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THE ABUNDANCE AND INFECTION STATUS OF ANOPHELES spp. MOSQUITOES AT THREE SITES IN LOUDOUN COUNTY, NORTHERN VIRGINIA

Priya Krishnan
Virginia Commonwealth University

THE ABUNDANCE AND INFECTION STATUS OF *ANOPHELES* spp. MOSQUITOES AT THREE SITES IN LOUDOUN COUNTY, NORTHERN VIRGINIA

A thesis submitted in partial fulfillments of the requirement for the degree of Master of Science at Virginia Commonwealth University

BY
PRIYA KRISHNAN
Bachelor of Science, University of Delhi, 1996

ADVISOR - Dr. D.C. GHISLAINE MAYER

Assistant Professor, Department of Biology, VCU

Virginia Commonwealth University
Richmond, Virginia,
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LIST OF ABBREVIATIONS

An.	*Anopheles*
Cx.	*Culex*
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
CDC	Center for Disease Control
TAE	Tris-acetate-EDTA
VCU	Virginia Commonwealth University
USA	United States of America
ELISA	Enzyme-linked immunosorbent assay
AIDS	Acquired Immunodeficiency Syndrome
SARS	Severe Acute Respiratory Syndrome
WNV	West Nile Virus
WHO	World Health Organization
TEA	Triethylamine
PAHO	Pan American Health Organization
GIS	Geographic Information Systems
NA	Not Available

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ABSTRACT

THE ABUNDANCE AND INFECTION STATUS OF *ANOPHELES* spp. MOSQUITOES AT THREE SITES IN LOUDOUN COUNTY, NORTHERNVIRGINIA

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Malaria is a re-emerging infectious disease with approximately half of the world's population at risk. In the US, since the 1950's the Center for Disease Control (CDC) has been reporting between 1,000 and 1,500 cases of malaria every year. A majority of these cases were among US travellers and were attributed to *Plasmodium falciparum.* In August 2002, two cases of human malaria due to *Plasmodium vivax* were reported in Loudoun County, Northern Virginia. The Center for Disease Control and Prevention concluded that these cases were acquired locally. This was because of an absence of other risk factors such as international travel, and blood transfusion. Pools of *Anopheles quadrimaculatus* and *Anopheles punctipennis* collected in Loudoun County, Northern Virginia in 2002 tested positive for *P. vivax* subtype 210 indicating local transmission of malaria in the area.

The purpose of this study is two-fold: 1) to determine the abundance of blood-fed *Anopheles* mosquitoes in the three sites close to the 2002 local transmission of human malaria in Loudoun County, Northern Virginia, and 2) to determine the infection status of the blood-fed *Anopheles* mosquitoes collected in the area. We observed a significant difference in the total

abundance of *Anopheles quadrimaculatus* and *Anopheles punctipennis* at all the three sites, with *Anopheles quadrimaculatus* being more abundant. We also found a significant difference in the total abundance of *Anopheles quadrimaculatus* across the years at each of the three sites. All the pools collected in 2009 and 2010 tested negative for human *Plasmodium* parasites. The pools collected in 2010 tested negative for avian *Plasmodium* and *Haemoproteus* parasites. However, in 2009, 20 (28%) pools out of 71 tested positive for avian *Plasmodium* and *Haemoproteus* parasites. Four (20%) pools tested positive for avian *Plasmodium*, three of which were composed of *Anopheles quadrimaculatus* and were collected at Algonkian Regional Park while, one pool of *Anopheles punctipennis* was collected at Youngs Cliff. In addition, one (5%) pool composed of *Anopheles quadrimaculatus* and collected from Algonkian Regional Park tested positive for *Haemoproteus.* Eleven (55%) pools tested positive for both *Haemoproteus* and *Plasmodium*. Of these, four pools were composed of *Anopheles quadrimaculatus* and were collected from Algonkian Regional Park, two pools composed of *Anopheles quadrimaculatus* and one pool composed of *Anopheles punctipennis* were collected from Youngs Cliff. Lastly, four pools collected at Potomac Drive were composed of *Anopheles quadrimaculatus.* In summary, Algonkian Regional Park showed a high number of mosquitoes with malarial parasites.

The maximum likelihood estimation (MLE) of mosquito infection rates based on the Biggerstaff (2006) method showed that among the three sites Youngs cliff had the overall highest infection rate (74.50%) for *Anopheles* mosquitoes compared to Algonkian Regional Park (53.86%) and Potomac drive (25.01%). *An. quadrimaculatus* had a higher infection rate compared at Algonkian Regional Park (57.95%) and Potomac drive (32.56%) compared to *An. puncipennis* (0%), while at Youngs cliff *An. puncipennis* had a higher infection rate (182.88%) compared to *An. quadrimaculatus* (56.06%).

INTRODUCTION

Emerging infectious diseases are those, whose incidence or geographical range has increased in recent times (Lederberg et al., 1992, Morse, 1993, and Daszak et al., 2000 as cited in for a review, see Daszak et al., 2001). They could be due to newly discovered pathogens or due to existing pathogens that are evolving (Lederberg et al., 1992, Morse, 1993, and Daszak et al., 2000 as cited in for a review, see Daszak et al., 2001, Fauci, 2006). Emerging infectious diseases can be attributed to increased international trade and travel, rapid urbanization and changes in land-use patterns (Morse, 1995).

Emerging infectious diseases among wildlife populations may be due to greater interaction between infected domestic animals and wildlife living in close proximity or may be due to movement of infected wildlife either naturally or through human intervention leading to the introduction of pathogens in new geographical locations (Daszak et al., 2000). For example, infected migratory seabirds crossing the Atlantic from the West African coast and legally imported infected zoo and pet birds such as ducks, turkeys have contributed to the rapid spread of West Nile disease along the east coast of the US (Rappole, Derrickson, and Hubálek, 2000).

In the last few decades, several vector-borne infectious diseases of humans have also emerged or re-emerged including mosquito borne diseases such as malaria, and dengue fever. Mosquitoes are arthropod vectors that spread diseases by transmitting pathogens between hosts through bites. When a mosquito vector bites a reservoir host it picks up the pathogens, which then completes its development in the mosquito gut and then reach the salivary glands. When the infected mosquito bites a healthy host it transmits the pathogens to it. Several of the mosquito vector-borne infectious diseases that have been emerging and re-emerging in the past few decades are given in Table 1.

Factors Affecting Mosquito-borne Diseases

Several factors affect mosquito vector-borne diseases including temperature, precipitation and humidity. These factors can either favor the introduction of novel pathogens and vectors or eliminate existing pathogens and vectors in an area. Higher temperatures can cause mosquito larvae to mature quickly thereby increasing the abundance of vectors (Rueda et al., 1990, for a review, see Githeko et al., 2000, Jelinek et al., 2002). Also, at higher temperatures, the adult female mosquitoes feed at regular intervals and digest blood meals quickly, thereby increasing the rate of transmission of the disease (Gillies, 1953, for a review, see Githeko et al., 2000). Additionally, at higher temperatures, malarial parasites complete their development rapidly in the mosquito vector thus increasing the abundance of infective vectors (Turell, 1989, for a review, see Githeko et al., 2000, Jelinek et al., 2002). However temperatures above 34^{o} C have an adverse affect on the survival of both the vectors and the parasites thus impacting the transmission of vector-borne diseases (Rueda et al., 1990, for a review, see Githeko et al., 2000). The amount of precipitation can also affect vector-borne diseases. Mosquito vectors prefer to breed in pools of water and so droughts should naturally lead to a decrease in the vector population and thus a decrease in disease transmission. However, Shaman et al., 2005 have shown in the case of West Nile Virus (WNV), droughts result in an increase in disease transmission by bringing hosts and mosquitoes into close contact in the small number of moist habitats that are left.

Malaria as a Re-emerging Infectious Disease of Humans and Birds

Malaria is a re-emerging infectious disease. Approximately half of the world's population is at risk of malaria (WHO, 2009). Each year 250 million cases of malaria occur worldwide and over 1 million people die. Young children in sub-Saharan Africa and Asia are the most vulnerable (WHO, 2009, and 2010). Ninety eight percent of malaria deaths across the world occur in thirty-five countries (30 in sub-Saharan Africa and 5 in Asia) (CDC, 2010). Malaria

has been eradicated in the US and most of Europe (Kuhn, Campbell-Lendrum, and Davies, 2002, Tanne, 2002, and Leon et al., 2005), though it is still endemic in several countries of South and Central America, Africa, the Middle East, South Asia and Southeast Asia (CDC, 2003). There are 5 *Plasmodium* species that are known to infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae*, *P. knowlesi* (for a review, see Zimmermann et al., 2004, Singh et al., 2008). Symptoms of malaria include fever, chills, headache, and vomiting. Drugs commonly used to treat malaria include chloroquine, primaquine, and doxycycline. In many countries, malarial parasites have gained resistance to antimalarial drugs thereby undermining malaria control.

