Hunters are at a higher risk for exposure to zoonotic pathogens due to their close interactions with wildlife and arthropod vectors. In this study, high throughput sequencing was used to explore the viromes of two tick species, *Amblyomma dissimile* and *Haemaphysalis juxtakochi*, removed from hunted wildlife in Trinidad and Tobago. We identified sequences from 3 new viral species, from the viral families *Orthomyxoviridae*, *Chuviridae* and *Tetraviridae* in *A. dissimile*.

1. Introduction

Compared to other vectors, ticks transmit the most diverse set of pathogens worldwide (Jongejan and Uilenberg, 2004; Pfafflé et al., 2013). Because of their clinical importance, numerous studies have explored the pathobiomes, the pathogenic agents and their integration within their biotic environment (Vayssier-Taussat et al., 2014), of tick species. Historically, this work has primarily focused on tick species with an established history of transmitting agents of human and veterinary vector-borne diseases (VBDs). However, other species may also contribute to the risk associated with tick-borne diseases (TBDs). The recent coronavirus pandemic highlights the importance of identifying novel viruses within wildlife populations. Hunters interact with wildlife at a unique interface, and often expose themselves to an increased risk of zoonotic transmission (Vayssier-Taussat et al., 2014), of tick species that typically do not parasitize humans (Karesh and Noble, 2009; Kilonzo et al., 2013; Paige et al., 2014; Wolfe et al., 2000). There is also an additional risk of exposure to TBDs through the butchering and consumption of bush meat parasitized by ticks. It is currently impossible to quantify this risk because the pathobiome of these tick species remains largely unexplored.

Recent pathobiome studies have identified a wide array of novel viruses, many of which are thought to be arthropod specific viruses (ASVs) and whose host-range or pathogenicity has yet to be determined (Brinkmann et al., 2018; Cholleti et al., 2018; Gondard et al., 2020a; Harvey et al., 2018; Kobayashi et al., 2020; Meng et al., 2019; Pettersson et al., 2017; Sameroff et al., 2019; Shi et al., 2016; Souza et al., 2018; Tokarz et al., 2018; Tokarz et al., 2014a,b; Wille et al., 2020; Xia et al., 2015). Some of them share homology with known pathogens. One such group are the quaranjaviruses (family *Orthomyxoviridae*), which are related to influenza viruses and thogotoviruses, both known human pathogens. (Walker et al., 2019) The first quaranjavirus was isolated from soft ticks as well as the blood of children suffering from unexplained febrile illness (Taylor et al., 1966). This study also revealed that neutralizing antibodies were present in about 8% of the endemic population(Taylor et al., 1966). Quaranjaviruses have also been associated with mass avian die-offs (Allison et al., 2015), highlighting their importance as an agent of disease. Quaranjaviruses have been historically isolated from soft ticks and their vertebrate hosts, primarily avian species (Briese et al., 2014; Cler et al., 1983; Mourya et al., 2019; Pinto Da Silva et al., 2005; Presti et al., 2009). However, recently quaranjavirus sequences were identified within the hard tick species *Rhipicephalus microplus*, and *Ixodes uriae* (Cholleti et al., 2018; Wille et al., 2020). Based on the known hosts and vectors of these viruses, the risk of exposure is highest at the sylvatic interface.

Hunting is common for both sport and sustenance in Trinidad and...
Tobago. Typical hunted game animals include iguana, agouti, lappe (lowland pacca), wild hog, and the red brocket deer. During tick collections for a previous study (Sameroff et al., 2019), we obtained anecdotal evidence of local hunters being parasitized by ticks, highlighting the need for exploring the pathobiome of tick species from this region. Partnering with local hunters, this pilot study employed high throughput sequencing to analyze the pathobiome of two tick species, *Amblyomma dissimile* and *Haemaphysalis juxtakochi*, removed from hunted game animals from three regions of Trinidad and Tobago.

2. Methods

2.1. Sample collection

Ticks were removed from wildlife (*Iguana iguana* and *Mazama americana*) postmortem by groups of hunters in the Cedros, Caura, and Caroni regions of Trinidad and Tobago in November of 2018. Samples were divided by animal and stored and transported at 4 °C, until arriving at The University of the West Indies, St. Augustine where they were frozen and stored at ~80 °C. Samples were then transported on dry ice to The Center of Infection and Immunity at Columbia University.

