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Rapid blood meal scoring in anthropophilic *Aedes albopictus* and application of PCR blocking to avoid pseudogenes

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ABSTRACT

Blood meal analysis (BMA) is a useful tool for epidemiologists and vector ecologists to assess which vector species are critical to disease transmission. In most current BMA assays vertebrate primers amplify DNA from a blood meal, commonly an abundant mitochondrial (mtDNA) locus, which is then sequenced and compared to known sequences in GenBank to identify its source. This technique, however, is time consuming and costly as each individual sample must be sequenced for species identification and mixed blood meals cloned prior to sequencing. Further, we found that several standard BMA vertebrate primers match sequences of the mtDNA of the Asian tiger mosquito, *Aedes albopictus,* making their use for blood meal identification in this species impossible. Because of the importance of *Ae. albopictus* as a vector of dengue and chikungunya viruses to humans, we designed a rapid assay that allows easy identification of human blood meals as well as mixed meals between human and nonhuman mammals. The assay consists of a nested PCR targeting the cytochrome b (cytb) mtDNA locus with a blocking primer in the internal PCR. The blocking primer has a 3′ inverted dT modification that when used with the Stoffel Taq fragment prevents amplification of nuclear cytochrome b pseudogenes in humans and allows for the continued use of cytb in BMA studies, as it is one of the most species-rich loci in GenBank. We used our assay to examine 164 blooded specimens of *Ae. albopictus* from suburban coastal New Jersey and found 62% had obtained blood from humans with 7.6% mixes between human and another mammal species. We also confirmed the efficiency of our assay by comparing it with standard BMA primers on a subset of 62 blooded *Ae. albopictus*. While this assay was designed for use in *Ae. albopictus*, it will have broader application in other anthropophilic mosquitoes.

1. Introduction

Aedes albopictus (Skuse), the Asian tiger mosquito, is native to Eastern Asia but has successfully expanded its range throughout much of the world especially into urban and suburban areas in both tropical and temperate regions (Benedict et al., 2007). Although on occasion an important vector of dengue to humans (Effler et al., 2005) and dog heartworm to dogs (Gratz, 2004), until recently this species was mainly considered to be a nuisance since it is a very aggressive human biter. After a single basepair mutation in the chikungunya virus (CHIKV) increased the vector competence of *Ae. albopictus* for the virus (Ng and Hapuarachchi, 2010; Tsetsarkin and Weaver, 2011) this mosquito has become the principal vector of the chikungunya epidemic occurring in Africa and the Indian Ocean Basin since 2004 (Enserink, 2007). Although chikungunya fever has not spread broadly in the temperate zone, an epidemic in northern Italy in 2007 sickened over 200 people (Moro et al., 2010) and small numbers of locally transmitted chikungunya fever cases have been identified in southern France since 2010 (Grandadam et al., 2011). The European expansion of CHIKV would not have been possible without the invasion of that continent by *Ae. albopictus* (Lo Presti et al., 2012).

Since the bloodfeeding habits of mosquitoes play a role in determining the vectorial capacity of pathogens to wildlife and humans, it is especially important to assay what proportion of their meals are human-derived, or how anthropophilic they are, because this translates directly to their potential role in disease epidemics. Further, for invasive mosquitoes such as *Ae. albopictus* bloodfeeding patterns are critically important in understanding how they may alter disease transmission pathways and trigger epidemics in new areas.

There are multiple techniques used for identifying blood meals in mosquitoes and other hematophagous arthropod species (see Kent, 2009 for a review) but the most recent methods use blood meal DNA. In this technique blood meal DNA is extracted,

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amplified, and sequenced with vertebrate-specific primers on multiple copy loci. By far the most commonly used locus has been the cytochrome b mitochondrial locus (Kent, 2009). Cytochrome b primers used in blood meal analysis include the primer pairs Mammal A (Ngo and Kramer, 2003), Avian A (Cicero and Johnson, 2001), L14841/H15149 (or BM, Kocher et al., 1989), Mammal B (Molaei and Andreadis, 2006), and reptilian/amphibian primers (Cupp et al., 2004), among others (Kent, 2009). The sequences obtained are then compared to other sequences of known origin in a GenBank BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), where species with >98% similarity to the query are identified as the source of the blood meal (Kent, 2009).

