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IDENTIFICATION OF MOSQUITO AVIAN-DERIVED BLOOD MEALS BY POLYMERASE CHAIN REACTION-HETERODUPLEX ANALYSIS

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Abstract. A polymerase chain reaction (PCR) heteroduplex assay (HDA) was developed to identify avian derived mosquito blood meals to the species level. The assay used primers amplifying a fragment of the cytochrome B gene from vertebrate but not invertebrate species. In *Culex tarsalis* fed on quail, PCR products derived from the quail cytochrome B gene were detected seven days post-engorgement. In an analysis of wild-caught mosquitoes, 85% of blood-fed mosquitoes produced detectable PCR products. Heteroduplex patterns obtained from bird-derived PCR products were found to permit the unambiguous identification of all species examined. No intraspecific variation in HDA patterns was found. The PCR-HDA was used to characterize blood meals in wild caught *Cx. tarsalis.* Of the 67 blood meals analyzed, 60% were derived from avian sources. Of the avian blood meals, 65% were derived from a single host, the common grackle.

INTRODUCTION

Eastern equine encephalomyelitis virus, Saint Louis encephalomyelitis virus, and western equine encephalomyelitis virus are important mosquito-transmitted pathogens of humans, equines, and game birds in North America.[1–3] These viruses all exhibit similar life histories. They are maintained primarily in an enzootic cycle of infection in local bird populations. Within the bird populations, transmission is primarily carried out through several ornithophilic mosquito species. However, the virus can also be transmitted by other mosquitoes that are more catholic in their feeding habits. These so called bridge vectors permit the virus to escape the enzootic cycle maintained in the avian population and begin infecting mammals, including humans. Despite the fact that the basic outlines of this process are understood, many aspects of the ecology of these viruses remain unclear. These include the relative role that different bird species play in the maintenance of the virus, the factors that result in enzootics, and the environmental conditions that allow the virus to escape the enzootic cycle and infect vertebrates, including humans.

Blood meal identification of mosquitoes provides information on host-feeding preferences or host-feeding patterns of mosquitoes in nature. Such information may provide indirectly data indicating what reservoirs are significant in vector-borne diseases.[4–6] Studies on blood meal identification of hematophagous insects have historically exploited antigen-antibody reaction based assays that require polyclonal antibodies raised against blood components from potential hosts.[7–11] Preparation of antibodies against each potential host is often laborious and difficult, and pre-adsorption steps are often necessary to prevent cross-reactions.[8] Furthermore, despite extensive pre-absorption steps, antibody-based assays are often capable of identifying blood meals only to the order level. Thus, these assays cannot be used to study the feeding profiles of ornithophilic mosquitoes to the species level.

Recently, Boakye and others developed a polymerase chain reaction (PCR)-heteroduplex assay (HDA) to distinguish blood meals derived from several species of vertebrates.[12] This assay was based upon specific amplification of the blood meal-derived cytochrome B sequences, followed by analysis of the heteroduplex products by heteroduplex analysis. The assay was capable of distinguishing vertebrate blood meals in black flies and tsetse flies at least to the genus level. Here we present a modification of this assay applicable to North American mosquitoes that is capable of identifying avian blood meals to the species level.

MATERIALS AND METHODS

Sample collection. Blood samples for the PCR-HDA were obtained from 16 avian species, including northern cardinal *(Cardinalis cardinalis),* house finch *(Carpodacus mexicanus),* American goldfinch *(Carduelis tristis),* eastern towhee *(Pipilo erythrophthalmus),* white-throated sparrow *(Zonotrichia albicolis),* chipping sparrow *(Spizella passerina),* field sparrow *(Spizella pusilla),* swamp sparrow *(Melospiza georgiana),* blue grosbeak *(Guiraca caerulea),* red-winged blackbird *(Agelaius phoeniceus),* brown-headed cowbird *(Molothrus ater),* orchard oriole *(Icterus spurius),* Carolina chickadee *(Poecile carolinensis),* tufted titmouse *(Baeolphus bicolor),* blue jay *(Cyanocitta cristata),* and house sparrow *(Passer domesticus).*

Laboratory-reared nulliparous mosquitoes *(Culex pipiens* and *Cx. tarsalis)* and *Cx. tarsalis* fed on northern bobwhite *(Colinus virginianus)* were held at 25°C and 80% relative humidity for different periods following engorgement. These were provided by Dr. Wayne A. Rowley (Department of Entomology, Iowa State University, Ames, IA).

