



A metatranscriptomic evaluation of viruses in field-collected bed bugs

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Abstract

Cimex lectularius, known as the common bed bug, is a widespread hematophagous human ectoparasite and urban pest that is not known to be a vector of any human infectious disease agents. However, few studies in the era of molecular biology have profiled the microorganisms harbored by field populations of bed bugs. The objective of this study was to examine the viruses present in a large sampling of common bed bugs and related bat bugs (*Cimex pipistrelle*). RNA sequencing was undertaken on an international sampling of > 500 field-collected bugs, and multiple workflows were used to assemble contigs and query these against reference nucleotide databases to identify viral genomes. Shuangao bed bug virus 2, an uncharacterized rhabdovirus previously discovered in *Cimex hemipterus* from China, was found in several bed bug pools from the USA and Europe, as well as in *C. pipistrelle*, suggesting that this virus is common among bed bug populations. In addition, Shuangao bed bug virus 1 was detected in a bed bug pool from China, and sequences matching Enterobacteria phage P7 were found in all bed bug pools, indicating the ubiquitous presence of phage-derived elements in the genome of the bed bug or its enterobacterial symbiont. However, viral diversity was low in bed bugs in our study, as no other viral genomes were detected with significant coverage. These results provide evidence against frequent virus infection in bed bugs. Nonetheless, our investigation had several important limitations, and additional studies should be conducted to better understand the prevalence and composition of viruses in bed bugs. Most notably, our study largely focused on insects from urban areas in industrialized nations, thus likely missing infrequent virus infections and those that could occur in rural or tropical environments or developing nations.

Keywords *Cimex lectularius* · *Cimex pipistrelle* · Bed bug · Virus · Arbovirus · Metatranscriptomics · RNAseq

Introduction

The family Cimicidae is comprised of ectoparasitic insects that feed exclusively on the blood of a variety of vertebrate hosts. At least eight species from four genera of Cimicidae

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are known to feed on humans, either as preferred hosts, when other preferred hosts (e.g., bats or birds) nest in human households, or when humans enter infested environments such as caves (Williams et al. 1976, Harlan et al. 2008, Doggett et al. 2012). However, *Cimex lectularius*, the common bed bug, and *Cimex hemipterus*, the tropical bed bug, are the two species that most frequently parasitize humans (Doggett et al. 2012).

Bed bugs have undergone a global resurgence in recent decades and are prevalent pests in diverse built environments including single and multi-family homes, hotels, homeless shelters, and poultry houses. They are also one of the likeliest human ectoparasites to be encountered by clinicians in healthcare settings (Totten et al. 2016, Sheele et al. 2016, 2017a, b, 2019a, b). One large medical center where multiple bed bug studies have been conducted reported finding a bed bug every ~2 days and ~4 days in the emergency department (ED), yet this likely underrepresented the true number of bed bug introductions into the hospital (Sheele et al. 2017a, b). ED surveys found that 1 in 50 patients reported an active home bed bug infestation, 15% of patients knew someone with a current bed bug infestation, 37% reported a previous bed bug feeding, and 59% knew someone with bed bugs in the last 5 years (Sheele et al. 2017c, 2019a).

A plethora of human blood-feeding arthropods, including mosquitoes, sand flies, fleas, kissing bugs, lice, and ticks are important vectors of bacterial, viral, and parasitic human disease agents. In contrast, while over 40 different pathogens have been sporadically detected in bed bugs from the field, bed bugs have never been concretely linked to the transmission of any human pathogens (Delaunay et al. 2011; Ho et al. 2016; Lai et al. 2016). Most investigations into associations between bed bugs and human pathogens took place before the advent of modern molecular biology. Further, the limited number of modern studies of this nature that have employed molecular biology have focused on detecting bacterial pathogens (Potts et al. 2021, Peta et al. 2022; Ndiaye et al. 2022, Sevestre et al. 2023). However, associations between bed bugs and arthropod-borne viruses (arboviruses) have gone largely unstudied (Adelman et al. 2013; Pietri 2020).