This technique has several disadvantages. First, having to sequence every sample is time- consuming and costly, and costs are compounded in the case of mixed blood meals (when the mosquito carries blood from more than one host) which have to be cloned and then sequenced. Second, this technique is prone to error when no reference sequence for the target locus exists in GenBank for the species being queried. A third problem is amplification of nuclear mitochondrial pseudogenes (numts), which are portions of the mitochondrial genome that have been copied into the nuclear genome (Zhang and Hewitt, 1996). Without careful analysis to avoid this problem the primers in use may amplify one or more nuclear pseudogenes of the mitochondrial locus, which may differ significantly from the mitochondrial sequence of that species in GenBank and may lead to misidentification of species. Pseudogene amplification is already known to complicate the use of mitochondrial DNA in phylogenetic and population genetic studies (Bensasson et al., 2001), including in mosquitoes (Hlaing et al., 2009). A fourth and final problem is that primers developed to work in one mosquito or arthropod species may not work in another: we found that several primers used successfully in other species of mosquitoes (specifically, the BM and Mammal B primers) were a perfect match for *Ae. albopictus* cytochrome b, and thus consistently amplified the more abundant mosquito DNA and not the blood meal DNA even after careful dissection and extraction of just the blood.

To address problems of pseudogene contamination in our assay, we used a technique first developed by Yu et al. (1997) which specifically inhibits a particular sequence of DNA from amplifying in a PCR reaction, called PCR blocking. PCR blocking has been applied for a variety of uses particularly in clinical studies (e.g. to detect the presence of mutations associated with disease) and more recently in ecological feeding studies (to block predator DNA allowing identification of its prey) (Chow et al., 2011; Deagle et al., 2009; Vestheim and Jarman, 2008).

The primary objective of this study was to develop a technique to allow easy identification in *Ae. albopictus* of human blood meals thus significantly reducing time and costs of blood meal analysis in this anthropophilic species. Secondarily, our strategy also allows the rapid identification of mixed blood meals involving human blood and non-human (but mammalian) blood without extensive sequencing. To do so, and because of the pervasive and disruptive presence of many human mitochondrial nuclear pseudogenes, we developed and optimized a new strategy for blood meal identification using blocking primers. We focused on mammalian blood because previous studies have shown that *Ae. albopictus*, like many invasive *Aedes* mosquitoes, feeds predominantly on mammals (Delatte et al., 2010; Ponlawat and Harrington, 2005; Richards et al., 2006; Valerio et al., 2008). To assess the accuracy of our rapid assay we examined blooded *Ae. albopictus* from suburban coastal communities in New Jersey, a U.S. state on the northernmost range of the distribution of this species across the world, using both our approach and standard assays.

2. Methods

We originally thought of optimizing a PCR multiplex for identification of particular species in mosquito blood meals similar to those developed to pick out major groups of birds (Ngo and Kramer, 2003) or identify an assortment of animals commonly present in a Kenyan village dwelling (Kent and Norris, 2005; Kent et al., 2007). However, we quickly realized from published blood meal analysis studies (Delatte et al., 2010; Ponlawat and Harrington, 2005; Richards et al., 2006; Valerio et al., 2008) as well as from our own preliminary results that *Ae. albopictus* often targets a broad range of blood hosts especially in suburban areas. Therefore we decided to develop a methodology focusing on the rapid identification of human blood meals (alone or mixed) that, however, also allowed us to amplify and identify by sequencing a broad array of possible mammalian (and as it turned out, also marsupial) targets.

2.1. Study site and collections

Monmouth County is located in central and eastern New Jersey, USA and we obtained specimens from municipalities located along the Raritan Bay specifically in Aberdeen, Keyport, Union Beach, Keansburg and Middletown. The sites we targeted are largely sub-urban, with residential housing interspersed with forest remnants (for detailed site information, see Unlu et al., 2011). As a result, prospective blood meal sources include the common suite of northeastern US suburban mammal and marsupial species, such as Virginia opossum (*Didelphis virginianus)*, white-tailed deer (*Odocoileus virginianus)*, eastern grey squirrel (*Sciurus carolinensis)*, domestic dog (*Canis domesticus)*, domestic cat (*Felis catus domesticus*), and of course, humans (*Homo sapiens*). We chose to test our assay on samples from suburban sites because we were interested in the behavior of *Ae. albopictus* in non-urban environments where human density is lower and they have a broader diversity of available sources of blood.