Wild-caught, blood-fed mosquitoes of various species were collected from resting boxes placed at sites in northeastern Mississippi and central Alabama in March-May 2001. Engorged *Cx. tarsalis* were collected from Centers for Disease Control and Prevention (CDC) miniature light traps baited with CO_2. *Culex tarsalis* collections were made bi-weekly from June through September during the period 1996–2000 at six sites in Goshen County, Wyoming and Scotts Bluff County, Nebraska. Mosquitoes collected were anesthetized with CO_2 and either frozen on dry ice and returned to the laboratory, or returned live to the laboratory. The mosquitoes were examined under a dissecting microscope to identify those that had taken a blood meal and the stage of blood meal digestion (e.g., fully blooded, partially blooded, or gravid). The DNA was then extracted from the blood-fed mosquitoes

as described in the next section. Mosquitoes from Alabama and Mississippi were used in studies of the sensitivity of the blood meal PCR, while the *Cx. tarsalis* from Nebraska and Wyoming were used in the HDA identification studies described in this report.

Extraction of DNA. Genomic DNA from avian blood samples and blooded mosquitoes was extracted with DNAzol-BD (Molecular Research Center, Cincinnati, OH). Briefly, specimens (5 μl of avian blood or individual mosquitoes) were disrupted by mechanical homogenization in 200 μl of DNAzol-BD solution. The DNA was precipitated by the addition of 80 μl of isopropanol followed by centrifugation at 6,000 × g for 6 min. The pelleted DNA was washed in 100 μl of DNAzol-BD and re-pelleted. The re-pelleted DNA was washed with 95% ethanol and centrifuged at 2,000 × g for 5 min. The DNA was resuspended in 100 μl of 10 mM Tris-HCl (pH 8.0), 1m M EDTA (TE).

Polymerase chain reaction-heteroduplex analysis. The PCR amplifications were conducted in 50 μl of a solution containing 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, 1.25 units of Taq DNA polymerase (Roche Biochemicals, Indianapolis, IN), and 1 μl of DNA template. The sequence of the primers used in the PCR were as follows: 5′-CCCCTCAGAATGATATTTGTCCTCA-3′ and 5′-CCATCCAACATCTCAGCATGATGAAA-3′. Reactions began with an incubation at 95°C for 3.5 min, followed by 36 cycles consisting of 30 sec at 95°C, 50 sec at 60°C, and 40 sec at 72°C. The reaction was completed by incubation at 72°C for 5 min. The products were analyzed using a modification of the heteroduplex protocol for the identification of blood meals in black flies.[12] Briefly, equal volumes of the sample and driver (either northern cardinal or Carolina chickadee) PCR products were mixed with 8 μl of TE and overlaid with 10 μl of mineral oil. The mixture was denatured at 99°C for 2.5 min and allowed to form heteroduplex products by slow cooling to room temperature. An aliquot (14 μl) of each heteroduplex solution was mixed with 6 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). A total of 10 μl of this mixture was loaded onto a 5% polyacrylamide/urea gel (29 acrylamide:1 bisacrylamide, 1 M urea) prepared in 108 mM Tris-boric acid (pH 8.3), 2.7 mM disodium EDTA. Electrophoresis was conducted on 20 cm × 20 cm Protean II Xii system (Bio-Rad, Hercules, CA) at 12 mA per gel for 18 hr in 89 mM Tris-boric acid (pH 8.3), 2.5 mM disodium EDTA. Gels were stained in 2 μl/ml of ethidium bromide and homoduplex and heteroduplex patterns were visualized under UV light. Samples were identified on the basis of a comparison of the relative mobility of the HDA bands with those of the standard samples derived from avian blood samples. In some cases, samples did not produce detectable products in the HDA, or produced an HDA pattern that did not match any of the standards. The PCR products from such samples were purified using the QiaAMP PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's instructions, and subjected to direct DNA sequence analysis on an Applied Biosystems 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The identity of the unknown samples was then determined by Blastn searches against the Genbank nucleic acid sequence database.