Avian blood protozoan parasites include *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. They are known to infect both wild and captive birds across the world (Murata, 2002, Hagihara et al., 2004, Nagata, 2006, and Ejiri et al., 2009). The life cycle of all haemosporidian parasites, involve asexual reproduction in the vertebrate hosts and sexual reproduction and asexual reproduction in the mosquito vector (Valkiunas 2005). When a mosquito takes a blood meal it injects sporozoites into the vertebrate host. The sporozoites invade liver cells and undergo asexual reproduction to produce merozoites. The merozoites invade erythrocytes and continue to produce more merozoites through asexual reproduction. Some merozoites differentiate into gametocytes and are picked up by the mosquito vector along with a blood meal. Sexual reproduction occurs in the mosquito vector to produce an oocyst. The oocyst undergoes asexual multiplication to produce sporozoites, which then reach the salivary glands (Valkiunas 2005). Blood sucking midges of the genus *Culicoides* are vectors for *Haemoproteus* spp. (Bonneaud et al. 2009, Kettle, 1982, Valkiunas and Iezhova, 2004, and Martinsen, Perkins and Schall, 2008), whereas *Leucocytozoon* spp. is transmitted by black flies of the genus *Simuliidae* (Martinsen et al., 2008). Avian malaria is a mosquito borne disease among birds and is caused by several species of *Plasmodium*. These include *P. relictum, P.*

cathemerium and P. elongatum (Sabrosky, Mc Daniel and Reider, 1946). Mosquitoes such as *Culex* spp. and *Aedes* spp. are vectors for avian *Plasmodium spp.* (Bennett, 1987, Valkiunas, 2005, and Ejiri et al., 2009). Studies on avian malaria are predominantly focused on the bird-parasite interactions and not so much on the vector-parasite interactions (van Riper et al., 1986, Beadell et al., 2004, 2006, 2009, Hellgren et al., 2004, Valkiunas, 2005, and Njabo et al., 2010). Avian malaria was introduced into the Hawaiian Islands and has since been implicated in the widespread decline and extinction of many native bird species (Warner, 1968, van Riper et al., 1986, Beadell et al 2006). The transmission of avian malaria in the Hawaiian Islands began after a competent vector, *Culex quinquefasciatus* was introduced in 1826 (Warner, 1968).

Malaria in the US

Malaria was eradicated in the US in the 1950's (Tanne, 2002, and Leon et al., 2005). Since then the Centers for Disease Control (CDC) has been reporting between 1,000 and 1,500 cases of malaria in the United States every year. A majority of them were acquired abroad (for a review, see Newman et al. 2004, Causer et al., 2002, AND PAHO 1969). Most of these cases were among travellers and were attributed to *P. falciparum*, due to failure to adhere to recommended chemoprophylaxis and to seek medical care after travel (for a review, see Newman et al., 2004).

In the past 15 years the number of cases of locally transmitted malaria in the US has been increasing (Mac Arthur et al., 2001). Travellers, migrant workers, refugees and US troops returning from malaria endemic countries are potential sources for establishing a local transmission cycle for malaria. The states in the US that have reported cases of locally transmitted malaria in the last 2 decades include California, Texas, New York, New Jersey, Georgia and Florida (Mac Arthur et al., 2001, Centers for Disease Control and Prevention, Malaria Surveillance Reports and other unpublished reports). Georgia reported a case of *P.*

vivax malaria in 1999, which was suspected to be a case of local transmission from migrant workers from Latin America living in close proximity to the patient (Mac Arthur et al., 2001).

Malaria in Virginia

The state of Virginia reported 156 imported cases of malaria to the CDC from1999 to 2001 (CDC, 2002, Leon et al., 2005). The risk of malaria transmission continues to increase owing to immigration, global travel, and the presence of competent *Anopheles* mosquito species throughout the eastern United States (Zucker, 1996, and Leon et al., 2005, PAHO, 1969). The Potomac River and Chesapeake Bay are known to favor breeding of *An. quadrimaculatus* and *An. punctipennis* (Good, 1946, and Leon et al., 2005).

In August 2002, Loudoun County in Northern Virginia reported 2 cases of human malaria due to *P. vivax* (CDC, 2002 as cited in Leon et al., 2005). They were locally acquired. The patients lived about half a mile apart. A study was carried out, where in; mosquito collections were made between September and October 2002 in Northern Virginia and Maryland areas. Two female pools composed of *An. quadrimaculatus* collected in Loudoun County, Virginia, one female pool composed of *An. punctipennis* collected in Fairfax County, Virginia, and two female pools composed of *An. quadrimaculatus* collected in Montgomery County, Maryland, tested positive for *P. vivax* subtype 210 with VecTest panel assay and enzyme-linked immunosorbent assay (ELISA) (Leon et al. 2005). A polymerase chain reaction (PCR) technique was used to confirm ELISA and VecTest-positive samples (Leon et al., 2005). The PCR results were mixed (Leon et al., 2005). Three out of the five mosquito pools that tested positive with ELISA and VecTest were found to be positive with PCR (Leon et al., 2005). Despite the inconclusive nature of the study carried out by Leon et al., 2005, the CDC concluded that the cases of malaria transmission in Loudoun county, Northern Virginia in August 2002 were locally acquired because there was a lack of other risk factors such as

international travel, blood transfusion, and organ transplantation (CDC, 2002 as cited in Leon et al., 2005).

Anopheles Vectors

There are about 400 species of *Anopheles* in the world of which only 30-40 are involved in transmitting the human malarial parasite (CDC, 2010). Some Anophelines are anthropophilic, they prefer to feed on humans while others are zoophilic, they prefer to feed on animals (CDC, 2010). *An. gambiae* is anthropophilic, while *An. funestus* and *An. arabiensis* are zoophilic (Highton et al., 1979, Serivce, 1970, Minakawa et al., 2002, and Githeko et al., 1994). The distribution of *Anopheles* species across the world is given in Table 2.

There are several species and subspecies of *Anopheles* found in North America. *An. quadrimaculatus, An. freeborni* and *An. albimanus* were the major vectors when malaria was endemic in the US (Zimmerman, 1992). *An. freeborni*, *An. quadrimaculatus,* belongs to *An. maculipennis* complex, which also consists of other subspecies such as *An. earlie* and *An. walkeri. An. punctipennis* complex consists of subspecies such as *An. crucians*, *An. bradleyi* and *An. punctipennis* (Floore, Harrison, and Eldridge, 1976). In the US as early as 1916, *An. crucians* has proven to be a competent vector of *P. falciparum* and *P. vivax* in laboratory conditions (King, 1916 and Mitzmain, 1916). The states of Florida (Metz, 1919) and Louisiana (Mayne, 1919) reported natural malaria infections in *An. crucians* before eradication. *An. bradleyi* was also found to be competent in transmitting *P. falciparum* in laboratory conditions (Boyd, Kitchen and Mulrennan, 1936).

An. quadrimaculatus s.l. and *An. punctipennis* are considered to be the vectors of malaria in the eastern part of the United States, (Leon et al. 2005), *An. quadrimaculatus* being the primary vector (Faust, 1949, and Gladney and Turner, 1969, and Leon et al., 2005). It feeds on humans as well as wild and domesticated animals such as cattle and deer. It breeds in ponds, irrigation ditches, and other semi-permanent water sources. It is abundant between mid to late

summer (O'Malley 1992, Leon et al., 2005, Goddard et al., 2010). *An. punctipennis* is considered to be a secondary vector (Carpenter and La Casse 1955, and Leon et al., 2005). It feeds on humans and other mammals. It breeds in streams, marshes and occasionally in containers. It is abundant in late spring and early summer (Hickman R and Brown J, Brunswick mosquito control, Carpenter and LaCasse 1955, Leon et al., 2005 and Goddard et al., 2010).

Anopheles and Avian Blood Parasites

It has been experimentally established that avian *Plasmodium* can be transmitted by *Anopheles* mosquitoes in laboratory conditions (Hunninen, Young, and Burgess, 1950). *An. quadrimaculatus, An. crucians,* and *Cx. pipiens* were allowed to feed on English sparrows (*Passer domesticus*) infected with *P. relictum*, an avian malarial parasite that has devastated native bird populations in the Hawaiian Islands (Warner, 1968, van Riper et al., 1986). A high percentage (43.3 %) of *An. quadrimaculatus* showed presence of oocysts in the gut as compared to *Culex pipiens* (23.2 %). However only small percentage of *An. quadrimaculatus* (6.7 %) showed presence of sporozoites in the salivary glands as compared to *Cx. pipiens* (31.5 %). Out of the few *An. crucians* that survived, none had fully developed sporozoites in the salivary glands (Hunninen et al. 1950). This study established that *Anopheles* species could act as a competent vector for avian malaria. In addition, the blood meal analysis of *An. quadrimaculatus* and *An. punctipennis* mosquitoes collected in Central Virginia in 2010 showed the presence of avian *Plasmodium* (Catherine Wallace, unpublished).