We collected blooded *Ae. albopictus* with BioGents Sentinel (BGS) traps (Biogents, AG, Regensburg, Germany) equipped with the BG-Lure (AgriSense, Pontypridd, South Wales, UK), which were used for surveillance within a larger project aimed at developing methods to control this nuisance mosquito (Unlu et al. 2011). While these traps are designed to capture questing females, they also yielded a small number of blooded *Ae. albopictus*, which were perhaps using them as sheltered resting areas. Between 60 and 72 BGS traps were deployed weekly in Monmouth County sites during the active seasons of 2008–2011 for a cumulative total of 5433 trap days yielding 164 blooded *Ae. albopictus* specimens. After the BGS catches were brought back to the lab, blooded *Ae. albopictus* were placed into individual microcentrifuge tubes and stored in a −80 °C freezer until extracted. Both because of the large volume of traps deployed and because the BGS collections were not performed specifically to capture blooded *Ae. albopictus*, specimens spent up to 24 hours in traps in the field and often died during this time period and/or became wet, likely impacting our ability to obtain good DNA from them.

2.2. Primer design

Cytochrome b sequences of *Didelphis virginianus*, *Odocoileus virginianus, Sciurus carolinensis, Canis domesticus,* and *Felis catus* were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and used as nonhuman targets in designing our primers. We also obtained sequences of *Homo sapiens* and *Ae. albopictus* cytochrome b from GenBank to aid in primer design, as well as control DNA

Table 1
Sequences of primers used to amplify a segment of cytochrome b in an assay developed for blood meal identification in *Aedes albopictus*. CytbVertR1 and UnvRev1C are used to produce a 571bp product in the external PCR amplification, followed by the internal multiplexed PCR with Human741F, NonHumanF1C, PossumSp1, UnvRev1C and NewPseudBlckdT.

Primer name	Sequence (5′–3′)	Product size w/UnvRev1C	w/NonhumanF1C
CytbVertR1	GGACGAGGACTATACTACGG	571	–
Human741F[a]	GGCTTACTTCTCTTCATTCTCTCCT	175	–
NonhumanF1C	GGGMTTCTCMGTWGAYAAAG	368	–
PossumR1	TGTGAGATGGGTCGGAATGC	–	468
UnvRev1C	CTAGTTTGTTAGGGATGGAKCG	–	368
NewPseudBlckdT	CGTAAGATTGCRTATGCAAACAA	–	–

[a] Primer from Kent and Norris (2005).

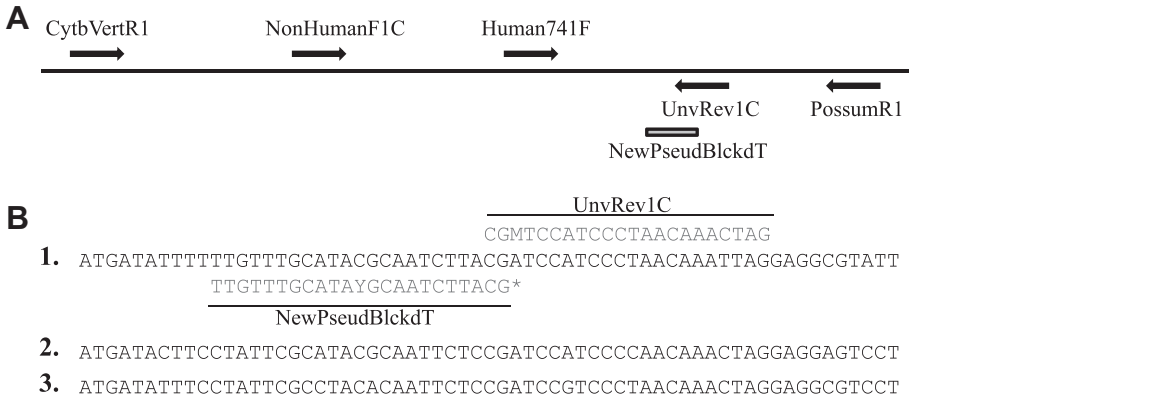

Fig. 1. (A) Schematic representation of assay depicting relative locations of primers. Arrows indicate direction of amplification. (B) Close-up of area where blocking primer anneals. Contains aligned sequences of cytochrome b from (1) human nuclear copy on Chromosome 1, (2) a nonhuman mammal, cat, and (3) human mitochondrial DNA, and binding sites of the universal primer (UnvRev1C) and blocking primer (NewPseudBlckdT). The sequence "TTGTTT" for which the blocking primer was designed is unique to all pseudogenes requiring blocking, not just chromosome 1, and is distinct from the sequences of nonhuman mammals and human mitochondrial DNA, ensuring it will only anneal to and block the pseudogenes. The asterisk (*) indicates the location of the inverted dT 3′ modification that prevents extension of the blocking primer.

samples of all species from local veterinarians, hunters, road kills, and human volunteers to use for optimization purposes.

