Autosomal Short Tandem Repeats and an *Alu* Insertion Polymorphism Are Detectable in *Cimex lectularius* L. (Hemiptera: Cimicidae) for 48 Hours after Human Blood Ingestion[1]

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ABSTRACT Human DNA has been identified successfully from several hematophagous and necrophagous insects. Because bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae), imbibe human blood and achieve high population densities in human habitations, we assessed the stability of human DNA in bed bugs over time and matched blood isolated from bed bugs to an individual human host. Using polymorphic autosomal short tandem repeat (STR) markers commonly employed in forensic investigations and an *Alu* insertion polymorphism, we unambiguously identified human DNA in bed bugs up to 48 h after blood ingestion and visualized faint bands from bed bugs fed 72 h prior. Using STR markers, we could not identify human DNA in bed bug excreta at any time point after blood ingestion. All blood samples matched identical STRs amplified from the host that the bed bugs had fed on, indicating the feasibility of this approach to identify a human host within 48–72 h of blood ingestion.

KEY WORDS Forensic science, forensic entomology, bed bug, DNA, microsatellite repeats

Identifying an individual based on blood or tissue isolated from insects can be used to implicate a suspect in the time and place of a crime. However, human DNA isolated from an insect would have to be (1) stable and intact long enough to be useful in a forensic investigation and (2) unambiguously identifiable to an individual host. Human DNA has been extracted and identified from individual mosquitoes for up to fifteen hours after ingestion of a blood meal (Coulson et al. 1990), and subsequent studies have confirmed the sensitivity and reproducibility of using DNA fingerprinting of mosquito blood meals to identify individual human hosts (Michael et al. 2001). Experiments with the human crab louse, *Pthirus pubis* (L.) (Phthiraptera: Pthiridae), and human head louse, *Pediculus humanus capitis* De Geer (Phthiraptera: Pediculidae), both obligate human ectoparasites, found that blood-fed lice and their excreta could yield DNA from

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single or multiple human hosts (Replogle et al. 1994, Lord et al. 1998, Mumcuoglu et al. 2004). Human-specific markers such as prostate-specific antigen from semen or mitochondrial DNA (mtDNA) from decomposing tissues have been isolated from necrophagous fly larvae, the most common insects associated with homicide investigations (Clery 2001, Wells et al. 2001, Linville et al. 2004). Remarkably, mtDNA from human bone has been isolated from beetle larvae, demonstrating the robustness of polymerase chain reaction (PCR) amplification even with degraded human tissue (DiZinno et al. 2002).

Bed bugs, *Cimex lectularius* L. and *Cimex hemipterus* (F.) (Hemiptera: Cimicidae), are globally resurgent pests that feed exclusively on vertebrate blood (Usinger 1966, Reinhardt & Siva-Jothy 2007). Both males and females feed on blood, and juveniles require blood to molt (Usinger 1966). Bed bugs readily feed on several avian and mammalian hosts (Usinger 1966, Reinhardt & Siva-Jothy 2007) and can achieve alarming population densities (Cater et al. 2011). In our field studies, we found hundreds of bed bugs per square meter in lower-income housing and more than 10,000 bed bugs in one bedroom alone (unpublished data). Because of their intimate association with humans, tendency to aggregate in harborages, frequent and obligate blood-feeding behavior, and high population density, bed bugs would be a valuable source of human DNA for forensic analysis. Two studies found that human DNA can be isolated from bed bugs (Szalanski et al. 2006a) and that a human mtDNA hypervariable region (HVR1) marker was recoverable seven days after blood-feeding (Szalanski et al. 2006b). The goals of our study were (1) to assess the stability of human DNA in bed bugs and their excreta over time, using five validated polymorphic autosomal short tandem repeat (STR) and one *Alu* insertion polymorphism marker commonly employed in forensic investigations and (2) to match blood isolated from bed bugs to the genotype of an individual human host.