Haemoproteus are parasitic protozoans found in birds, reptiles and amphibians. They are transmitted by blood sucking midges of the genus *Culicoides* (Bonneaud et al. 2009, Kettle, 1982, Valkiunas and Iezhova, 2004, and Martinsen, Perkins and Schall, 2008). The blood meal analysis of *An. quadrimaculatus* and *An. punctipennis* mosquitoes collected in Southern Virginia in 2010 has shown the presence of avian *Haemoproteus* parasites (Catherine Wallace, unpublished).

Blood meal Analysis

Blood meal identification of arthropod vectors is important to understand the pathogen, host and vector interactions. It enables us to know the specificity of pathogens and their vectors and hosts and also allows us to assess the risk of transmission of the pathogen and outbreak of disease (Kent, 2009). PCR based techniques using the mitochondrial gene cytochrome b are increasingly being used for this purpose (Ngo, and Kramer, 2003). The identification of blood meal of mosquito vectors provides information about host preference of the vector (Ngo et al., 2003). Ngo et al., 2003 used blood meal analysis to identify mosquito species that were transmitting WNV from the enzootic cycle between birds and *Culex* mosquitoes to humans and horses. Blood meal analysis can be used to identify avian malarial parasites and the source of the blood meals to understand the pathway of pathogen transmission (Kim et al., 2009). Analysis of blood meals of arthropod vectors could provide information on the kinds of pathogens that are being carried by hosts in an area. This information can be used to estimate the kinds of pathogens that can be transmitted in an area. Arthropod vectors of disease can therefore be used as biosensors for predicting risk of outbreaks of disease in an area.

OBJECTIVES

OBJECTIVE 1- DETERMINING THE ABUNDANCE OF *ANOPHELES* spp. MOSQUITOES AT THREE SITES IN LOUDOUN COUNTY, NORTHERN VIRGINIA

To determine the vectors of malaria in Loudoun County, Northern Virginia, where there was an outbreak of locally acquired human malaria in 2002. We determined the abundance of *Anopheles* mosquito species in three sites (Algonkian regional park, Youngs Cliff, and Potomac Drive) from 2008-2010.

OBJECTIVE 2- DETERMINING THE INFECTION STATUS OF *ANOPHELES* spp. MOSQUITOES AT THREE SITES IN LOUDOUN COUNTY, NORTHERN VIRGINIA

We used blood-meal analysis to determine the infection status of blood-fed *Anopheles* mosquitoes in the three sites (Algonkian Regional Park, Youngs Cliff, and Potomac Drive) in Loudoun County, Northern Virginia, in order to ascertain whether there was a risk of local transmission of malaria in the area.

METHODS

Field Collection

The collection and pooling of mosquitoes was carried out by Andrew Lima (Clarke, Manassas, VA). The collection was done from three sites in Northern Virginia over three years, between 2008 and 2010 using resting boxes. The sites chosen Potomac Drive, Youngs Cliff, Algonkian Regional Park were within one km of the Potomac river. Collections were done from early August through early October during 2008-2010. The collection dates are listed in Table 3. Algonkian Regional Park is a public park less than 1000 acres with vacation cottages, picnic shelters. Youngs Cliff is a 2-acre lot private property and has a house on it and a surrounding residential neighborhood. Potomac Drive is also a 2-acre lot private property with no house and a residential neighborhood in close proximity. The collection area chosen were each about 5 m long and 2 m wide. Resting boxes made of plywood with a volume of 38 cm^3 were used in groups of ten at each site. The boxes were arranged in two linear rows of five each and placed one m apart. The resting boxes were open at one end and were placed in such a way that the open end faced the west to prevent sunlight from entering the boxes during the day. The boxes were painted in black on the outside and red on the inside. The boxes remained in the same spot for the entire collection period. Resting mosquitoes were aspirated from the boxes between 12:00 -16:00 hours. Triethylamine (TEA) (Fisher Scientific, Fair Lawn, NJ) was administered to each box to anesthetize the mosquitoes. The mosquitoes were collected using forceps or a battery operated aspirator. They were then placed on dry ice and brought to the laboratory.

The female blood-fed *Anopheles* spp. mosquitoes were selectively separated into pools (10 mosquitoes in most pools with some having 2, 3, 5 and 8 mosquitoes). All mosquitoes collected on a particular collection date were pooled into ten in one pool and any remaining mosquitoes left on that date were pooled together. Hence, the last pool on each collection date

had less than ten mosquitoes. They were then decapitated and stored in a freezer at – 15^{o} C until DNA extraction was performed.

Statistical analysis

Statistical analysis was carried out using JMP.version.8 software. A student's *t* test was used to compare the mean abundance of blood-fed *An. quadrimaculatus* and *An. punctipennis* between Algonkian Regional Park, Youngs Cliff, Potomac Drive during the time of collection. Since there were 3 sites, Bonferroni correction was applied for each site. Bonferroni corrected α for each site was determined to be $\alpha = 0.05/3=0.0167$. Statistical significance was set at $p < 0.0167$. A significant p-value indicated a statistical difference between the abundances of the two species of *Anopheles* mosquitoes at the three sites in the three years.

DNA Extraction

Blood-fed mosquitoes were ground using sterile forceps to prepare for extraction of DNA according to the previously described protocol of Kim et al. (2009). The heads of the mosquitoes were removed to eliminate inhibitory effect on PCR (Arez et al., 2000). For mosquito pools collected in 2008 and 2009 both the thorax and abdomen were used, while for pools in 2010 only the abdomens were used. Total DNA from pools of blood-fed mosquitoes was extracted using Sigma red extract blood and tissue kit for 2008, 2010 (Sigma, St. Louis, MD) with the following modifications: the volume of the extraction solution, tissue preparation solution and neutralization solution were doubled the incubation was done at 55^{o} C for 10 minutes followed by 95^{o} C for 3 minutes. The Qiagen blood and tissue kit was used for extraction of 2009 samples (Qiagen, Valencia, CA).

PCR Amplification of *Anopheles* DNA

Extracted DNA was tested to see if it was of the quality required for further molecular analysis by Polymerase Chain Reaction (PCR) using primers against the white gene of *An. gambiae* 5'AACACGGACGACCAGTATG 3' and 5' TGTTGTCGGGCTTCATTATC 3'

according to the method of Rafferty et al. 2002. The reaction mixture included 15 mM $MgCl_2$, 2 mM dNTPs and 1 unit *Taq* polymerase in a final volume of 25 µl. The PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, then 72°C for 10 min. 70 pools out of 194 in 2008 were tested.

PCR Amplification of Human *Plasmodium* DNA

Human *Plasmodium* infection was detected by using a multiplex PCR assay targeting species-specific sequences in the small subunit r RNA genes of four *Plasmodium* species that infect humans (Padley et al., 2003). Multiplex PCR is a variant of PCR, in which more than a single target sequence can be amplified using more than a pair of primers in a single reaction. This reduces effort and cost (Elnifro et al., 2000). Primers specific for human *Plasmodium (vivax, falciparum, malariae, and ovale)* were used in a single reaction. *P. knowlesi* was not tested because there has never been a report of *P. knowlesi* in North America. A single reverse primer 5'GTA TCT GAT CGT CTT CAC TCCC 3' conserved in all four *Plasmodium* species, and four species-specific forward primers: one each for *P. falciparum* 5' AAC AGA CGG GTA GTC ATG ATT GAG 3', *P. vivax* 5'CGG CTT GGA AGT CCT TGT 3', *P. ovale* 5'CTG TTC TTT GCA TTC CTT ATGC 3' and *P. malariae* 5'CGT TAA GAA TAA ACG CCA AGCG 3'were used. Hot Star Taq Master Mix: (Qiagen Valencia, CA) that contained 3.0 mm $MgCl_2$, 400 mm of each dNTP, 2.5 units of Hot Start Taq DNA polymerase, 15 pmol of the reverse primer, 15 pmol of *P. falciparum* forward primer, 17.5 pmol each of the *P. ovale* and *P. malariae* forward primers, 7.5 pmol of the *P. vivax* forward primer and 5 µl of extracted DNA, were used in a final volume of 25 µl. The PCR conditions were as follows-: an initial 15-min incubation at 95°C followed by 43 cycles, each of 45 seconds at 95°C followed by 90 seconds at 60°C, and then a final step at 72°C for 5 min. The amplicons were electrophoresed on 2% agarose gels and stained with ethidium bromide. *P. vivax* DNA was used as positive control. The expected product sizes were 276 bp for *P. falciparum,* 300 bp for *P. vivax,* 375 bp for *P.*

ovale, 412 bp for *P. malariae.*

PCR Amplification of haemosporidian DNA

Haemosporidian parasites (*Haemoproteus* and *Plasmodium*) were detected by nested PCR using Illustra PuReTaq Ready-to-Go PCR beads (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) and 1.5 µl of each of the primers HaemF 5'ATG GTG CTT TCG ATA TAT GCA TG 3' and HaemR2 5'GCA TTA TCT GGA TGT GAT AAT GGT 3' and 20 µl of DNase/RNase free water according to the method of Waldenstrom et al. (2004). The PCR was performed in a final volume of 25 µl. The PCR conditions were as follows: 94˚C for 3 minutes, 35 cycles of 94˚C for 30 seconds, 50˚C for 30 seconds, 72˚C for 45 seconds, and a final extension step of 72˚C for 10 minutes after which the samples were held at 4˚ C. The samples were electrophoresed on 1.2% agarose gels and stained with ethidium bromide. Blood from bird that tested positive for Haemosporidia was used as the positive control (Grillo, In press). The expected product size was 580 bp. The amplicons were used as template for subsequent PCR reaction.