We employed a nested PCR design to enrich the DNA concentration of the region of interest and prevent multiplex primers from annealing elsewhere in the genome. First, external primers CytbVertR1 and UnvRev1C amplify a 571bp fragment (Table 1, Fig. 1A). Utilization of an external forward primer distinct from the internal forward primers prevents issues with contamination due to the nested design. Next, in the internal multiplex, one primer amplifies in humans only (Human741F, developed by Kent and Norris, 2005); another amplifies only in nonhuman mammals (NonHumanF1C); and the reverse primer is universal (UnvRev1C) (Table 1, Fig. 1A). A second reverse primer specific to opossum (PossumR1) amplifies with NonHumanF1C (Table 1, Fig. 1A). All primers other than Human741F were developed for this study. The assay results in the amplification of a small (175bp) band if the blood meal was human-derived, a larger band (368bp) if the blood meal was from a nonhuman mammal, an even larger band (468bp) if the blood meal was from an opossum, and multiple bands if the blood meal contained a mix of human and nonhuman, opossum and nonhuman, opossum and human blood, or even one of each (Fig. 2). An opossum-specific primer was needed because UnvRev1C did not amplify well in opossum, as this marsupial has a somewhat different sequence from the mammals at the primer site. However, researchers not interested in opossum blood meals may omit this primer from the multiplex. The small size of the human-only band (175bp) increases the likelihood of amplification in more degraded blood meals (shorter fragments are detectable over a longer time course, as illustrated by Fornadel et al., 2010). All primers were explicitly designed not to amplify in *Ae. albopictus*. As mentioned, our primers target mammalian blood meals, which means that blood meals from avian or reptilian/amphibian sources will be missed with our methodology.

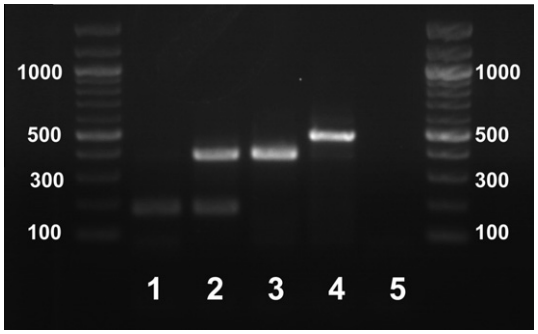

Fig. 2. Gel visualization of assay on some control DNA samples. Lane 1 is human (175bp); lane 2 is a mix between human and dog (175bp and 368bp); lane 3 is squirrel (368bp); lane 4 is opossum (468bp); and lane 5 is the negative control. Outside lanes are 100bp ladder (lines are 100bp apart, starting at 100bp as indicated).

2.3. Blocking technique

During primer design and optimization, the universal primer UnvRev1C, which was specifically designed not to amplify in human *mitochondrial* cytb, was found to amplify several human *nuclear* cytochrome b pseudogenes. This resulted in amplification of both the nonhuman and human bands in controls that contained only human DNA, and thus potential misidentification of human meals as mixes. To prevent this, we implemented a PCR blocking technique following Yu et al. (1997). This technique utilizes a

Table 2
List of *Homo sapiens* nuclear pseudogenes of cytochrome b identified in the course of this study, with GenBank accession number, annotation info, and whether or not they matched the primers well enough to require blocking. Accession numbers whose GenBank annotation identifies the sequence as containing a pseudogene are bolded.

H. sapiens chromosome #	Accession #	Annotation	Blocker required?
1	AL590396.13	Sequence from clone RP11–193H5 on chromosome 1 Contains...a cytochrome b (MTCYB) pseudogene	*Y*
2	**AC062016.5**	BAC clone RP11–59P4 from 2	*N*
4	**AC110766.5**	BAC clone RP11-93M12 from 4	*N*
5	AF196982.1	Cytochrome b pseudogene	*Y*
6	NG_032377.1	MT-CYB pseudogene 4 (MTCYBP4) on chromosome 6	*N*
6	AL356774.13	sequence from clone RP11–203B4 on chromosome 6 Contains a cytochrome b (MTCYB) pseudogene	*N*
6	AL078595.12	sequence from clone RP3–399J4 on chromosome 6q15–16.3 Contains a cytochrome B pseudogene	*N*
7	**AC083875.1**	chromosome 7 clone RP11–699P13	*N*
8	**AC022861.4**	chromosome 8, clone RP11–343B22	*Y*
11	**AP000676.6**	chromosome 11 clone:RP11–665E10	*Y*
13	NG_032321.1	MT-CYB pseudogene 3 (MTCYBP3) on chromosome 13	Y
15	**AC066616.7**	chromosome 15 clone RP11–925D8 map 15q21.3	*N*
16	**AC007595.9**	chromosome 16 clone RP11–109M19	*Y*
22	Z95114.19	sequence from clone CTA-212A2 on chromosome 22q12 Contains the gene for cytochrome b (CYTB) pseudogene	*N*
X	AL158141.14	sequence from clone RP11–351K23 on chromosome *X* Contains ...a cytochrome b family pseudogene	*N*
X	AL391375.11	sequence from clone RP11–375A20 on chromosome *X* Contains a cytochrome b (MTCYB) pseudogene	*N*
X	AL590407.8	sequence from clone RP11–522L3 on chromosome *X* Contains...a mitochondrial cytochrome B (MTCYB) pseudogene	*N*
Y	NG_023458.1	MT-CYB pseudogene 1 (MTCYBP1) on chromosome *Y*	*N*