Materials and Methods

Bed bugs. *C. lectularius* were maintained in a colony established from a natural population collected in 1973 in Fort Dix, New Jersey. We maintained bed bugs at 28°C, at a photoperiod of 16 h of light to 8 h of dark. Adult female bed bugs were fed simultaneously on a human volunteer (SRI Human Subjects Committee Approval, Case 1199), and engorged bed bugs were maintained in glass scintillation vials with a 1-cm^2 section of cotton wicking under the conditions detailed above. Three bed bugs and the cotton were removed 0, 12, 24, 48, 72, and 96 h after feeding and stored immediately at −20°C until DNA isolation was performed. The cotton wicking was replaced at each time point. This experiment was repeated three times (1 d between each experiment) on a single human host, yielding nine bed bugs and three pieces of cotton per time point. Our positive controls included DNA from a buccal swab of the human volunteer amplified with STR primers and bed bug DNA amplified with primers for the gene of the α-subunit of the voltage-gated sodium channel were included on every gel with bed bug samples (Zhu et al. 2010). To ensure that our STR primers were specific for human DNA and not amplifying bed bug genes, we also attempted to amplify DNA from unfed bed bugs with human STR primers.

DNA isolation. We isolated DNA from blood-fed bed bugs (n = 54), soiled cotton cloth (n = 18), and a single buccal swab from the human host that the bed

Table 1. Bed bug, human STR, and *Alu* primer sets. Numbers in front of primer names for human microsatellites designate the chromosome on which the gene is located.

Primer set name	Sequence
C. lectularius V419F	5' ATTCCTGGGATCATTCTACCTCG 3'
C. lectularius V419R	5' TGATGGAGATTTTGCCACTGATG 3'
2p TPOX F	5' ACTGGCACAGAACAGGCACTTAG 3'
2p TPOX R	5' GGAGGAACTGGGAACCACACAGG 3'
4q FIBRA F	5' ATTATCCAAAAGTCAAATGCCCC 3'
4q FIBRA R	5' ATCGAAAATATGGTTATTGAAGT 3'
5q CSF1PO F	5' AACCTGAGTCTGCCAAGGACTAG 3'
5q CSF1PO R	5' TTCCACACACCACTGGCCATCTT 3'
8p12 ALU F	5' GTAAGAGTTCCGTAACAGGACAGCT 3'
8p12 ALU R	5' CCCCACCCTAGGAGAACTTCTCTTT 3'
11p THO1 F	5' GTGGGCTGAAAAGCTCCCGATTA 3'
11p THO1 R	5' ATTCAAAGGGTATCTGGGCTCTG 3'
12p VWA F	5' CCCTAGTGGATGATAAGAATAAT 3'
12p VWA R	5' GGACAGATGATAAATACATAGGATGGATGG 3'

bugs had fed on. Cheek cells were washed in deionized water and pelleted. Individual bed bugs, cloth, or cheek cells were resuspended in 100 μL of Edward's buffer (200 mM Tris, 250 mM NaCl, 25 mM EDTA) at room temperature and pH 8.0 (Edwards et al. 1991). Samples were homogenized with a handheld pestle in a 1.5 mL microcentrifuge tube, and an additional 600 μL of Edward's buffer was added to each sample and mixed vigorously. Each microcentrifuge tube lid was perforated, and the samples were heated for 5 min at 100°C. The samples were then centrifuged at 16.2 × *g* for 3 min to pellet debris; 600 μL was transferred to a new tube with 800 μL of isopropanol at room temperature. The samples were centrifuged at 16.2 × *g* for 5 min, and the alcohol was discarded. All tubes were then heated at 37°C for 5 min and dried further by speed vacuum for 10 min. Samples were then treated with 100 μL of ice-cold Tris-EDTA with RNase A (Carolina Biological). Concentrations were determined with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA), and samples were stored at −20°C until use.

PCR. We conducted PCR on all samples, with 3 μL of DNA template, 10 μL of Master Mix, 2 μL of dye, 3 μL of deionized water, 1 μL of forward primer, and 1 μL of reverse primer (Qiagen Fast Cycling PCR Kit, Alameda, CA). We used seven primer pairs (Table 1). The reaction cycle consisted of 95°C for 5 min followed by 29 cycles of [96°C for 5 sec, 54°C for 5 sec, 68°C for 15 sec], and a final step of 72°C for 15 sec. The samples were maintained at 10°C and visualized by 2% agarose gel electrophoresis with ethidium bromide staining. A 100 basepair ladder was run simultaneously (New England Biolabs, Ipswich, MA).