Testing for Avian *Plasmodium* and *Haemoproteus* DNA

Avian *Plasmodium* was detected by using Illustra PuReTaq Ready-to-Go PCR beads (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) and 1.5 µl of each of the primers against the cytochrome b gene of *Plasmodium* parasite: FP3 5' TAT ATA ACT TTT TTG ATA TG 3' and RP3 5' GTT ATT GCA TTA TCT GGA TGT GA 3' and 20 µl of DNase/RNase free water according to the method of Waldenstrom et al. (2004). Two µl of the PCR products were used. For avian *Plasmodium* the PCR was performed in a final volume of 25 µl. The PCR conditions were as follows: an initial 94˚C for 1 minute, followed by 40 cycles of 94˚C for 20 seconds, gradient 52.9˚C for 20 seconds, and 72˚C for 30 seconds, and then a final extension step at 72˚C for 10 minutes after which the samples were held at 4˚ C. For avian *Haemoproteus* the following primers were used: FH3 5' GAT TRA ACT CAT TTT TTG TTT TTA CT 3' and

RH3 5' ACA ATT GCA TTA TCA GGA TGA GC 3'. The PCR conditions were as follows: 94˚C for 1 minute, 40 cycles of 94˚C for 20 seconds, gradient 52˚C for 20 seconds, and 72˚C for 30 seconds, followed by a final extension at 72˚C for 10 minutes after which the samples were held at 4˚ C. The samples were electrophoresed on a 1.2% agarose gel. Blood from a bird that tested positive for avian *Plasmodium* and *Haemoproteus* was used as the positive control (Grillo, In press). The expected product size was 524 bp.

The maximum likelihood estimation (MLE) of mosquito infection rates was calculated according to the method of Biggerstaff (2006) as cited in Kent et al., 2009.

RESULTS

Abundance of vectors of malaria in Loudoun County, Virginia

To determine the abundance of vectors of malaria at three sites in Loudoun County in Northern Virginia, a total of 10,197 blood fed, non-blood fed and gravid mosquitoes were collected and pooled, as described earlier, from the three sites in Northern Virginia in a period of three years. The size of the pools is given in Table 4. The total mosquitoes collected in 2008 from all the three sites were 5708, with 2153 collected in 2009 and 2336 collected in 2010 (Table 5). Mosquito species collected from the three sites included *An. quadrimaculatus, An. punctipennis, Cx. erraticus, Cx. territans,* and *Cx. pipiens.*

Mosquito abundance at Algonkian Regional Park

In 2008, the total mosquitoes collected from Algonkian Regional Park were 2888, with 912 (32 %) blood-fed *An. quadrimaculatus* and 6 (0.2 %) blood-fed *An. punctipennis* (Table 5). In 2009, the total mosquitoes collected from Algonkian Regional Park were 1299, with 370 (28.4 %) blood-fed *An. quadrimaculatus* and 15 (1.2 %) blood-fed *An. punctipennis* (Table 5). In 2010, the total mosquitoes collected from Algonkian Regional Park were 808, with 371 (50 %) blood-fed *An. quadrimaculatus* and 4 (0.5 %) blood-fed *An. punctipennis* (Table 5). The total blood-fed *An. quadrimaculatus* mosquitoes collected from 2008 to 2010 at Algonkian Regional Park was 912 in 2008, 370 in 2009 and 371 in 2010 and *An. punctipennis* was 6 in 2008, 15 in 2009 and 4 in 2010 (Figure 1 A). In Algonkian Regional Park, there was a decrease in the mean abundance of *An. quadrimaculatus* collected from 2008 to 2009, which stabilized from 2009 to 2010. There was a significant difference in the total abundance of blood-fed *An. quadrimaculatus* collected in 2008 and 2009 ($t = 2.877$, $df = 75$, P value = 0.0052) and those collected in 2008 and 2010 ($t = 2.874$, $df = 75$, P value = 0.0053). There was no significant difference in the mean abundance of blood-fed *An. quadrimaculatus* collected in 2009 and 2010. The mean abundance of blood-fed *An. punctipennis* was also stable across 2008, 2009

and 2010 (Figure 1A). Additionally, there was a significant difference in the mean abundance of blood-fed *An. quadrimaculatus* and *An. punctipennis* collected in 2008 (t = 3.848, df = 49, P value = 0.0003), with blood-fed *An. quadrimaculatus* being more abundant as compared to blood-fed *Anopheles punctipennis* (Figure 1A). There was no significant difference in the mean abundance of blood-fed *An. quadrimaculatus* and blood-fed *An. punctipennis* collected in 2009 and 2010 (Figure 1A).

A closer look at the total abundance during the collection dates in 2008 shows a spike in blood-fed *An. quadrimaculatus* on 27August in 2008 when a huge number of mosquitoes were collected on one collection date (Figure 1B). Blood-fed *An. punctipennis* abundance was relatively stable across the collection dates in 2008, 2009, and 2010 (Figure 1B).

Mosquito abundance at Youngs Cliff

In 2008, the total mosquitoes collected from Youngs Cliff were 1696, with 342 (20.2 %) blood-fed *An. quadrimaculatus* and 7 (0.4 %) blood-fed *An. punctipennis* (Table 5). In 2009, the total mosquitoes collected from Youngs Cliff were 331, with 104 (31.4 %) blood-fed *An. quadrimaculatus* and 12 (3.6 %) blood-fed *An. punctipennis* (Table 5). In 2010, total mosquitoes collected from Youngs Cliff were 668, with 449 (67.2 %) blood-fed *An. quadrimaculatus* and 5 (0.7 %) blood-fed *An. punctipennis* (Table 5). The total blood-fed *An. quadrimaculatus* collected from 2008 to 2010 at Youngs Cliff was 342 in 2008, 104 in 2009 and 449 in 2010 and for *An. punctipennis* 7 in 2008, 12 in 2009 and 5 in 2010 were collected (Figure 2 A). In Youngs Cliff, there was a decrease in the mean abundance of blood-fed *An. quadrimaculatus* collected from 2008 to 2009 and then an increase from 2009 to 2010 (Figure 2A). The decrease in the mean abundance of blood-fed *An. quadrimaculatus* collected from 2008 to 2009 and then the increase from 2009 to 2010 was not significant. There was also no significant difference in the mean abundance of blood-fed *An. quadrimaculatus* collected in 2008 and 2010. The mean abundance of blood-fed *An. punctipennis* was also stable across

2008, 2009 and 2010 (Figure 2A). Additionally, there was a significant difference in the mean abundance between blood-fed *An. quadrimaculatus* and *An. punctipennis* collected in 2008 (t = 3.353, df = 51, P value = 0.0015) and 2010 (t = 4.073, df = 51, P value=0.0002), with blood-fed *An. quadrimaculatus* being more abundant as compared to *Anopheles punctipennis*. There was no significant difference in the mean abundance of blood-fed *An. quadrimaculatus* and blood-fed *An. punctipennis* collected in 2009.

A closer look at the total abundance during the collection dates in 2008 showed a spike in blood-fed *An. quadrimaculatus* on 27 August in 2008, 24 September 2009, 13 August 2010 and 9 September 2010 (Figure 2B). Blood-fed *An. punctipennis* abundance was relatively stable across the collection dates in 2008, 2009, and 2010 (Figure 2B).

