and Veterinary Authority of Singapore. All samples were noninvasive.
Data availability
The datasets supporting the conclusions of this article are available in National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), BioProject ID PRJNA561193 and under GenBank accession numbers MT081488-MT081490.

Supplementary data
Supplementary data are available at *Virus Evolution* online.

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