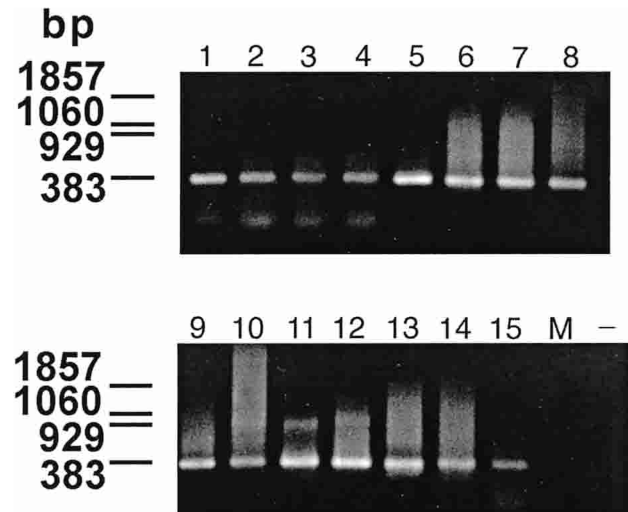

FIGURE 1. Polymerase chain reaction (PCR) amplification of avian and mosquito cytochrome B sequences. Preparation of DNA and PCR amplifications were carried out as described in the Materials and Methods. Template DNAs for each lane were as follows: 1 = Carolina chickadee *(Poecile carolinensis)*; 2 = eastern towhee *(Pipilo erythrophthalmus)*; 3 = white-throated sparrow *(Zonotrichia albicolis)*; 4 = chipping sparrow *(Spizella passerina)*; 5 = field sparrow *(Spizella pusilla)*; 6 = blue jay *(Cyanocitta cristata)*; 7 = house finch *(Carpodacus mexicanus)*; 8 = blue grosbeak *(Guiraca caerulea)*; 9 = house sparrow *(Passer domesticus)*; 10 = tufted titmouse (Baeolophus bicolor); 11 = swamp sparrow *(Melospiza georgiana)*; 12 = orchard oriole *(Icterus spurius)*; 13 = brown-headed cowbird *(Molothrus ater)*; 14 = red-winged blackbird *(Agelaius phoeniceus)* (L.); 15 = northern cardinal *(Cardinalis cardinalis)*; M = house mosquito *(Culex pipiens)*; – = PCR negative control (no template). Values on the left are in basepairs (bp).

RESULTS

To confirm the applicability of the blood meal-specific PCR to North American mosquitoes and their avian hosts, the assay was tested for its ability to amplify cytochrome B sequences from several native North American bird species. The specificity of the assay was also evaluated using DNA prepared from unfed mosquitoes as a template. As shown in Figure 1, the specific amplification conditions were found to support the amplification of detectable PCR products from all 15 of the bird species tested. In contrast, no amplification was detected in reactions using unfed mosquito DNA as a template (Figure 1).

To determine the sensitivity of the avian cytochrome PCR in detecting blood meals in North American mosquitoes, DNA was extracted from individual quail blood-fed *Cx. tarsalis* that were housed for different periods following acquisition of a blood meal. The quail cytochrome B sequence was detected within individual mosquitoes for up to seven days post-feeding (Figure 2). As expected, no signal was detected in a nulliparous mosquito (Figure 2).

To evaluate the sensitivity of the blood meal-specific PCR for the detection of blood meals in wild-caught mosquitoes, DNA was prepared from 52 wild-caught, blood-fed mosquitoes of several different species collected at sites in Alabama and Mississippi. The individual mosquitoes were classified by microscopic inspection as fully blooded, containing a partial blood meal, or gravid. These DNA samples were then used as substrates in the vertebrate-specific PCR. More than 90% of