Mosquito abundance in Potomac Drive

In 2008, the total mosquitoes collected from Potomac Drive were 1124, with 215 (19.2 %) blood-fed *An. quadrimaculatus* and 18 (1.6 %) blood-fed *An. punctipennis* (Table 5). In 2009, the total mosquitoes collected from Potomac Drive were 523, with 157 (30 %) blood-fed *An. quadrimaculatus* and 60 (11.5 %) blood-fed *An. punctipennis* (Table 5). In 2010, the total mosquitoes collected from Potomac Drive were 860, with 434 (50.5 %) blood-fed *An. quadrimaculatus* and 11 (1.3 %) blood-fed *An. punctipennis* (Table 5). The total blood-fed *An.quadrimaculatus* collected from 2008 to 2010 at Potomac Drive was 215 in 2008, 157 in 2009 and 434 in 2010 and *An. punctipennis* was 18 in 2008, 60 in 2009 and 11 in 2010 (Figure 3 A). In Potomac Drive, there was a decrease in the mean abundance of blood-fed *An. quadrimaculatus* collected from 2008 to 2009 and then an increase from 2009 to 2010. The decrease in the mean abundance of blood-fed *An. quadrimaculatus* collected from 2008 to 2009 and then the increase from 2009 to 2010 was not significant. There was also no significant difference in the mean abundance of blood-fed *An. quadrimaculatus* collected in 2008 and 2010. There was no significant difference in the mean abundance of blood-fed *An. punctipennis*

collected in 2008, 2009 and 2010 (Figure 3A). Additionally, there was a significant difference in the mean abundance between blood-fed *An. quadrimaculatus* and blood-fed *An. punctipennis* only in 2010 (t = 3.529, df = 52, P value = 0.0009), with blood-fed *An. quadrimaculatus* being more abundant as compared to blood-fed *An. punctipennis*. There was no significant difference in the mean abundance and the total abundance of blood-fed *An. quadrimaculatus* and blood-fed *An. punctipennis* collected in 2008 and 2009 (Figure 3A).

A closer look at the total abundance during the collection dates in 2008 showed a spike in blood-fed *An. quadrimaculatus* around the 5 September in 2008, 12 August 2009, 6 August 2010 and 3 September 2010 (Figure 3B). Blood-fed *An. punctipennis* abundance was relatively stable across the collection dates in 2008, 2009, and 2010 (Figure 3B).

Seventy pools out of 194 in 2008 that were tested to see if they are of the quality required for PCR by using primers against the white gene of *An. gambiae* did not produce amplicons. Therefore all the 2008 samples were not available for PCR to detect human *Plasmodium* and avian *Plasmodium* and *Haemoproteus.*

Presence of human *Plasmodium* in mosquitoes collected in Loudoun County, Virginia

To determine whether the mosquitoes collected in 2009 and 2010 were infected with human *Plasmodium vivax, falciparum, ovale and malariae*, 71 pools of blood-fed *Anopheles* spp. collected in 2009 and 143 pools collected in 2010 were tested with primers for human *Plasmodium vivax, falciparum, ovale* and *malariae*. We found that all the pools of mosquitoes collected in 2009 and 2010 tested negative for presence of human *Plasmodium.*

Presence of avian blood parasites in mosquitoes collected in Loudoun County, Virginia

To determine whether the mosquitoes collected in 2009 and 2010 were infected with avian *Plasmodium* and *Haemoproteus*, 71 pools collected in 2009 and 143 pools collected in 2010 were tested by PCR. None of the 143 pools in 2010 tested positive with primers for haemosporidian parasites (Table 7). In contrast, in 2009, 20 (28%) pools out of 71 pools tested

positive with primers for haemosporidian parasites (Table 8). Out of these 20 pools, 10 (50%) pools were from Algonkian Regional Park, 6 (30%) pools were from Youngs Cliff, and 4 (20%) pools were from Potomac Drive (Table 7). The pools that tested positive at Algonkian Regional Park were all made up of *An. quadrimaculatus.* At Youngs Cliff, 4 (20%) pools were of *An. quadrimaculatus* and 2 (10%) pools were of *An. punctipennis* (Table 8). At Potomac Drive 4 (20%) pools were of *An. quadrimaculatus* having 10 mosquitoes each (Table 7). At Algonkian Regional Park, 9 pools had 10 mosquitoes each while 1 pool had 6 mosquitoes (Table 8). At Youngs Cliff, 3 pools had 10 mosquitoes each while 1 pool had 8 mosquitoes of *An. quadrimaculatus* and the 2 pools of *An. punctipennis* had 1 mosquito each (Table 9). At Potomac Drive, all 4 pools had 10 mosquitoes each (Table 8). Out of the 20 pools in 2009 that tested positive with primers for haemosporidian parasites, only 1 (5%) pool tested positive with primers for *Haemoproteus* (Table 9). This pool was from Algonkian Regional Park and was of *An. quadrimaculatus* and had 6 mosquitoes. Out of the 20 pools in 2009 that tested positive with primers for haemosporidian parasites, 4 (20%) pools tested positive with primers for *Plasmodium* (Table 9). Of these, 3 pools were from Algonkian Regional Park and were of *An. quadrimaculatus* having10 mosquitoes each and 1 pool was from Youngs Cliff and was of *An. punctipennis* having 1 mosquito. Out of the 20 pools in 2009 that tested positive with primers for haemosporidian parasites, 11 (55%) pools tested positive with primers for *Haemoproteus* and *Plasmodium* (Table 9). Of these, 4 pools were from Algonkian Regional Park and were of *An. quadrimaculatus* having 10 mosquitoes each, 3 pools from Youngs Cliff, of which, 2 pools were composed of *An. quadrimaculatus* having 8 and 10 mosquitoes, respectively. One pool was of *An. punctipennis* with 1 mosquito. 4 pools were from Potomac Drive and were of *An. quadrimaculatus* having 10 mosquitoes each (Table 9). Out of the 20 pools in 2009 that tested positive with primers for haemosporidian parasites, 4 (20%) pools tested negative with primers for both *Haemoproteus* and *Plasmodium.* Two of these pools were collected at Algonkian

Regional Park and the other 2 were collected at Youngs Cliff. All of them were composed of *An. quadrimaculatus* and had 10 mosquitoes each. In summary, Algonkian Regional Park had a high number of mosquitoes infected with *Haemoproteus* and avian *Plasmodium* and *Haemoproteus* as compared to Youngs Cliff and Potomac Drive.

Anopheles **Mosquito Infection Rates**

The total infection rate for *Anopheles* mosquitoes regardless of the site and species was 47.74% (Table 10). When considering species regardless of sites, *An. quadrimaculatus* had a higher infection rate of 50.17% compared to *An. punctipennis* with 31.92% (Table 10). When considering sites regardless of species, Youngs cliff had the highest infection rate of 74.50% followed by Algonkian Regional Park with 53.86% and then Potomac drive with 25.01% (Table 10). When considering individual sites, at Algonkian regional park, *An. quadrimaculatus* had an infection rate of 57.95% compared to 0% for *An. punctipennis*, at Potomac drive *An. quadrimaculatus* had an infection rate of 32.56 % compared to 0% for *An. punctipennis* and at Youngs cliff *An. punctipennis* had a higher infection rate of 182.88% compared to 56.06% for *An. quadrimaculatus* (Table 10).

DISCUSSION

Blood-fed *Anopheles quadrimaculatus* was higher in abundance as compared to *An. punctipennis* at all the three sites. *An. quadrimaculatus* is more abundant during mid to late summer (O' Malley, 1992) whereas *An. punctipennis* is more abundant in spring and early summer (Goddard et al, 2010). This was also confirmed in our study. Mosquito collections can be carried out around spring and early summer to assess whether there is any shift in the abundance of *An. quadrimaculatus* and *An. punctipennis* at a different time of the year.

The pools of blood-fed *Anopheles* spp. mosquitoes collected in 2010 from the three sites in Loudoun County, Northern Virginia, tested negative for avian *Plasmodium* and *Haemoproteus* parasites. Twenty (28%) pools out of 71 in 2009 tested positive for avian *Plasmodium* and *Haemoproteus* parasites. We found that in 2009 Algokian Regional Park had a high number of mosquito pools infected with avian *Plasmodium* and *Haemoproteus* as compared to Youngs Cliff and Potomac Drive. Also, majority of the pools that were infected with avian *Plasmodium* and *Haemoproteus* parasites were of blood-fed *An. quadrimaculatus.*

Blood meal analysis can provide information on host abundance, diversity, and preference (Kent, 2009 and Molei et al 2006). Therefore, identification of host blood meals in addition to detection of parasites in the vector helps us understand disease transmission. This information can be used to control current outbreaks and predict the risk of future outbreaks.

Since only the mosquito blood meals were analyzed, the only conclusion that can be drawn from this study is that the blood that mosquitoes carry is infected. We have not determined their capacity to transmit the infection. For this purpose the salivary glands of the mosquitoes in the pools that tested positive will have to be analyzed.

A major limitation of this study was that pooling of mosquitoes reduced the sample size considerably. Statistical analysis using repeated measures ANOVA is not an appropriate

statistical test for this study since the mosquitoes collected across the three sites each year was different. Only a student *t* test could be performed to obtain p-values and compare the total and mean abundance of blood-fed *An. quadrimaculatus* and *An. punctipennis* at the three sites over the three years.