special oligonucleotide that precisely matches the sequence targeted for PCR inhibition with the exception of a 3′ modification that prevents its extension. We chose to use an inverted dT modification as it was the most cost-effective of the three best 3′ modifications as tested by Dames et al. (2007). While not all blocking approaches require the Stoffel fragment, we found it greatly improved blocking efficacy in our study. The Stoffel fragment of Taq polymerase is an enzyme missing a stretch of amino acids at the N-terminus resulting in a lack of exonuclease activity and higher specificity than regular Taq polymerase (Lawyer et al., 1993).

We designed NewPseudBlckdT (Table 1) to anneal to human cytb pseudogenes at the UnvRev1C binding site and block annealing and extension by this primer, while being unable to be extended itself due to the inverted dT and use of Stoffel Taq (see Fig. 1B). To design the blocking primer, we obtained pseudogene sequences from GenBank both by searching in the nucleotide database using the string "cytochrome b pseudogene AND (homo sapiens[Organism])" and by performing BLAST searches on pseudogene sequences amplified by our primers. In total, we identified 18 different nuclear copies of cytochrome b in the human genome (Table 2) including seven not yet annotated in GenBank as such. However, the majority of the pseudogenes did not match one or more of our blood-meal primers, so we designed a blocking primer matching six pseudogenes on chromosomes 1, 5, 8, 11, 13, and 16. The blocking primer targets an area where the nuclear copies differ from mitochondrial DNA sequences and overlaps with UnvRev1C by two basepairs at the 3′ end preventing it from annealing and extending (Fig. 1B).

2.4. Optimization and testing

The assay was optimized using the following control samples: deer, dog, cat, and human blood; human hair follicle; squirrel ear tissue; opossum hair follicle; and larval (non-bloodfed) *Ae. albopictus* mosquitoes obtained from a Rutgers Center for Vector Biology colony. Control samples were extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen Sciences, Germantown, MD) and yielded between 10 and 100 ng/μl DNA. Optimized final concentrations were as follows, for the external reaction: 1× PCR Buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KCl), 2.5 mM $MgCl_2$, 200 μM each dNTP, 50 nM each primer (CytbVertR1 and UnvRev1C), and 1 unit of AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA) along with 2 μl template DNA in a 20 μl reaction. The assay was optimized to run at an initial denaturing temperature of 95 °C for 3 min 50 s, followed by 20 cycles of a denaturing step of 95 °C for 40 s, an annealing step of 59 °C for 50 s, and an extension step of 72 °C for 40 s, with a final extension of 72 °C for 5 min. The internal multiplex was run with final concentrations of 1× Stoffel PCR Buffer (10 mM Tris-HCl, pH 8.3, and 10 mM KCl), 2.5 mM MgCl2, 200 μM each dNTP, 150 nM each of Human741F and PossumR1, 500 nM of NonHumanF1C, 300 nM of Unrev1C, 1200 nM of Pseudblck1, and 2 units of AmpliTaq DNA Polymerase, Stoffel Fragment (Applied Biosystems, Foster City, CA) along with 1 μl PCR product in a 20 μl reaction. Internal PCR reactions were carried out with an initial denaturing temperature of 95 °C for 5 min, followed by 35 cycles of a denaturing step of 95 °C for 40 s, an annealing step of 59 °C for 40 s and an extension step of 72 °C for 40 s, with a final extension of 72 °C for 7 min. All PCRs were run on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Life Technologies, Carlsbad, CA) and visualized with Ethidium Bromide on a 1% agarose gel. DNA fragments matching the expected size of the nonhuman mammal band were purified directly with Exo-Sap-IT (USB Products, Cleveland, OH) or, in the case of a mixed meal that needed to be excised from the gel, with a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Cleaned PCR products were cycle-sequenced with NonHumanF1C and run on capillary automated sequencers. The resulting sequences were compared to known sequences in a GenBank BLAST search to determine their most likely origin. To test the pseudogene blocking primer, we created artificial mixed meals by mixing together 1 μl (33 ng/μl) of control human DNA with 1 μl (51.8 ng/μl) of control dog DNA and, in a separate reaction, 1 μl (33 ng/μl) of control human DNA with 1 μl (14.3 ng/μl) of control cat DNA. These artificial mixes were then run with our assay as above, and the nonhuman bands were excised from the gel, gel extracted, and sequenced, and the chromatograms were examined for evidence of pseudogene contamination. In addition, we conducted tests of the efficacy of our primers on mixed DNA samples by combining equal amounts of human and cat DNA in the following ratios (Human: Cat): 10:90, 25:75, 40:60, 50:50 (14.3 ng/μl of each), 60:40, 75:25, 90:10. We also made serial dilutions in water (1:5, 1:10, 1:20, 1:50, 1:100, 1:200) of the 50:50 mix of human and cat DNA