Arboviruses mostly belong to the viral families Peribunyaviridae, Flaviviridae, Reoviridae, and Togaviridae. There are more than 530 described arboviruses, with over a hundred known to be pathogenic to humans (CDC 2023). Unlike bed bugs, other species in the family Cimicidae are known to be arboviral vectors. For example, swallow bugs (*Oaeciacus vicarius*) transmit the alphavirus Fort Morgan virus among their bird hosts, and bat bugs (*Cimex insuetus* and *Striticimex parvus*) have been implicated in both enzootic and zoonotic transmission of the bunyavirus Kaeng Khoi virus (Williams 1976, Hayes et al. 1977; Rush et al. 1980). Thus, the potential for bed bugs to harbor and transmit arboviruses

may be underappreciated and merits additional exploration (Adelman et al. 2013).

Several relatively recent studies have analyzed the viromes of some bed bug samples (Li et al. 2015, Ling et al. 2020, Stanojevic et al. 2020, Xu et al. 2022). These studies have been geographically restricted to single regions, and some generated mixed libraries from different species of insects, not allowing for identification of viruses derived specifically from bed bugs. Yet, some results of interest have been obtained. For instance, a novel reovirus of unknown pathogenicity was identified in bed bugs from Serbia (Stajenovic et al. 2020). Hepatitis C virus was also found in a pool of bed bugs collected from Sweden and Norway, although these insects had recently blood fed and the possibility that the virus was simply a transient contaminant could not be excluded (Ling et al. 2020). With the present study, we sought to expand current knowledge of the viruses that can be associated with bed bugs, which remains meager. We performed a large-scale metatranscriptomic evaluation of viruses in bed bugs by conducting RNA sequencing on a geographically diverse sampling, with a particular interest in identifying any viruses of potential public health significance.

Materials and methods

Bed bug sampling

Between December 2016 and December 2018, 242 bed bug samples containing 361 individual bed bugs, plus 142 samples which contained pools of multiple uncounted bed bugs, were collected by entomologists, pest management professionals, or hospital personnel. The bed bug samples came from the following geographical locations: United States (USA) ($n = 165$) (Connecticut ($n = 7$), Delaware ($n = 1$), Illinois ($n = 5$), California ($n = 2$), Pennsylvania ($n = 5$), New Jersey ($n = 17$), Ohio ($n = 108$; 10 from Cleveland-Akron community and 118 from University Hospitals Cleveland Medical Center (UHCMC))), United Kingdom (UK) ($n = 51$), Czech Republic ($n = 21$), France ($n = 14$), Slovakia ($n = 4$), and China ($n = 12$). Given that the collection locations were all at temperate latitudes, all the samples were presumed to be *C. lectularius*, but this was not confirmed. Eight samples of the bat bug *Cimex pipistrelle* were also captured in 2014 and subsequently raised in the laboratory on human blood. All bed bugs were collected alive and placed into 1.5-mL sterile nuclease-free microcentrifuge tubes containing RNALater and stored at $-80\text{ }^{\circ}\text{C}$, with the exception of four *C. pipistrelle* samples which were placed directly at $-80\text{ }^{\circ}\text{C}$ without RNALater. Unless otherwise noted, only insects without evidence of recent blood feeding were studied to avoid detection of transient viruses ingested in a blood meal.

RNA extraction

Bed bug samples were divided into six groups for sequencing based on their origin as follows: OH, USA ($n = 98$); UK ($n = 52$); US non-Ohio ($n = 37$); Dongguan or Shenzhen in China ($n = 12$); Czech Republic, Slovakia, and France ($n = 39$); and *C. pipistrelle* from the Czech Republic ($n = 8$). A list of all samples sequenced and their relevant details is provided in Supplementary File 1. RNA was extracted from whole bed bugs using a RNeasy Mini Kit (Qiagen, Germantown, MD). Bed bugs were homogenized in RLT buffer using a Mikro-Dismembrator S at 3000 rpm for 45 s, and then the protocol from the manufacturer was followed.