Additionally, while mosquito collection was being carried out it was important to place resting boxes at different locations in the three sites to obtain representative populations of mosquitoes. However, for this study, all resting boxes were placed at one spot at the three sites for the entire collection period and therefore the *Anopheles* spp. mosquitoes collected might not have been representative of the entire area.

Geographical information systems (GIS) analysis (John Deemy, unpublished) revealed that Algonkian Regional Park had more standing water bodies as compared to Youngs Cliff and Potomac Drive (John Deemy, unpublished). Algonkian Regional Park is also very close to large tracts of farmland indicating extensive use of pesticides and insecticides (John Deemy, unpublished). But this does not appear to have had any impact on mosquito breeding at this site (John Deemy, unpublished). There is a huge spike in the number of mosquitoes collected at Algonkian Regional Park in 2008 in August as compared to Youngs Cliff and Potomac Drive. Also, the mosquito abundance in August 2009 and 2010 at Algonkian Regional Park appears to be lower as compared to 2008.

Although we found no evidence of human malaria parasites in the blood-fed *Anopheles* spp. mosquitoes collected from the three sites in Loudoun County, Northern Virginia, we cannot conclude that *Anopheles* spp. mosquitoes in the area are not infected with human *Plasmodium,* since the resting boxes collected only a few of the *Anopheles* spp. mosquitoes in the area.

The pools of blood-fed *Anopheles* spp. mosquitoes collected in 2010 from the three sites in Loudoun County, Northern Virginia, tested negative for avian *Plasmodium* and *Haemoproteus* parasites. Out of the twenty pools (28%) in 2009 that tested positive for avian

Plasmodium and *Haemoproteus* parasites we found that Algokian Regional Park had a high number of mosquito pools infected with avian *Plasmodium* and *Haemoproteus* as compared to Youngs Cliff and Potomac Drive. This might be important since Algonkian Regional Park is the nearest to the site of the 2002 local human malaria transmission (1.16 miles). Although the number of blood-fed *An. quadrimaculatus* pools which tested positive for avian *Plasmodium* and *Haemoproteus* were higher as compared to *An. punctipennis* pools, we cannot conclude that *Anopheles quadrimaculatus* showed a higher infection rate as compared to *An. punctipennis* since the pool size for *An. quadrimaculatus* was larger as compared to *An. punctipennis*. For 11 (55%) pools that tested positive for both avian *Plasmodium* and *Haemoproteus*, we cannot determine if they were mixed infection since the pools had several mosquitoes. The four pools that tested positive for haemosporidian parasites but tested negative for *Haemoproteus* and *Plasmodium*, could be infected with *Leucocytozoon,* since the order *Haemosporidiae* include the genera *Haemoproteus, Plasmodium,* and *Leucocytozoon.*

The maximum likelihood estimation (MLE) of mosquito infection rates based on the Biggerstaff (2006) method showed that among the three sites Youngs cliff had the overall highest infection rate for *Anopheles* mosquitoes compared to Algonkian Regional Park and Potomac drive. At Algonkian Regional Park and Potomac drive, *An. quadrimaculatus* had a higher infection rate compared to *An. puncipennis,* while at Youngs cliff *An. puncipennis* had a higher infection rate compared to *An. quadrimaculatus.*

Anopheles spp. may be competent vectors for avian malaria as demonstrated by Hunninen et al. 1950. Blood meal analysis carried out in this study show that the *Anopheles* spp. *mosquitoes* are feeding on hosts infected with *Heamoproteus* spp. parasites. As stated earlier, *Haemoproteus* spp. parasites are transmitted by blood sucking midges of the genus *Culicoides* (Bonneaud et al. 2009, Kettle, 1982, Valkiunas and Iezhova, 2004, and Martinsen, Perkins and Schall, 2008). Mosquitoes are not known to be vectors for these parasites.

Leucocytozoon, another haemosporidian parasite infecting birds is transmitted by black flies of the genus *Simuliidae* (Martinsen et al., 2008). Presence of *Haemoproteus* and suspected *Leucocytozoon* parasites in some of the mosquito pools collected in Loudoun County, Northern Virginia indicates the presence of hosts in the area carrying these parasites. This could shed information about the risk of outbreaks of different kinds diseases that affect birds in the area. Also, *Plasmodium* spp. and in particular, *Haemoproteus* spp. are highly host specific (Atkinson and van Riper, 1991 as cited in Waldenström et al., 2002). Sequencing to determine the species of *Plasmodium* and *Haemoproteus* could therefore provide information about the hosts in the area.

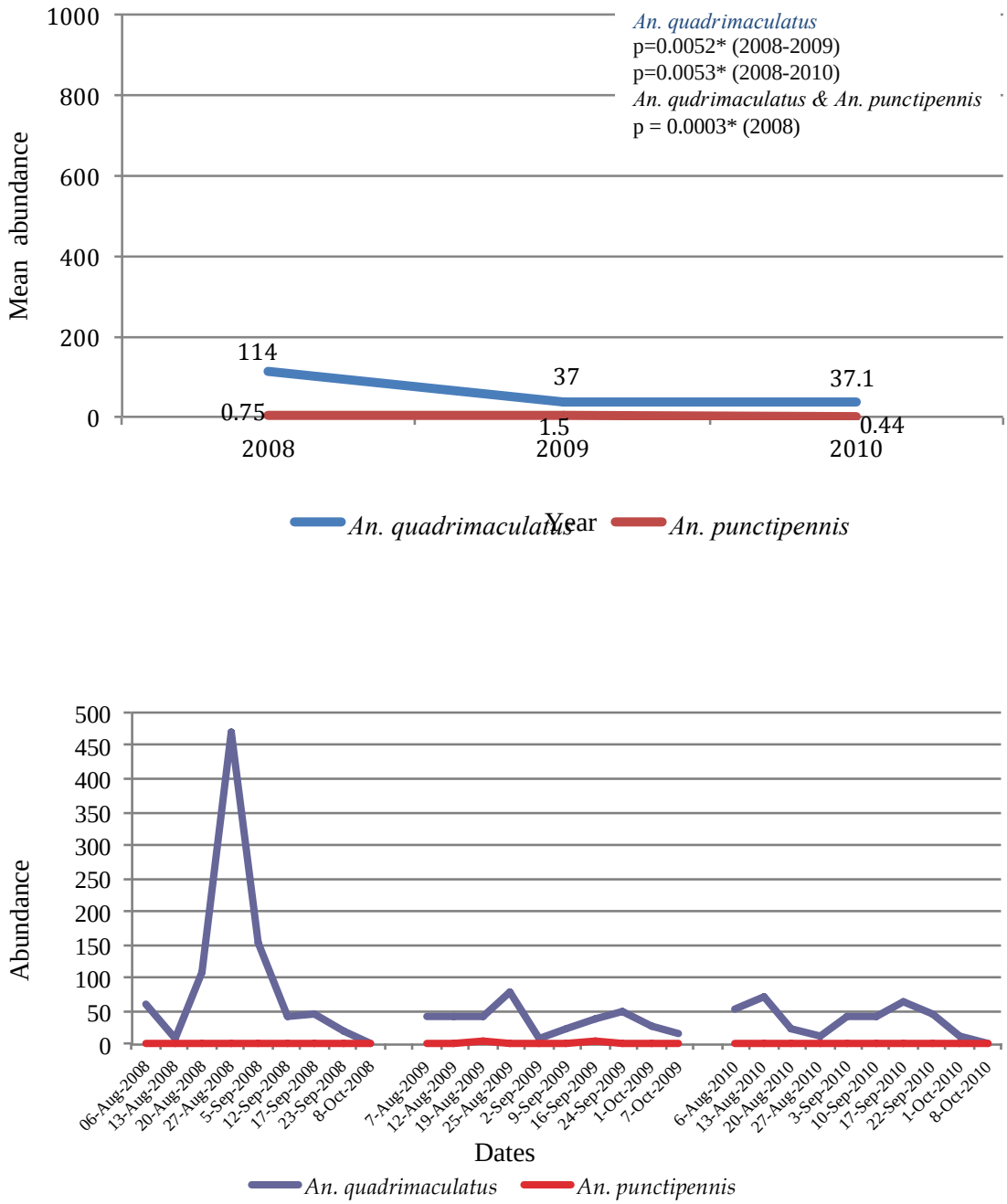

Figure 1 A. Mean abundance of blood-fed *Anopheles quadrimaculatus* and *Anopheles punctipennis* collected at Algonkian between 2008 and 2010. *Anopheles quadrimaculatus* is the dominant species in Algonkian during the collection period. B. Number of mosquitoes collected at Algonkian on each collection date. *Anopheles quadrimaculatus* is the dominant species in Algonkian during the collection period.