to examine the sensitivity of the assay. In all PCRs, 2 μl of DNA was tested with our assay to uncover differences in the likelihood that each blood source (human vs. non-human) would be detected.

Following optimization on control DNA samples, we used the assay to identify the blood sources of 164 blooded *Ae. albopictus* specimens. Blooded *Ae. albopictus* abdomens were dissected out from each sample then extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and re-suspended in 60–100 μl of buffer AE (Qiagen) depending on the visual size of the blood meal. A subset of these samples, those collected in 2008 ($N = 62$), were also tested with Mammal A primers (Ngo and Kramer, 2003) to compare with our assay. Of the 62, several samples that did not amplify with Mammal A were run with Mammal B (Molaei and Andreadis, 2006) and BM (Kocher et al., 1989) primers, but use of these two primer sets was soon suspended due to amplification of *Ae. albopictus* DNA. Therefore we switched to using Reptile/Amphibian primers as these amplify not only reptiles and amphibians but also some bird and mammal species (Cupp et al., 2004). The number of samples identified after using these four primer pairs was compared to the number identified from our assay. All 62 samples were also tested with Avian A primers (Cicero and Johnson, 2001) to further ensure we would not miss any bird blood meals due to the mammal specificity of our primers. To confirm that the human and opossum specific primers amplify correctly in field-collected specimens, we sequenced the resulting bands from several specimens using UnvRev1C.

3. Results

The assay successfully amplified on control samples of human, dog, cat, squirrel, deer, and opossum DNA and allowed easy identification of human vs. nonhuman mammalian or opossum blood meals without need for cloning (as in Fig. 2). As intended, our primers do not amplify *Ae. albopictus* DNA. The artificial mixes used to test the efficacy of the blocking primer when sequenced produced clean chromatograms of the nonhuman mammal sequence alone. When the blocking primer was not used, the chromatograms showed a mix of several sequences. This indicates the blocker is successful at preventing pseudogenes from amplifying. The mixes created in different ratios for evaluating primer efficacy indicated that our assay amplified control human and cat DNA at every relative concentration level, including the lowest levels of 90:10 cat:human and 10:90 cat:human. Furthermore, even 1:200 dilutions of the mix of both DNA types (down to concentrations of 0.075 ng/μl) resulted in the amplification of both bands. These DNA concentrations are much lower than those we recovered from a volume of vertebrate blood roughly equivalent to that found in a blooded *Ae. albopictus* (approximately 1 ng/μl). We found no evidence that the 193bp difference between the human and non-human products affected the likelihood of their amplification.

Examination of the 164 field collected blooded *Ae. albopictus* yielded 79 successful blood meal identifications (48.2%): 55 humans, 12 domestic cats, 8 domestic dogs, 3 Virginia opossum, 3 eastern gray squirrel, 2 eastern cottontail (rabbit), 1 white-footed mouse, and 1 white-tailed deer (Fig. 3). Mixed meals between human and another mammal accounted for 7.6% of those identified. When a subset of 62 specimens was examined with 4 standard cytb primer pairs, 33 samples were identified to species (53.2%) including cat, human, opossum, dog, and squirrel. Our assay identified 39 samples to species (62.9%), adding a blood meal from deer and 6 mixed blood meals that were not identified by the other primer pairs since we did not clone mixed blood meals. Importantly, we only had to sequence 16 fragments as opposed to 33 and immediately identified mixes of human and non-human blood, while identification of those mixes with the other methods would have involved cloning.