RNA sequencing

RNA concentration was determined using a Qubit fluorometer (Invitrogen), and quality was assessed using an Agilent 2100 bioanalyzer. An Illumina TruSeq Stranded Total RNA kit with Ribo-Zero Gold for rRNA removal was used for library preparation. All samples showed some degradation (DV 200 > 58%) so alternate Illumina protocol steps were followed for degraded/FFPE RNA. The protocol involved using the Ribo-Zero kit to remove ribosomal RNA (rRNA) from 400 ng of total RNA using a hybridization/bead capture procedure that selectively binds rRNA species using biotinylated capture probes. The resulting purified mRNA was used as the input for Illumina TruSeq libraries. Final libraries were validated on an Agilent 2100 bioanalyzer, quantified via qPCR and pooled at equimolar ratios. Pooled libraries were diluted, denatured, and loaded onto an Illumina NextSeq 550 System for a paired-end 150 cycle run. The sequencing took place at the Genomics Core at Case Western Reserve University (CWRU).

Data analysis

Raw fastq files were uploaded to the Galaxy Webserver (Afgan et al. 2018) and first processed with Trimmomatic v. 0.38.1 (Bolger et al. 2014) to remove adapter sequences and low-quality reads using default parameters and the universal

Illumina adapter option. After processing, two data analysis workflows were performed. In workflow 1, trimmed reads were aligned to the *C. lectularius* genome (accession number: NW_019942502.1) using the mapping application HISAT2 (Kim et al. 2019) with default parameters. Unaligned reads that did not map to the bed bug genome were used to assemble viral contigs using metaviralSPAdes v. 3.15.4 (Antipov et al. 2020) with default parameters, and the resulting contigs were then searched against the NCBI Virus database (Hatcher et al. 2017) using the blastN module v. 2.10.1 (Camacho et al. 2009; Cock et al. 2015). In workflow 2, Trinity (Grabherr et al. 2011) was used for de novo assembly of contigs without subtraction of host reads prior to searching against the NCBI virus database.

In addition, the raw reads were analyzed directly on the open-source, cloud-based platform Chan Zuckerberg ID (CZ ID, formerly IDseq) (Kalantar et al. 2020) for detection of human pathogens from metagenomic data.

Results

A summary of the sequencing reads generated and analyzed is provided in Table 1. Between 1,477,486,334 and 194,936,088 raw reads were generated for each pooled bed bug sample.

In workflow 1, this resulted in between 857,492,203 and 83,360,994 processed reads that did not map to the bed bug genome. The assembly of these reads into viral genome contigs using metaviralSPAdes produced between 8 and 47 contigs per pooled sample. BLAST analysis of these contigs against the NCBI Virus database identified the complete or nearly complete genome of Shuangao bed bug virus 2 in 3/6 pools (~88–97% identity). Specifically, this virus was detected in one bed bug pool from the USA (OH), as well as in the Czech Republic, Slovakia, and France pool. Shuangao bed bug virus 2 was also detected in the *C. pipistrelle* pool, but not in common bed bug pools from non-Ohio locations in the USA, from China, or from the UK. In addition to Shuangao bed bug virus 2, sequences between 673 and 1209 base pairs in length that strongly aligned to Enterobacteria

Table 1 Summary of sequencing read data

Sample	Total reads	Quality controlled processed reads	Unmapped reads	Contigs assembled by metaviral SPAdes
US non-Ohio	194,936,088	192,127,994	110,597,817	8
China	211,759,716	207,897,556	165,637,280	15
Cimex pipistrelle	211,227,716	207,903,712	89,490,012	12
Czech Republic, Slovakia, and France	210,122,152	205,986,922	83,360,994	11
US Ohio	1,477,486,334	1,452,827,038	857,492,203	47
UK	197,532,404	192,096,002	100,064,714	10

phage P7 (> 99% identity) were found in bed bug pools from all locations. However, other portions of the P7 phage genome were not detected, indicating that these sequences are likely phage-derived elements present in the genome of the bed bug or its enterobacterial symbiont (Sakamoto and Rasgon 2006). Although the workflow subtracted reads mapping to the bed bug genome and used an assembly method tailored for virus discovery, some of the contigs nonetheless yielded significant matches to portions of the bed bug, *Wolbachia*, or human genomes. The results are included in Table 2, and raw sequences and analysis outputs for workflow 1 are provided in Supplementary File 2.