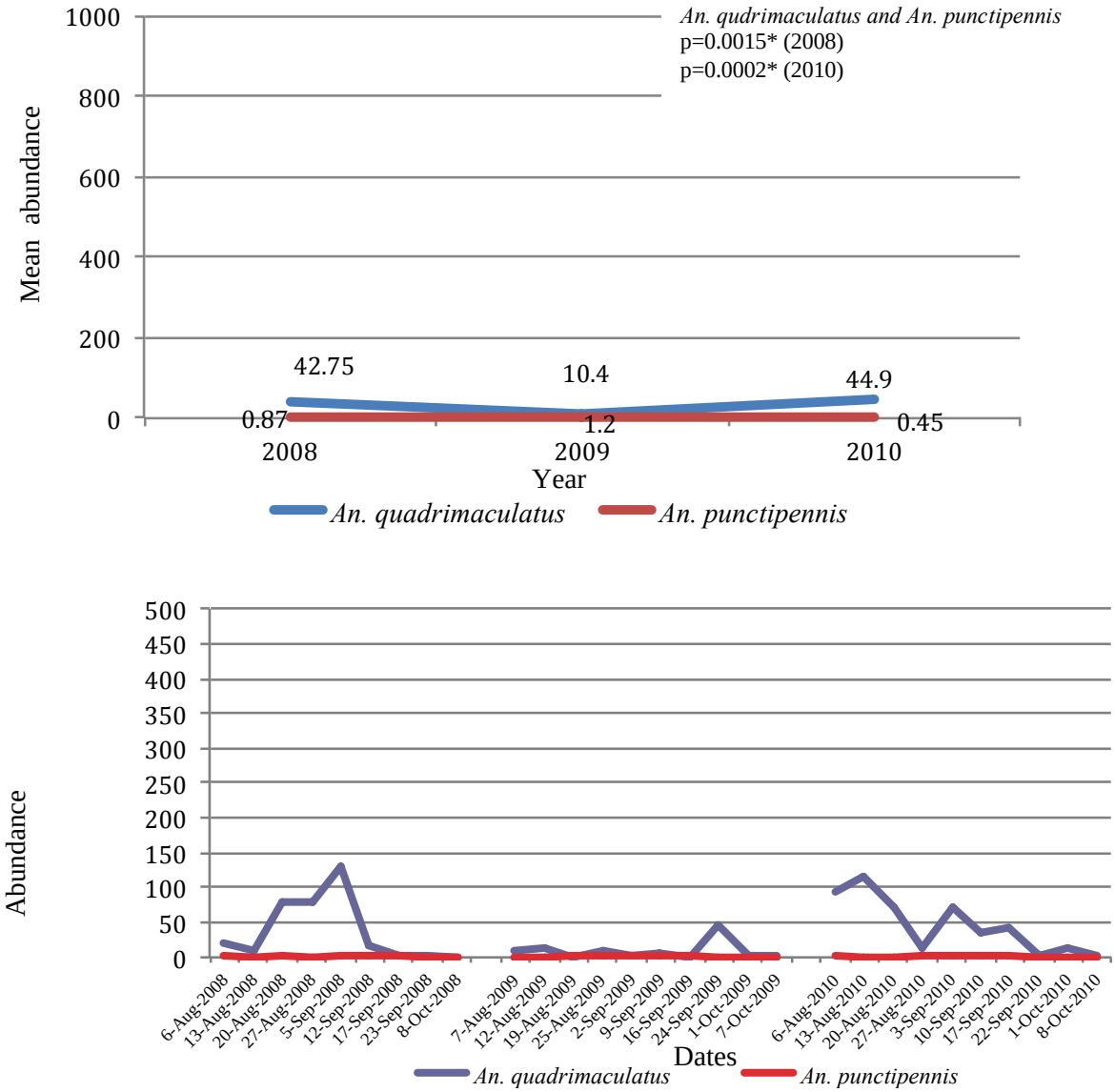

Figure 2 A. Mean abundance of blood-fed *Anopheles quadrimaculatus* and *Anopheles punctipennis* collected at Youngs Cliff between 2008 and 2010. *Anopheles quadrimaculatus* is the dominant species in Youngs Cliff during the collection period. B Number of mosquitoes collected at Youngs Cliff on each collection date. *Anopheles quadrimaculatus* is the dominant species in Youngs Cliff during the collection period.

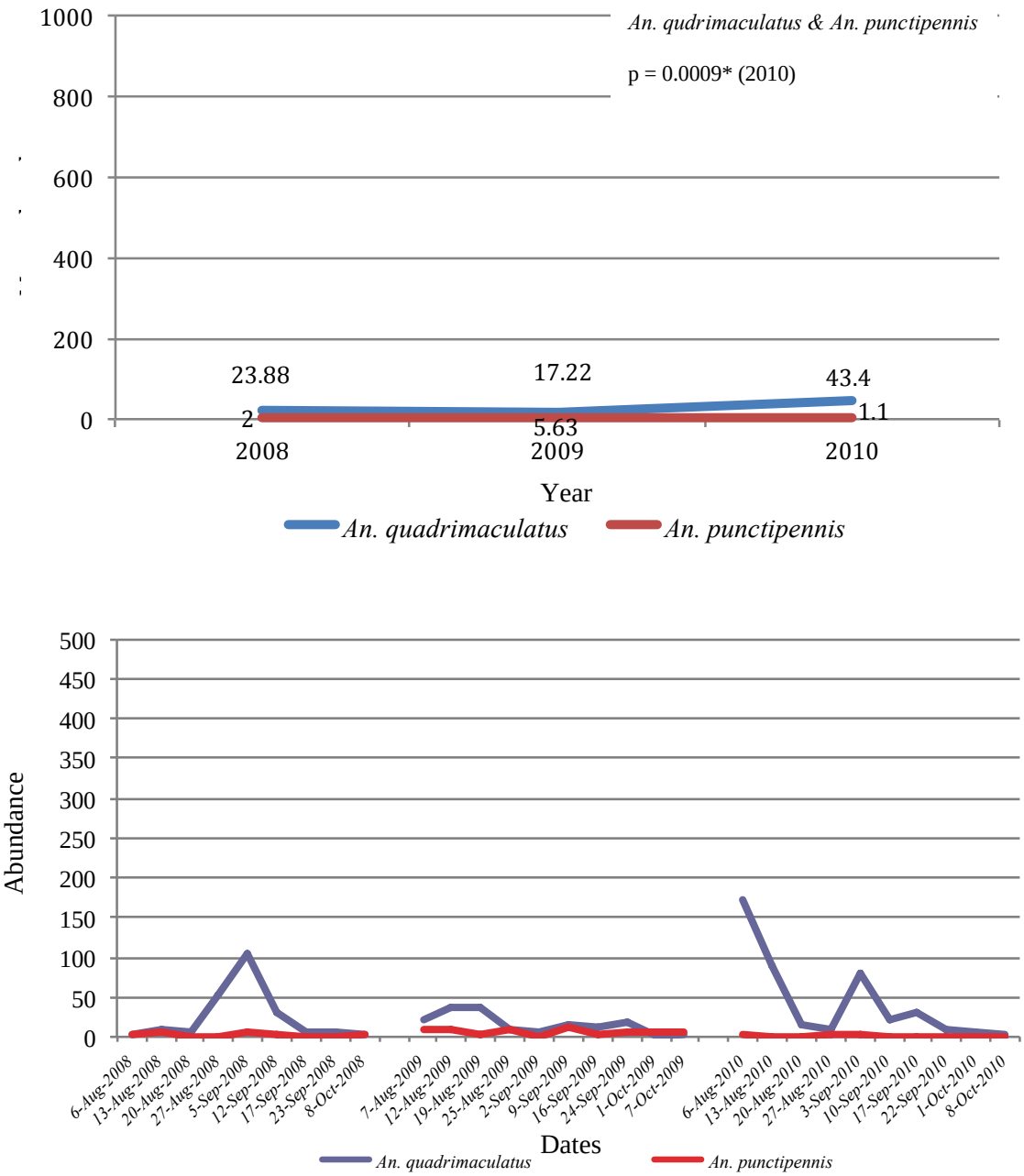

Figure 3 A. Total abundance of blood-fed *Anopheles quadrimaculatus* and *Anopheles punctipennis* collected at Potomac Drive between 2008 and 2010. *Anopheles quadrimaculatus* is the dominant species in Potomac Drive during the collection period. B. Number of mosquitoes collected at Potomac Drive on the each collection date. *Anopheles quadrimaculatus* is the dominant species in Potomac Drive during the collection period.