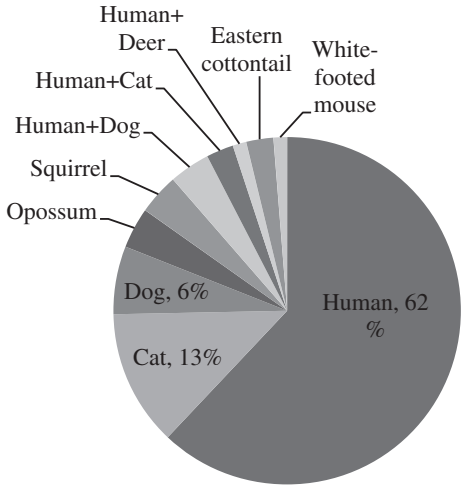

Fig. 3. Sources of blood from 79 blooded *Aedes albopictus* collected in coastal Monmouth County, NJ, USA from 2008 to 2011. Labels with more than one name, e.g. "Human+Dog" indicate a mixed blood meal containing DNA from both sources.

4. Discussion

We developed a mixed strategy that reduces costs while maximizing generality and likelihood of identification of critical mammalian blood meal hosts. When compared with standard blood meal primer sets, our assay was as effective as others while requiring significantly less time and cost. Although our approach still requires cloning in the case of mixes between two different nonhuman mammals, sequencing costs and effort are reduced by a large margin since all sequencing of human DNA (found in 69.6% of the specimens examined) was eliminated. Our approach also allows the rapid identification of mixed meals between humans and nonhuman mammals, which can have important implications for understanding disease transmission pathways. Additionally, though our assay was not optimized using DNA from white-footed mouse or eastern cottontail, these species were amplified from field-collected specimens indicating the assay works for a wide range of mammal species. However, as we have not exhaustively tested our primers on all mammal species, it is strongly recommended that researchers working with very different suites of mammal fauna test the assay on control samples of species likely to be found in their study sites.

Our study underlines a serious problem with blood meal analysis methods as they are currently carried out—pseudogenes. We report amplification of several human nuclear cytochrome b pseudogenes with primers designed specifically to amplify in nonhuman mammals. This is not surprising given that the entire human mitochondrial genome has been shown to be integrated into the nuclear genome (Parr et al., 2006). Some of these pseudogenes were remarkably similar to the mitochondrial cytochrome b sequences of nonhuman mammals, and quite distinct from the mitochondrial sequence of humans, particularly at the primer sites used. Further, any attempt to make NonHumanF1C or UnvRev1C more degenerate to engender amplification in a wider array of nonhuman species (such as marsupials or birds) caused them to amplify even more human cytochrome b pseudogenes than the six matched at present. The issue of pseudogenes is a serious one whenever mitochondrial DNA is being amplified, whether in studies of population genetics, phylogenetics or blood meal analysis

(Zhang and Hewitt, 1996). Even when using our assay it is possible to encounter nonhuman mammal pseudogenes that masquerade as mtDNA from a different nonhuman mammal, as our blocker only removes human pseudogenes. Proper steps must be taken to ensure that the sequence obtained is truly mitochondrial in origin (e.g., comparison with control DNA samples and high match cut-offs) and not from a pseudogene, to avoid incorrect conclusions.

Previous studies have used PCR blocking oligos for ecological purposes such as blocking study organism DNA when assessing its stomach contents (Chow et al., 2011; Deagle et al., 2009; Vestheim and Jarman, 2008), blocking contamination from modern humans when working with ancient DNA (Boessenkool et al., 2012; Gigli et al., 2009), and identifying the bacterial community within algal cultures (Powell et al., 2012). Blocking approaches have also been successfully used in clinical research: to target rare mutant sequences associated with disease (Dominguez and Kolodney, 2005; Lee et al., 2011; Morlan et al., 2009), prevent competition with probe binding in DNA chip hybridization assays (Tao et al., 2003), allow sequencing of hairpin loops (Esposito et al., 2003), and block competing rRNA sequences during RNA virus discovery (de Vries et al., 2011). Our analysis represents a novel application of this technique, used here to specifically block nuclear pseudogenes and prevent incorrect species identifications when working with mitochondrial DNA.