In workflow 2, which did not perform subtraction of bed bug reads and used Trinity (Grabherr et al. 2011) for de novo assembly of contigs, portions of the Shuangao bed bug virus 2 genome were detected in additional bed bug pools. These results are also included in Table 2, and raw sequences and analysis outputs for workflow 2 are provided in Supplementary File 3. Notably, while both workflows identified numerous isolated short sequences with high similarity to sequences from a variety of known viruses, these matches lacked significant coverage.

Analysis in CZ ID (Kalantar et al. 2020) did not detect any viral human pathogens, but additionally identified the nearly complete genome of Shuangao bed bug virus 1 in the bed bug pool from China. This virus has been previously detected in *C. hemipterus* from this country (Li et al. 2015).

Discussion

This study is the first large scale metatranscriptomic study that focused solely on virus discovery in bed bugs by sampling hundreds of individual bugs from unique sites across different continents. Despite a large sampling of bed bugs, we failed to detect many viruses. The significance of this finding is not fully clear, but it suggests that viral infections in bed bugs may be infrequent and lack diversity, providing valuable new insight into the potential of these insects as vectors of viruses.

Although our results were mostly negative, we identified the ubiquitous presence of Shuangao bed bug virus 2. Shuangao

bed bug virus 2 is an uncharacterized rhabdovirus that was first detected in *C. hemipterus* from China (Li et al. 2015). Rhabdoviruses are a diverse family of negative strand RNA viruses which includes a number of arboviruses and human pathogenic viruses. However, much remains unknown about the biology of Shuangao bed bug virus 2. Our study adds to the limited knowledge of this virus, indicating that it is common across geographically diverse bed bug populations and should be further investigated to understand its transmission as well as its implications for bed bug biology and control.

It is critical to acknowledge that our study had several important limitations. First, bed bugs are global pests, but our investigation was limited to insects mostly from urban areas in industrialized nations, thus potentially missing infrequently acquired viruses, especially if they are more likely to occur in rural or tropical environments or developing nations. Additionally, we pooled large numbers of bed bugs for sequencing and sequencing depth was variable, which, coupled with RNA degradation, may limit virus detection via the methods used. Therefore, future work to better understand the prevalence and composition of viruses in bed bugs in different environments should still be encouraged. Such work should focus on geographic regions where arbovirus acquisition and transmission are more likely to occur (Adelman et al. 2013), employ long read technology or methods for viral sequence enrichment to enhance detection of low abundance viruses in pools, or perform sequencing on individual insects to increase sampling depth. Lastly, as mining and discovery of viral sequences from metagenomic/metatranscriptomic data faces multiple challenges, the improvement and development of new bioinformatics tools for this purpose may extend the usefulness of our data in the future.

Conclusions

This study suggests that viral infections in bed bugs are infrequent and lack diversity. However, our investigation had several limitations, and additional studies should be conducted to better understand the prevalence and composition of viruses in bed bugs from different environments.

Table 2 Summary of viruses detected in bed bug samples

US non-Ohio	China	<i>C. pipistrelle</i>	Czech Republic, Slovakia, and France	US Ohio	UK
Shuangao bed bug virus 2	Shuangao bed bug virus 2	Shuangao bed bug virus 2	Shuangao bed bug virus 2	Shuangao bed bug virus 2	Shuangao bed bug virus 2
Enterobacteria phage P7	Enterobacteria phage P7	Enterobacteria phage P7	Enterobacteria phage P7	Enterobacteria phage P7	Enterobacteria phage P7
	Shuangao bed bug virus 1				

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00436-023-08049-1>.

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Author contribution JS: investigation, conceptualization, methodology, validation, supervision, funding acquisition, resources, writing—original draft, writing—review and editing. VP: formal analysis, writing—original draft, writing—review and editing. AM: conceptualization, methodology, resources. OB: resources. DC: resources. SE: conceptualization, methodology. TM: conceptualization, methodology, resources. JP: investigation, conceptualization, methodology, validation, supervision, funding acquisition, writing—original draft, writing—review and editing.

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Data availability Raw sequencing reads are publicly available for additional mining and analysis. These can be accessed from the NCBI SRA under BioProject ID: PRJNA972449.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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