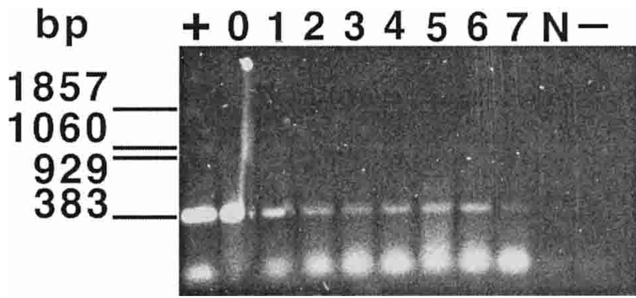

FIGURE 2. Time course of detection of blood meals in mosquitoes. *Culex tarsalis* were fed on a Japanese quail and held at 25°C, 80% relative humidity for various periods of time post-feeding. Lane + = polymerase chain reaction (PCR) positive control (northern cardinal); lane 0 = a mosquito at day 0 post-feeding; lane 1 = a mosquito at day 1 post-feeding; lane 2 = a mosquito at day 2 post-feeding; lane 3 = a mosquito at day 3 post-feeding; lane 4 = a mosquito at day 4 post-feeding; lane 5 = a mosquito at day 5 post-feeding; lane 6 = a mosquito at day 6 post-feeding; lane 7 = a mosquito at day 7 post-feeding; lane N = a nulliparous mosquito; lane − = PCR negative control (no template). Values on the left are in basepairs (bp).

the fully engorged mosquitoes produced detectable PCR products in the blood meal cytochrome B PCR (Table 1). The sensitivity of the assay was somewhat less in partially engorged mosquitoes (83%) and in gravid specimens (71%). Overall, 85% of the blood-fed specimens tested showed positive results in the blood meal PCR assay (Table 1).

The PCR products from a number of avian and mammalian sources were subjected to the HDA using the corresponding sequences from Carolina chickadee (CC) or northern cardinal drivers (NC) as the drivers. As shown in Figure 3, all 17 avian species tested produced specific heteroduplex patterns that were distinguishable from each other. In contrast, a human cytochrome B PCR product did not produce detectable HDA patterns with either the CC or NC drivers (Figure 3). This finding was representative of all of the mammalian PCR products analyzed, none of which produced detectable HDA patterns with the avian drivers.

Intraspecific variation in the cytochrome B gene might complicate identification of blood meals by the HDA. To determine if this might be a potential problem, intraspecific variation was explored in 18 house finches obtained from two geographically distinct locations (Figure 4). No noticeable intraspecific variation was found in the finch HDA, either with the NC or CC drivers (Figure 4).

To demonstrate the utility of the PCR-HDA for the identification of blood meals in wild-caught mosquitoes, the blood meal-specific PCR was used to amplify blood meal-encoded cytochrome B sequences from recently blood-engorged *Cx. tarsalis* collected in Wyoming and Nebraska. The PCR products from 68 individuals producing positive results in the blood meal-specific PCR were then classified by the HDA. In 41 of the 68 samples, HDA patterns were obtained, while no HDA patterns were obtained from 27 samples, suggesting that these products were derived from non-avian species.

The 41 samples producing detectable HDA bands were classified by comparison to the avian standards shown in Figure 3. Samples producing HDA patterns that did not correspond to the standards, as well as those that did not produce detectable HDA patterns were further analyzed by direct DNA sequence analysis. Using a combination of HDA mobility analysis and direct DNA sequencing, it was possible to identify 67 of 68 of the blood meals to the species level. One of the samples produced a complex pattern consisting of four HDA products, suggesting that this mosquito contained a mixed blood meal. Because this sample produced HDA bands that did not match those of the standards and direct DNA sequencing of the mixed PCR product population would have produced equivocal results, this sample was not characterized further. The results obtained from the remaining 67 samples are summarized in Table 2. The 27 samples that did not produce detectable HDA patterns were all found to have been derived from mammalian species. The most common mammalian blood meals were from humans, domestic cattle, and pigs (Table 2).

TABLE 1

Sensitivity of blood meal cytochrome B gene amplification in wild-caught, blood-fed, and gravid mosquitoes

Mosquito blood meal status	Number positive	Number negative
Fully engorged	19	2
Partially engorged	20	4
Gravid	5	2