Results and Discussion

Human mtDNA has been identified from the excreta of the human pubic louse (Replogle et al. 1994) and from the excreta of adult blow flies (*Lucilia cuprina*)

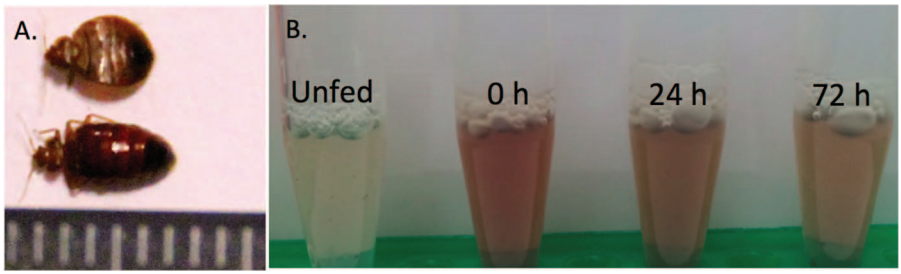

Fig. 1. A. Unfed (top) and recently fed (bottom) female bed bugs (*Cimex lectularius*) illustrate the difference in appearance after blood ingestion. Scale is in 1 mm increments. B. From left to right: a single female bed bug unfed, 0, 24, and 72 h after feeding. Each sample was homogenized in 800 µL of Edward's buffer, and debris was pelleted by centrifugation for 3 min at 13,000 x *g*.

(Durdle et al. 2009). In addition, full DNA profiles have been obtained from fly excreta two years after deposition (Durdle et al. 2013). However, we could not identify human DNA from cotton cloth stained with bed bug excreta at any of our time points. Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) readings ranged from 2 ng/µl to 12 ng/µl. PCR followed by gel electrophoresis yielded no bands in all 12 samples (2 from each time point). This is likely due to our testing for specific STRs, which, unlike mtDNA, might not have persisted through bed bug digestion and excretion. In addition, bed bugs are gregarious, feeding and excreting in large colonies. While our samples were obtained from individual bed bugs, a collection of excreta from multiple bed bugs would have potentially yielded more DNA and increased the chance of a positive detection. Further studies aimed at detecting human DNA in bed bug excreta should examine collections of excreta from multiple bed bugs in addition to those from individual bed bugs.

Crime scene investigators should recognize the difference between an engorged and unengorged bed bug, because isolation of human DNA is possible only from a bed bug that has imbibed blood. Recently fed bed bugs are larger and bright red, compared to unfed bed bugs (Figure 1A). When bed bugs were homogenized in Edward's buffer with isopropanol, recently fed individuals yielded a red solution that could be used to successfully isolate human DNA (Figure 1B). A brown solution contained a bed bug that fed 72 h prior, and PCR resulted in a faint amplification product or none at all. Likewise, an unfed bed bug yielded a straw-colored solution, and human DNA could not be isolated for forensic purposes. From this we were able to roughly correlate color and presence of human blood to PCR results. Thus, we proceeded with the reddest samples. This varied from time point to time point, whether a bed bug had fed or not, and the degree to which it had engorged.

We found that the concentration of human DNA in a bed bug at any time point ranged in concentration from 10 ng/µl to 110 ng/µl. In general, the longer time past feeding, the lower the DNA concentration per sample observed (Figure 2). There is size variation among adults, and larger females will imbibe a larger blood meal (Reinhardt & Siva-Jothy 2007). In addition, humans may yield meals

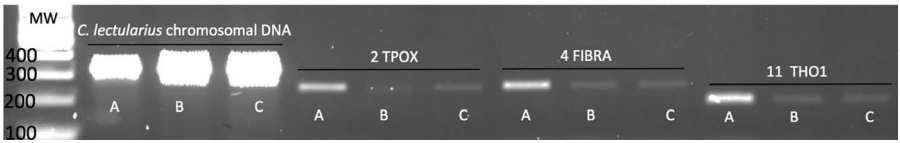

Fig. 2. Variance in concentration of human DNA among bed bugs collected at 24 h after blood ingestion. A 100 basepair (bp) molecular weight (MW) ladder is shown on the left. Total DNA isolated from bed bugs A, B, and C was amplified using *C. lectularius*-specific primers, followed by primer sets 2 TPOX, 4 FIBRA, and 11 THO1. Total DNA contained both bed bug and human DNA. Note that human DNA template isolated from bed bug A yielded significantly stronger signals than the other two isolates.