2.2. Nucleic acid extraction and species determination

Prior to nucleic acid extraction, ticks (separated according to individual animal source) were each washed in 1 ml of hydrogen peroxide followed by three washes with 1 ml of ultraviolet-irradiated, nuclease-free water and then air-dried. Individual ticks were then transferred into a 1.7 ml microcentrifuge tube containing 100 µl of viral transport media (VTM) (Becton Dickinson) and homogenized. Total nucleic acid (TNA) was extracted from 33 µl of tick homogenate on the EasyMag platform (BioMerieux)(Loens et al., 2007) and eluted in 40 µl. From each sample, 11 µl of the TNA was aliquoted for RT-PCR while the remainder was stored at ~80 °C.

To identify the tick species, a barcoding PCR was performed using primers for targeting the 16s rRNA mitochondrial gene (Black IV and Piesman, 1994). However, these established primers for genotyping tick species did not amplify a PCR product for all samples, so new primers were designed using Primer3Plus (Untergasser et al., 2007) on MAFFT sequence alignment generated in Generous software (v 10.2.4) that consisted of all the complete tick 16s rRNA mitochondrial gene sequences deposited in GenBank as of June 2019 with duplicate species removed to prevent bias. The alignment allowed for the identification of conserved regions flanking the previously described primers to amplify products from all tick species. The specificity of these primer sequences was confirmed from Blast alignments showing that these primers matched only to tick sequences and were discriminatory without amplifying sequences from non-tick species. The designed primers used were 5′-GGGTCATTGACATCRIATCAWGTRGAR-3′ and 5′-GGGCG GCCTGRTATTTRACTAT-3′, with 1 µl of cDNA as template. The cycling conditions included 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 40 s, and 72 °C for 40 s, and finished with a final step of 72 °C for 5 min. All PCR products were confirmed using Sanger sequencing.

2.3. Library preparation and genome assembly

Following species confirmation, 33 µl of original VTM homogenate from individual ticks were pooled according to species (*A. dissimile* n = 9 and *H. juxtakochi* n = 4) to create libraries for high-throughput sequencing (HTS). Before extraction on the EasyMag platform (BioMerieux), 300 µl of pooled material was purified to enrich for viral particles. Pools were filtered (0.45 µm) then treated with RNase A (15 min at room temperature) and Turbo DNase and Benzonase (30 min at room temperature). This method degrades nucleic acids that are not protected by the presence of a viral capsid. TNA (11 µl) from each tick pool was subjected to first and second-strand cDNA synthesis with Super Script IV reverse transcriptase (Invitrogen) and exo- Klenow fragment (NEB) respectively. Double-stranded DNA was processed for the library construction using KapaHyperPlus kit (Roche) following manufacturer guidance. Sequencing was performed on the Illumina NextSeq 550 system (Illumina, San Diego, CA). The demultiplexed FastQ files were adapter trimmed using the Cutadapt program (v 3.0) (Martin, 2011). Adapter trimming was followed by generation of quality reports using FastQC software (v0.11.5) (Anders, 2015), which were used to determine filtering criteria based on the average quality scores of the reads, read length, homopolymeric reads, nucleotide bias and quality scores at the ends of the reads. The reads were quality filtered and end trimmed with PRINSEQ software (v0.20.3) (Schmieder and Edwards, 2011). Host background levels were determined by mapping filtered reads against a tick reference database (consisting of all *Ixodes scapularis, Amblyomma americanum*, and *Dermacentor variabilis* sequences present in GenBank as of August 2019) using Bowtie2 mapper (v2.2.9) (Langmead and Salzberg, 2012). The host-subtracted reads were de novo assembled using the MIRA (4.0) and MEGAHIT (1.2.8) assemblers (Chevreux, 2005; Li et al., 2015a). Contigs and unique singletons were subjected to homology search using MegaBLAST against the GenBank nucleotide database. Sequences that showed low or no homology at the nucleotide level were subjected to a BLASTX homology search against the complete GenBank protein database. Sequences from viral BLASTX analysis were submitted to a second round of BLASTX homology search against the complete GenBank protein database to correct for biased E values and taxonomic misassignments.

2.4. Phylogenetic analysis

Protein sequences were aligned using ClustalW in Geneious v 10.2.4. Phylogenetic trees were constructed with MEGAX 10.1.7 (Kumar et al., 2018), and the robustness of each node was determined using 1000 bootstrap replicates using a maximum likelihood (ML) method employing an LG+G+I model with nearest-neighbor interchange (NNI) determined to be the best model through a ML fit of 56 different amino acid substitution models.