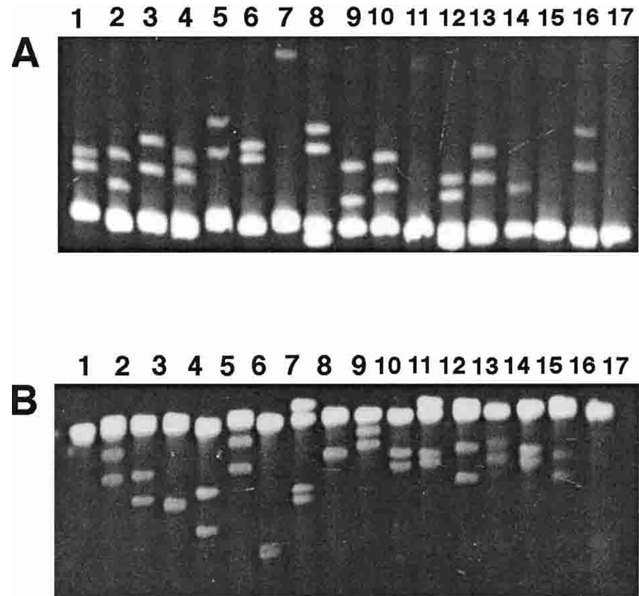

FIGURE 3. Analysis by heteroduplex assay (HDA) of cytochrome B polymerase chain reaction (PCR) products from avian and human samples. The HDAs were performed using a PCR product derived from either northern cardinal (A) or Carolina chickadee (B) as the heteroduplex driver. In each panel, the PCR products used in heteroduplex formation with an HDA driver were as follows: lane 1 = Carolina chickadee *(Poecile carolinensis)*; lane 2 = eastern towhee *(Pipilo erythrophthalmus)*; lane 3 = white-throated sparrow *(Zonotrichia albicolis)*; lane 4 = field sparrow *(Spizella pusilla)*; lane 5 = blue jay *(Cyanocitta cristata)*; lane 6 = house finch *(Carpodacus mexicanus)*; lane 7 = house sparrow *(Passer domesticus)*; lane 8 = swamp sparrow *(Melospiza georgiana)*; lane 9 = orchard oriole or northern oriole *(Icterus spurius)*; lane 10 = tufted titmouse (Baeolophus bicolor); lane 11 = blue grosbeak *(Guiraca caerulea)*; lane 12 = brown-headed cowbird *(Molothrus ater)*; lane 13 = red-winged blackbird *(Agelaius phoeniceus)* (L.); lane 14 = American goldfinch *(Carduelis tristis)*; lane 15 = northern cardinal *(Cardinalis cardinalis)*; lane 16 = northern bobwhite *(Colinus virginianus)* from an engorged mosquito *(Culex tarsalis)*; lane 17 = human *(Homo sapiens)*.

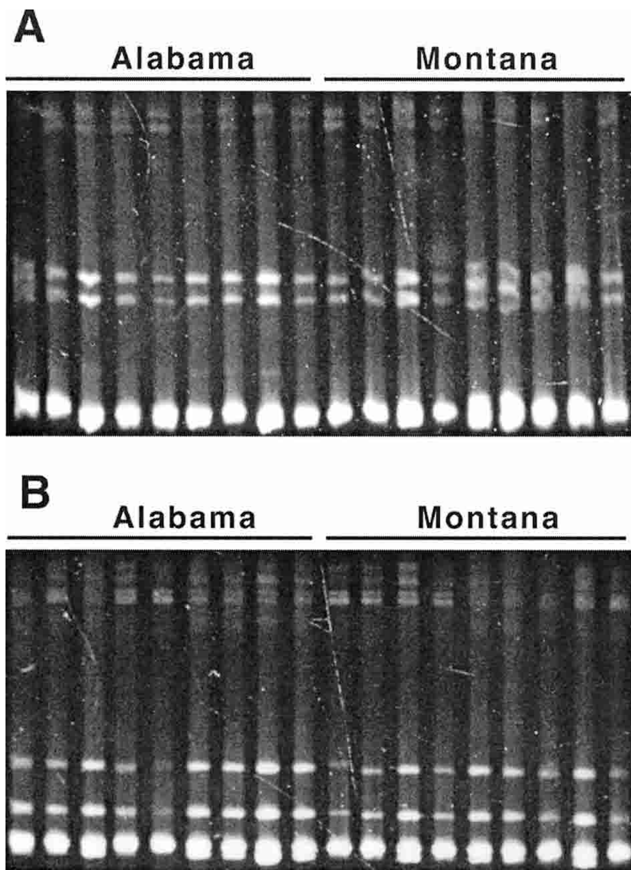

FIGURE 4. Intraspecific variation of cytochrome B sequences in house finches. The DNA from 18 house finches (9 from Alabama and 9 from Montana) were used as a template for amplification of the cytochrome B polymerase chain reaction products, and the products analyzed by heteroduplex analysis using northern cardinal **(A)** or Carolina chickadee **(B)** as the heteroduplex driver. Samples are labeled to indicate their state of origin.