of different molecular variables, such as DNA and protein concentrations, because of host defensive behavior or variation in blood protein content (Lehane 2005). The amount of blood ingested and the amount of DNA extracted varied among bed bugs that had fed on human blood 24 h prior (Figure 2). Similar results were seen with other time points; as more time passed after feeding, the probability that amplifiable human DNA would be recovered from bed bugs was reduced. Using primer sets 2, 4, and 11 as examples, we show that attempting to amplify DNA from more than one bed bug will increase the probability of yielding at least one unambiguous signal. Human STR primers did not amplify bed bug chromosomal DNA, so we were confident that products using STR or *Alu* primers represented actual human DNA amplification (Figure 3).

We demonstrated (1) the feasibility of amplifying five STRs and one *Alu* insertion polymorphism commonly used in forensic investigations and (2) unambiguously identifying DNA from an individual human host. PCR yielded robust amplification products, as visualized by gel electrophoresis, up to 48 h after blood feeding, and all six primer pairs yielded faint bands from all 9 samples

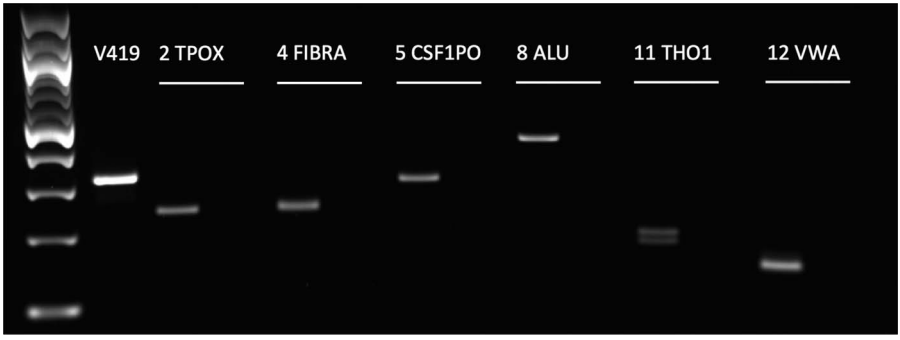

Fig. 3. STR and ALU verification using human and bed bug templates. A bed bug control, V419, was resolved to the right of the MW marker. All subsequent primers were used to amplify human DNA from a buccal swab in the first lane and bed bug DNA in the second. Human STR and ALU primers amplified products with human DNA template only, indicating that these regions were not found in the bed bug genome.