3. Results

Ticks were removed from three iguanas (*Iguana iguana*) and one red brocket deer (*Mazama americana*). A total of 13 ticks were collected. We speciated them using two PCR assays targeting the 16s RNA mitochondrial gene. Nine ticks were *A. dissimile*. Four ticks were *H. juxtakochi*

A total of two tick pools were sequenced, one for all nine *A. dissimile* and one with the four *H. juxtakochi*, on one lane of Illumina Nextseq resulting in 57,574,043 and 49,535,108 raw reads respectively. After quality filtration and host subtraction 12,201,410 reads for the *A. dissimile* pool and 6,098,179 reads for the *H. juxtakochi* pool were used for assembly, which generated 173,841 and 89,661 contigs respectively. A total of 188 and 416 contigs from the *A. dissimile* and *H. juxtakochi* pools, respectively, were identified as possible viral in origin through BLASTN and BLASTX of the viral protein GenBank database. After re-examining these contigs against the complete GenBank database, only 33 contigs from *A. dissimile* were identified as viral in origin and none of the *H. juxtakochi* contigs were identified as viral. From these contigs three putative novel viral species were identified, all from different viral families.

3.1. Orthomyxoviridae

Sequences for a novel quaranjavirus, tentatively designated Granville quaranjavirus (GQV), were identified in the *A. dissimile* pool. These sequences represent the complete coding sequences, however ends were not confirmed through RACE-PCR. The GQV sequences comprise five
segments with homology to known quaranjaviruses: the nucleoprotein (NP), the hemagglutinin (HA), and the polymerase subunits (PB1, PB2, and PA). Related viral sequences have been identified in previous tick virome studies (Cholleti et al., 2018; Wille et al., 2020). Within the PB1 protein, GQV was most similar to Zambezi tick virus (ZTV) (79% amino acid [aa]), a novel quaranjavirus identified in Rhipicephalus spp. ticks in Mozambique (Cholleti et al., 2018), and Uumaja virus (UMV) (61% aa), a novel quaranjavirus identified in Ixodes uriae ticks in the Artic (Pettersson et al., 2020). Additional segments for ZTV and UMV were not available for analysis. The other segments of GQV have lower homology to other characterized viruses. The NP and HA shared 34% and 37% aa identity to Wellfleet Bay virus, respectively, the PA has 32% aa identity to Johnston Atoll quaranjavirus, and the PB2 has 33% aa identity to Tjuloc virus (Briese et al., 2014; Lvov et al., 2014; Mourya et al., 2019; Presti et al., 2009). Phylogenetic analysis (Fig. 1) shows that GQV clustered with other recently identified tick-borne quaranjaviruses that form a secondary branch to the well characterized type species of the genus.

3.2. Chuviridae

We recovered the complete genome sequence for a novel mivirus, tentatively designated Amblyomma dissimile mivirus (ADM), from the A. dissimile pool. Within the RNA-dependent RNA polymerase (RdRp), ADM shared 59% aa identity with the next closest mivirus, Lonestar mivirus, also found in a tick belonging to the genus Amblyomma (Tokarz et al., 2018). The genome consisted of 3 open reading frames (ORFs) representing the RNA-dependent RNA polymerase (RdRp), the glycoprotein, and the nucleoprotein. The genome orientation was determined to be circular by PCR. Phylogenetic analysis revealed that ADM clustered with the lone other mivirus (Lonestar mivirus) identified within ticks of the genus Amblyomma (Fig. 2), while also within the larger monophyletic clade of all known tick-borne miviruses.

3.3. Tetraviridae

Partial genome sequences with homology to tetraviruses were identified within the Amblyomma dissimile pool. The recovered sequences (approximately 90% coverage spaced across the polyprotein) were most similar to with tick-borne tetravirus-like virus (65% aa identity) (Tokarz et al., 2018) and other more distantly related viruses including Vovk virus (32% aa identity) and Bulatov virus (31% aa identity) (Wille et al., 2020).

4. Discussion

Due to extended periods of time spent at the sylvatic interface, hunters have been shown to have a higher risk of exposure to zoonotic diseases (Karesh and Noble, 2009; Kilonzo et al., 2013; Paige et al., 2014). Anecdotal evidence suggests that hunters within Trinidad and Tobago are bitten more frequently by ticks compared to non-hunters in the regions that they reside. Therefore, their exposure to vector-borne pathogens is also presumably increased. The characterization of the pathobiomes of the ticks that are encountered during hunting would shed light on the disease risk.

The total number of ticks collected in this study was low, as it was difficult to find hunters who were willing to aid in the collections. Hunters were approached prior to the hunting season and given supplies to remove and store the ticks, however only a small subset of the hunters returned samples. Additionally, hunters in Trinidad and Tobago are often reluctant to share information due to concern that their hunting sites or practices will be exposed. Due to the limited sample size this data does not reflect the complete virome for these tick species.

Tick species identification is crucial for any tick-based research.