Blood from eight different avian species were identified in the 40 blood meals that produced detectable products in the HDA. Interestingly, 65% (26 of 40) of the avian blood meals were found to have been derived from a single species, the common grackle. Other common avian blood meals included the American robin and the Japanese quail (Table 2).

TABLE 2
Blood meal identification in wild-caught *Culex tarsalis*

Common name	Latin name	Number found
Avian		
Common grackle	*Quiscalus quiscala*	26
American robin	*Turdus migratorius*	5
Japanese quail	*Coturnix coturnix*	3
Mourning dove	*Zenaida macroura*	2
Blue jay	*Cyanocitta cristata*	1
Northern cardinal	*Cardinalis cardinalis*	1
American kestrel	*Falco sparverius*	1
Ring-necked pheasant	*Phasianus colchicus*	1
Mammalian		
Human	*Homo sapiens*	10
Cow	*Bos taurus*	9
Pig	*Sus scrofa*	6
Cat	*Felis catus*	1
Raccoon	*Procyon lotor*	1

DISCUSSION

Epidemics and/or epizootics and maintenance of mosquito-transmitted encephalitis viruses seem to be closely associated with resident birds in enzootic foci.[13,14] Identification of avian blood meals to the species level in both the enzootic and potential bridge vectors may provide important information as to the ecologic factors involved in maintaining an avian enzootic and in allowing the virus to escape the enzootic cycle and infect mammals, including humans. The data presented in this report demonstrate that the blood meal PCR-HDA is capable of reliably identifying avian blood meals to the species level.

In previous studies describing the application of the PCR-HDA to medically important African hematophagous insects, it was found that cytochrome B sequences derived from a human blood meal were detectable in *Simulium damnosum s.l.* held at ambient temperatures up to three days post-engorgement.[12] In the current study, PCR amplification of quail cytochrome B sequences in the blood meal of *Cx. tarsalis* was possible up to seven days post-feeding. There are two explanations for this difference. First, blood meal digestion is likely to be faster in the tropics than in temperate climates, where ambient temperatures are lower. This would have been reflected in this and our previous studies,[12] since in both the laboratory blood engorged insects were held at ambient temperatures following feeding. Second, unlike mammalian erythrocytes, avian erythrocytes are nucleated. Thus, it is likely that the greater amount of DNA in the avian blood meal persists for a longer period than does the DNA in a mammalian blood meal. In support of this hypothesis, recent studies have demonstrated that it is possible to detect DNA derived from human blood meals in *Aedes aegypti.*[15] However, DNA levels in blood meals of laboratory-reared mosquitoes fed on humans and maintained at 29°C decreased 90% at 24 hr post-engorgement. When taken together with the data presented earlier, these results suggest that it is possible to detect PCR-amplifiable DNA from avian blood meals for a longer period of time than from mammalian-derived meals.

Peak oviposition of *Cx. tarsalis* held at 25°C occurs 4–5 days after taking a blood meal, and the majority (greater than 90%) of females begin host seeking 2–4 days following oviposition (Lee, JH, unpublished data). It is thus possible that an individual wild-caught mosquito might contain residues from two different blood meals. Because the HDA is capable of detecting mixtures of PCR products, it should be capable of classifying mixed blood meals, since the PCR product population produced from a mixed blood meal should produce a complex HDA pattern containing 2^n HDA bands, where n is the number of different blood samples present in the blood meal. The fact that the HDA may be capable of identifying such mixed blood meals is supported by the finding that one of the 68 *Cx. tarsalis* samples produced an HDA pattern with four bands, suggestive of a mixed blood meal. In practice, the ability to detect and characterize a mixed blood meal will depend in the relative concentration of the DNAs from each host present in the blood meal, as well as stochastic processes, such as PCR selection and PCR drift, which can affect the

relative efficiency of the PCR amplification of two targets in a mixed template. Furthermore, if one of the samples present in the mixed blood meal does not correspond to a previously characterized species, an unambigious identification of the sample could only be obtained by cloning the mixed product population and determining the DNA sequence of the resulting clones, a laborious process.