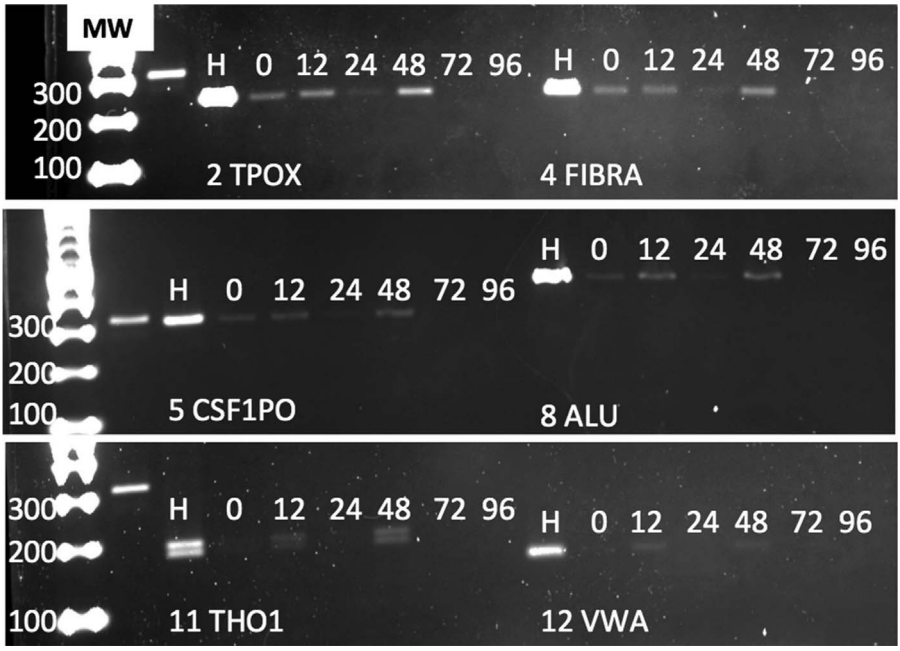

Fig. 4. Amplification of human STRs and an *Alu* insertion polymorphism from bed bugs fed 0, 12, 24, 48, 78, and 96 h prior on a human host. All samples yielded strong amplification products up to 48 h and a faint band at 72 h. Each gel image shows a 100 bp molecular weight (MW) ladder. The first band to the right of each ladder is the bed bug V419 product, which is our internal control for PCR, and the second band (H) is the amplification product from a human buccal swab.

collected 72 h after blood feeding (Figure 4). Previous studies have successfully detected DNA from a single human host in mosquitoes 15 h after blood ingestion (Coulson et al. 1990) and from the pooled blood meals of two body lice up to 20 h after blood ingestion (Mumcuoglu et al. 2004). Szalanski et al. (2006b) identified a mtDNA marker 7 d post-blood feeding and a 271 bp STR marker 60 d post-blood feeding.

When attempting to detect rabbit DNA in human body lice, *Pediculus humanus humanus* L. (Phthiraptera: Pediculidae), a 199 basepair (bp) amplicon had a significantly slower decay rate than a 283 bp amplicon, suggesting that amplicon size may be inversely proportional to decay rate (Davey et al. 2007). We did not calculate decay rates for human DNA in bed bugs, because we found that recovery for any one time point was variable and a function of the blood meal or the individual bed bug. However, we did recover amplifiable human DNA up to 72 h after blood feeding with all 9 samples, as shown previously for rabbit DNA from body lice (Davey et al. 2007).

Every primer set corresponded to a different chromosome, indicated by the primer set number. For example, the *Alu* gene is on the eighth chromosome, and is amplified by primer set 8. Forensic analysis based on STR technology evaluates

specific regions found on chromosomal DNA. The polymorphic variability of the STR regions that are analyzed for forensic testing intensifies the discrimination between the DNA profiles of two different individuals. The probability that any two random individuals will have the same 13-loci DNA profile is approximately one in 1 billion (Butler 2006). The U.S. Federal Bureau of Investigation started the Combined DNA Index System (CODIS) in 1998 and has used 13 specific STR loci to serve as the standard for CODIS since 2006. The purpose of establishing a core set of STR loci is to ensure that all forensic laboratories can establish uniform DNA databases (Bloom 1994). By testing five STR primer pairs that are part of a 13 to 16 set of STR primer pairs routinely used in forensic investigations, this study enables investigators multiple, validated targets for amplification.

Our results are specific for female bed bugs fed on one host and maintained at a constant temperature and photoperiod. The feasibility of this approach will need to be assessed with male or immature bugs, different temperatures and photoperiods, and storage conditions following collection. Szalanski et al. (2006b) found that human DNA could be identified from both male and female bed bugs using a HVR1 and STR marker. Mixed blood meals (from more than one human host) have been identified from a single crab louse (Replogle et al. 1994). We expect our STR markers could be identified from males and that blood meals from more than one host could be identified from a single bed bug. However, bed bugs commonly feed to repletion on one host, and the likelihood of obtaining two blood meals from one bed bug may be slim. Field and laboratory evidence indicate a feeding frequency of 3 to 7 d (Reinhardt & Siva-Jothy 2007). Our collections from lower-income housing always yielded fully engorged, recently fed bed bugs (unpublished data). Considering infestations harbor mixed populations of different instars that overlap in their feeding, at least some of the bed bugs collected in the course of a forensic investigation will have likely imbibed blood within the previous 72 h.

DNA degradation is a consequence of time, temperature, and the initial blood meal volume. A fed bed bug does not guarantee successful amplification of host DNA. Therefore, we recommend that a forensic investigation collect multiple blood-fed bed bugs, including both sexes and immatures, to estimate the time since feeding and to identify the individual human host.

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