Fig. 1. Phylogenetic relationships of Orthomyxoviridae based on a 204-aa alignment of the polymerase subunit (PB1) gene along with the genome organization of Granville quaranjavirus. Nodes with >80% bootstrap support are indicated.
Identifications keys are often difficult to use and can often lead to misidentification of related tick species (Barker et al., 2014; Chitimia et al., 2009). Regions within the 16s rRNA mitochondrial gene or the cytochrome oxidase (COI) gene have typically been employed as the targets for molecular speciation of ticks (Abouelhassan et al., 2019; Lv et al., 2014). We designed our assay on the 16s rRNA mitochondrial gene because of the availability of a greater number of complete sequences in GenBank. We tested several assays from the literature on previously identified ticks (data not shown). The assay described by Black and Piesman was determined to be the most reliable (Black IV and Piesman, 1994). Nevertheless, when we used this assay on the tick species from this study, only four out of the 13 ticks were identified. Accordingly, we developed an additional assay that enabled speciation of the remaining nine ticks.

All the viral sequences identified in this study originated from *A. dissimile*. No viral sequences were identified in *H. juxtakochi*. Previous work in the region highlights the fact that tick species may vary in viral abundances (Abouelhassan et al., 2019; Lv et al., 2014). We designed our assay on the 16s rRNA mitochondrial gene because of the availability of a greater number of complete sequences in GenBank. We tested several assays from the literature on previously identified ticks (data not shown). The assay described by Black and Piesman was determined to be the most reliable (Black IV and Piesman, 1994). Nevertheless, when we used this assay on the tick species from this study, only four out of the 13 ticks were identified. Accordingly, we developed an additional assay that enabled speciation of the remaining nine ticks.

All the viral sequences identified in this study originated from *A. dissimile*. No viral sequences were identified in *H. juxtakochi*. Previous work in the region highlights the fact that tick species may vary in viral abundances (Sameroff et al., 2019). A recent study in the US reported the absence of viruses in invasive *Haemaphysalis longicornis* ticks (Tufts et al., 2020). This was suggested to be partly due to the fact that *H. longicornis* can reproduce parthenogenetically, so if the progenitor was devoid of any viral endosymbionts the offspring would also lack them. Whether viral abundance within tick species impacts vector competence, or the reasons for differing viral abundances, is not currently understood.

GQV is a species of potential importance in the region because of known associations of quaranjaviruses with disease in vertebrates (Foster et al., 2018; Kosoy et al., 2015; Mourya et al., 2019). GQV clusters with other recently identified hard-tick associated quaranjaviruses (Cholleti et al., 2018; Pettersson et al., 2020). The viruses pertaining to this cluster are distributed across wide geographic ranges and may suggest that there are more unidentified novel tick-borne orthomyxoviruses to be classified. Additionally, this group currently has an unknown pathogen potential, however quaranjaviruses from soft-tick species are known to cause disease in humans, and within avian species, have led to mass die-offs (Allison et al., 2015; Mohammed et al., 1970; Shearn-Bochsler et al., 2017). All essential components for viral replication and infection (Neumann et al., 2004) have been identified. However quaranjaviruses, and orthomyxoviruses can have additional segments. Thus, we cannot rule out the possibility that our genomic sequence data are incomplete.

The other viral sequences identified are believed to be ASVs. While these viruses are part of the tick microbiome, they are most likely not part of the pathobiome. These viruses may however play an important role within the tick microbiome. It has been suggested that ASVs can influence vector competency, potentially inhibiting the acquisition and
transmission of other viruses (Öhlund et al., 2019). The recently clas-
sified order Jinghuitaevirales, currently consists of one family, Chuviridae,
and one genus, Mivirus, containing all currently identified miviruses (Walker et al., 2019). Over 30 species of mivirus have been identified, all
within the last couple of years (Brinkmann et al., 2018; Gomez et al., 2020; Gondard et al., 2020a, 2020b; Li et al., 2015a; Samaroff et al., 2019; Souza et al., 2018; Temann et al., 2019; Tokarz et al., 2018; W. M. et al., 2018). Little is known about these viruses, however they can have
variable genome organization consisting of either linear or circular
genes, and monoparitite or segmented genomes (Li et al., 2015b). To date all miviruses derived from ticks, including ADM, have circular
monoparitite genomes. The role these viruses play within the tick host
is currently unknown. Until recently tetraviruses were believed to have a
very narrow host range; however, with the recent surge in virome
analyses, tetraviruses have begun to be identified in many insect species
(Dorrington et al., 2020).

5. Conclusion

These findings highlight the diversity of the tick virome and the potential for as-yet-unknown invertebrate viruses to contribute to TBD.

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CRediT authorship contribution statement


Declaration of Competing Interest

None.

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