The cytochrome B gene is one of the most conserved regions of the mitochondrial genome.[16] In previous studies, no intraspecific variation in pigs, cattle, and water buffalo samples were found by the PCR-HDA.[12] Similarly, among 350 human samples tested by the PCR-HDA, only 4% had minor intraspecific polymorphisms, none of which were sufficient to affect species identification by the HDA.[12] In this study, little intraspecific polymorphism was found in house finch samples. These results, when taken together, suggest that any intraspecific polymorphisms that exist in the vertebrate cytochrome B sequences are not sufficient to interfere with their unambiguous identification by the HDA.

The blood meal-specific PCR assay produced positive results in approximately 85% of the wild-caught specimens that had taken a blood meal. The sensitivity of the assay was greatest in specimens containing fresh blood meals and decreased in mosquitoes with partial blood meals and in gravid individuals. This result was to be expected, since presumably the DNA in the blood meal is degraded over time in the midgut of the mosquito. Furthermore, it is likely that some proportion of the blood meals in the wild-caught specimens were derived from non-avian sources. As discussed earlier, it is also likely that the limit of detection of the PCR will be less with mammalian blood meals than with avian blood meals. Finally, it is possible that some of the blood meals not detected by the PCR assay may have been derived from other vertebrate hosts (such as reptiles and amphibians) that might not be amplified using the PCR conditions described here. More work will be necessary to determine if this is the case.

Identification of the blood meals in wild-caught blood-fed *Cx. tarsalis* demonstrated that the majority of these were derived from avian hosts. This finding agrees with previous studies that suggest that *Cx. tarsalis* is ornithophilic but also feeds on mammals, particularly in the latter part of the year.[17–19] The reasons for this host switch are unclear, but it may involve a change in abundance of the host species or in some life history change on the part of the avian hosts.[19] For example, *Cx. tarsalis* feeds more readily upon nestling birds than adults, a difference thought to be related to the relative immobility and defenseless nature of the young.[20]

Sixty-five percent of the avian blood meals identified in this population were derived from a single species, the common grackle. Previous studies of the avian host feeding preferences of *Cx. tarsalis* have suggested that feeding behavior may be influenced by the abundance of a particular bird species in the area under study.[19] As mentioned earlier, host life history variables may also play a role in the selection process. The finding that the common grackle was a preferred host of *Cx. tarsalis* in this study may therefore reflect the overall abundance of this species at the study sites where the samples were collected, or may reflect some aspect of the biology or behavior that make it a particularly desirable host for *Cx. tarsalis.* The ability to identify avian blood meals to the species level will allow longitudinal studies of the feeding preferences of *Cx. tarsalis* to be carried out, providing data to address these questions.

Apart from the common grackle, one of the more common avian hosts seen in the *Cx. tarsalis* sampled in this study was the Japanese quail. This bird is not native to North America, but it was used as a sentinel bird in the study sites from which the mosquitoes were collected. The finding that blood meals derived from the Japanese quail were common at these sites suggests that the sentinel flocks were serving as an important host for the local mosquito population, and therefore should be reliable indicators of when viral transmission is initiated.

A second potential advantage of the PCR-HDA over conventional assays resides in the ability of the assay to identify samples for which a standard is not available. This is due to the fact that a blood meal for which a standard has not been identified will produce a novel pattern upon gel analysis. The PCR product that produces such a novel pattern can then be subjected to DNA sequence analysis and its identity determined by comparison to sequences deposited in the Genbank nucleic acid sequence bank. If an exact match to the unknown sequence is not found, the identity of the unknown can be surmised through molecular phylogenetic analysis of the unknown sequence in conjunction with other known sequences. This approach has previously been used to identify sibling species of *S. damnosum s.l.* producing novel patterns in an HDA.[21] Once a phylogenetic analysis has narrowed down the likely identity of the host, field collections can be carried out to confirm the identity of the unknown sample. Because the PCR is so sensitive, such identification requires only a small amount of biological material (e.g., in our hands 5 μl of avian blood is sufficient to provide DNA for more than 20 PCRs). Thus, non-destructive and non-lethal methods can be used in sampling to definitively identify samples producing novel HDA patterns.

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