An obvious shortcoming of our assay is that it currently does not identify avian, reptilian or amphibian blood meals, however our comparative analyses using primers that target such blood sources indicate that *Ae. albopictus* does not derive a significant proportion of blood meals from these species in the suburban sites we examined in New Jersey. In locations where non-mammalian vertebrates may be considered an important source of blood, DNA from blooded *Ae. albopictus* should be tested with other primers as well. For this purpose we recommend screening samples that did not amplify in our multiplex with the 'Reptile/Amphibian' primers developed and used by Cupp et al. (2004) or with 'Avian A' primers first published by Cicero and Johnson (2001) for use in avian phylogenetics and subsequently used by Molaei et al. (2008) and Hamer et al. (2009) in blood meal analyses. These two sets of primers due to the species groups they target form a nice complement to the mammalian primers in this assay and we have confirmed they do not amplify *Ae. albopictus* DNA.

Our success in identifying blood meals in *Ae. albopictus* samples was relatively low (48%) compared to other studies that often identify closer to 80–90% when using enzyme-linked immunosorbent assay (ELISA) tests (Kamgang et al., 2012; Niebylski et al., 1994; Ponlawat and Harrington, 2005; Richards et al., 2006; Savage et al., 1993) although success is more variable when using PCR based methods (from 17.4%, Munnoz et al., 2011, to 92.3%, Sawabe et al., 2010). Considering how proficient our assay is at amplifying small amounts of DNA, either human or non-human, this difference likely reflects DNA degradation due to the way in which these samples were obtained, as mentioned earlier. Blood meals are notorious for degrading quickly and even a delay of a few hours may be the difference between an identifiable blood meal and one that will not amplify (Fornadel and Norris, 2008). Importantly, in our tests our rapid assay was able to identify a larger number of blood meals than other primer sets, particularly Mammal A. Part of the reason for this difference is likely the comparatively small product sizes we are targeting, as smaller products are detectable over a longer time course of degradation than longer ones (Fornadel and Norris, 2008): in our assay the human band is only 175bp and the nonhuman band 368bp, compared to the target size of 772bp with Mammal A primers.

Based on our results we are able to conclude that *Ae. albopictus* is primarily targeting mammalian species in suburban NJ, and in particular, that over 2/3 of its blood meals are derived from humans (Fig. 3). This is largely consistent with its feeding habits elsewhere in the world, where it has been observed to feed mainly on humans and other mammals (Niebylski et al., 1994; Richards et al., 2006; Savage et al., 1993; Sawabe et al., 2010) and in some cases, overwhelmingly on humans (Kamgang et al., 2012; Munnoz et al., 2011; Ponlawat and Harrington, 2005). Therefore despite the fact that our primers favor identification of human meals in very dilute or degraded samples, our results are in line with other studies indicating that this bias will not impact the usefulness of our assay. In fact, *Ae. albopictus* females consistently preferred to feed on humans over other domestic mammal species in laboratory choice experiments (Delatte et al., 2010) although in the field they appear to be more opportunistic, shifting from deriving nearly all their meals from humans in heavily human-dominated urban centers to obtaining only a quarter to a half of their meals from humans in more rural areas (Valerio et al., 2008). This is consistent with our finding that *Ae. albopictus* food choices in suburban NJ reflect a high density of humans and their associated companions (dogs and cats). In these areas forest fragmentation has greatly decreased the number of wild species and thus *Ae. albopictus* as an opportunistic feeder obtains fewer meals from them. A perhaps surprising outcome is the lack of any avian-derived meals among our samples, despite screening with Avian primers. Prior studies have found *Ae. albopictus* to feed on birds albeit at very low frequencies (Niebylski et al., 1994; Richards et al., 2006; Savage et al., 1993; Sawabe et al., 2010; Valerio et al., 2008). One reason for this may be the method of collection, as the majority of these studies used aspiration to collect blooded females off of vegetation and resting areas, whereas our blooded samples were obtained via BGS traps, which sit on the ground. Further studies using our blood meal assay are being developed to address this question.

In addition to a better understanding of the bloodfeeding habits of *Ae. albopictus* for ecological and disease transmission purposes, our assay may also be used to test the efficacy of various anti-mosquito measures. For example, Fornadel et al. (2010) examined the efficacy of insecticide-treated bed nets at reducing the proportion of human blood meals taken by *Anopheles gambiae* in Africa, using the multiplex designed by Kent and Norris (2005). Future studies could investigate other aspects of mosquito control, such as source reduction or avoidance education aimed at homeowners and the general public. A significant decrease in the proportion of human blood meals following execution of these measures would provide substantial support for their implementation. Our rapid blood meal assay is highly suited for such an application as we are able to detect human DNA even in low amounts and with relatively less time and expense.

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