Table 1 Emerging Infectious Diseases of the 21st century

Disease	**Hosts**	**Causative agent**	**Vector**
Malaria	Humans	*Plasmodium vivax, Plasmodium falciparum, Plasmodium ovale, Plasmodium malariae*	*Anopheles spp.* (CDC, 2010)
	Birds	*P. gallinaceum, P. hermansi, P. relictum, P. cathemerium, P. elongatum*	*Culex spp.* (USGS, 2011) (Talat, 2005) (Valkiunas, 2005)
West Nile disease	Birds (amplifying hosts), Mammals (dead-end hosts)	West Nile virus	*Culex pipiens, Culex quinquefasciatus* (Hayes et al., 2005)
Dengue	Humans, other primates	Dengue virus	*Aedes ageypti, Aedes albopictus* (WHO, 2011) (CDC, 2011)
Rift Valley fever	Cattle, sheep, humans	Rift Valley fever virus	*Aedes spp.* (CDC, 2007)
Filarial diseases	Humans	*Wuchereria bancrofti, Brugia malayi*	*Culex spp., Aedes spp., Anopheles spp., Mansonia spp.* (CDC, 2010)
Chicungunya	Humans, other primates	Chicungunya virus	*Aedes ageypti, Aedes albopictus* (Staples et al., 2009)
Yellow fever	Humans, other primates	Yellow fever virus	*Aedes ageypti,* (CDC, 2007)
Japanese encephalitis	Birds (amplifying hosts), Humans, cattle, horses (dead-end hosts)	Japanese encephalitis virus	*Culex tritaeniorhynchus* group (CDC, 2010)

Table 2 *Anopheles* species distribution

Region	**Species**
US, Canada, Mexico	*An. quadrimaculatus, An. punctipennis, An. freeborni, An. walkeri, An. earlie*
Central and South America	*An. albimanus, An. pseudopunctipennis, An. darlingi, An. aquasalis*
Africa	*An.gambiae, An. funestus, An. arabiensis*
Asia	*An. stephensi, An. fluviatilis, An. annularis*
Southeast Asia	*An. dirus, An. maculatus, An. fluviarostris*

Source – CDC - 03/27/2008

Table 3 Collection dates of mosquitoes from 3 sites in 3 years in Loudoun County, Northern Virginia between 2008-2010

	Algonkian Regional Park	**Youngs Cliff**	**Potomac Drive**
Time of collection	(1200 to 1600 hrs.)	(1200 to 1600 hrs.)	(1200 to 1600 hrs.)
2008			
	8/06	8/06	8/06
	8/13	8/13	8/13
	8/20	8/20	8/20
	8/27	8/27	8/27
	9/05	9/05	9/05
	9/12	9/12	9/12
	9/17	9/17	9/17
	9/23	9/23	9/23
	10/8	10/08	10/08
2009			
	8/07	8/07	8/07
	8/12	8/12	8/12
	8/19	8/19	8/19
	8/25	8/25	8/25
	9/02	9/02	9/02
	9/09	9/09	9/09
	9/16	9/16	9/16
	9/24	9/24	9/24
	10/01	10/01	10/01
	10/07	10/07	10/07
2010			
	8/06	8/06	8/06
	8/13	8/13	8/13
	8/20	8/20	8/20
	8/27	8/27	8/27
	9/03	9/03	9/03
	9/10	9/10	9/10
	9/17	9/17	9/17
	9/22	9/22	9/22
	10/01	10/01	10/01
	10/08	10/08	10/08

Table 4 Pool sizes of blood fed *Anopheles* mosquitoes collected from 3 sites in Loudoun County, Northern Virginia over 3 years

Pool size	**2008**		**2009**		**2010**	
	An. quadrimaculatus	*An. punctipennis*	*An. quadrimacultus*	*An. punctipennis*	*An. quadrimacultus*	*An. punctipennis*
1	0	6	4	6	4	5
2	2	3	2	6	5	3
3	3	2	2	1	4	1
4	3	0	1	2	6	1
5	5	2	4	1	3	0
6	2	0	3	2	0	0
7	1	0	3	1	3	0
8	1	0	8	3	1	0
9	1	0	4	0	1	0
10	172	0	45	0	115	0

Table 5 Mosquito species collected in the 3 sites in Loudoun County, Northern Virginia over 3 years

Sites	***Anopheles species***	**2008**	**2009**	**2010**
Algonkian	Total mosquitoes collected	5708	2153	2336
	Total	2888	1299	808
	An. quadrimaculatus (bf)	912	370	371
	An. punctipennis (bf)	6	15	4
Youngs Cliff	Total	1696	331	668
	An. quadrimaculatus (bf)	342	104	449
	An. punctipennis (bf)	7	12	5
Potomac	Total	1124	523	860
	An. quadrimaculatus (bf)	215	157	434
	An. punctipennis (bf)	18	60	11

Table 6

Mean abundance of blood-fed *Anopheles* mosquitoes at 3 sites in Loudoun County, Northern Virginia between 2008 and 2010.

Sites	***Anopheles species***	**2008**		**2009**		**2010**	
		Mean	SE	Mean	SE	Mean	SE
Algonkian	*An. quadrimaculatus*	114	20.8095	37	18.612	37.10	18.612
	An. punctipennis	0.75	20.8095	1.50	18.612	0.44	19.619
Youngs cliff	*An. quadrimaculatus*	42.75	8.828	10.40	7.896	44.90	7.896
	An. punctipennis	0.87	8.828	1.20	7.896	0.45	7.529
Potomac							
	An. quadrimaculatus	23.88	8.932	17.22	8.932	43.40	8.473
	An. punctipennis	2	8.932	5.63	8.079	1.10	8.473

Table 7 Presence of human *Plasmodium* in the *Anopheles* mosquitoes collected between 2008-2010

Year	**2008**	**2009**	**2010**
P. falciparum	NA	0	0
P. vivax	NA	0	0
P. ovale	NA	0	0
P. malariae	NA	0	0

Table 8 Haemosporidian parasites in the *Anopheles* mosquitoes collected between 2009-2010

	2009		**2010**	
Haemosporidiae	20/71=28%		0/143=0%	
	An. quadrimaculatus	*An. punctipennis*	*An. quadrimaculatus*	*An. punctipennis*
Algonkian	10/20=50%	0/20=0%	0%	0%
Youngs Cliff	4/20=20%	2/20=10%	0%	0%
Potomac	4/20=20%	0/20=0%	0%	0%

Table 9 Haemosporidian parasites *Haemoproteus***,** *Plasmodium* in *Anopheles* mosquitoes collected in 2009

Sites	*Haemoproteus* only		*Plasmodium* only		*Haemoproteus* and *Plasmodium*	
	An. quadrimaculatus	*An. punctipennis*	*An. quadrimaculatus*	*An. punctipennis*	*An. quadrimaculatus*	*An. punctipennis*
Algonkian	1 (6%)	0	3 (19%)	0	4 (25%)	0
Youngs Cliff	0	0	0	1 (6%)	2 (13%)	1 (6%)
Potomac	0	0	0	0	4 (25%)	0

Table 10 *Anopheles* Mosquito Infection Rates (MIR)

Total (irrespective of site and species)	Infection rate	Lower limit	Upper limit	Scale	Point estimate method	CI method	Number of pools	Number of positive pools	Number of individuals
	47.74	30.28	72.19	1,000	Bias Corrected MLE	Corrected Score	71	20	500
Species regardless of site									
An. punctipennis	31.92	6.19	98.08	1,000	Bias Corrected MLE	Corrected Score	16	2	60
An. quadrimaculatus	50.17	30.93	77.76	1,000	Bias Corrected MLE	Corrected Score	55	18	440
Site regardless of species									
Algonkian	53.86	27.77	96.25	1,000	Bias Corrected MLE	Corrected Score	30	10	229
Potomac	25.01	8.17	60.37	1,000	Bias Corrected MLE	Corrected Score	25	4	175
Youngs cliff	74.50	32.34	152.11	1,000	Bias Corrected MLE	Corrected Score	16	6	96
Algonkian by species									
An. punctipennis	0.00	0.00	178.84	1,000	MLE	Score	4	0	13
An. quadrimaculatus	57.95	30.00	103.50	1,000	Bias Corrected MLE	Corrected Score	26	10	216
Potomac by species									
An. punctipennis	0.00	0.00	74.42	1,000	MLE	Score	7	0	37
An. quadrimaculatus	32.56	10.68	78.79	1,000	Bias Corrected MLE	Corrected Score	18	4	138
Youngs cliff by species									
An. punctipennis	182.88	40.63	467.57	1,000	Bias Corrected MLE	Corrected Score	5	2	10
An. quadrimaculatus	56.06	18.69	136.82	1,000	Bias Corrected MLE	Corrected Score	11	4	86

Figure 4. Representative ethidium bromide stained agarose gel showing DNA from mosquito blood meal amplified with human *Plasmodium* (*vivax, falciparum, ovale* and malariae) primers. Lane 1(DNA marker), lane 2 to lane 12 (DNA extracted from blood meals of *Anopheles* mosquitoes collected in 2009 amplified with primers for human *Plasmodium* (*vivax, falciparum, ovale* and malariae), lane16 (Positive control *Plasmodium vivax* DNA, expected product size 300 bp), lane 17 (Positive control *Plasmodium falciparum* DNA, expected product size 276 bp), lane 18 (Positive control *Plasmodium malariae* DNA, expected product size 412 bp), lane 19 Negative control.

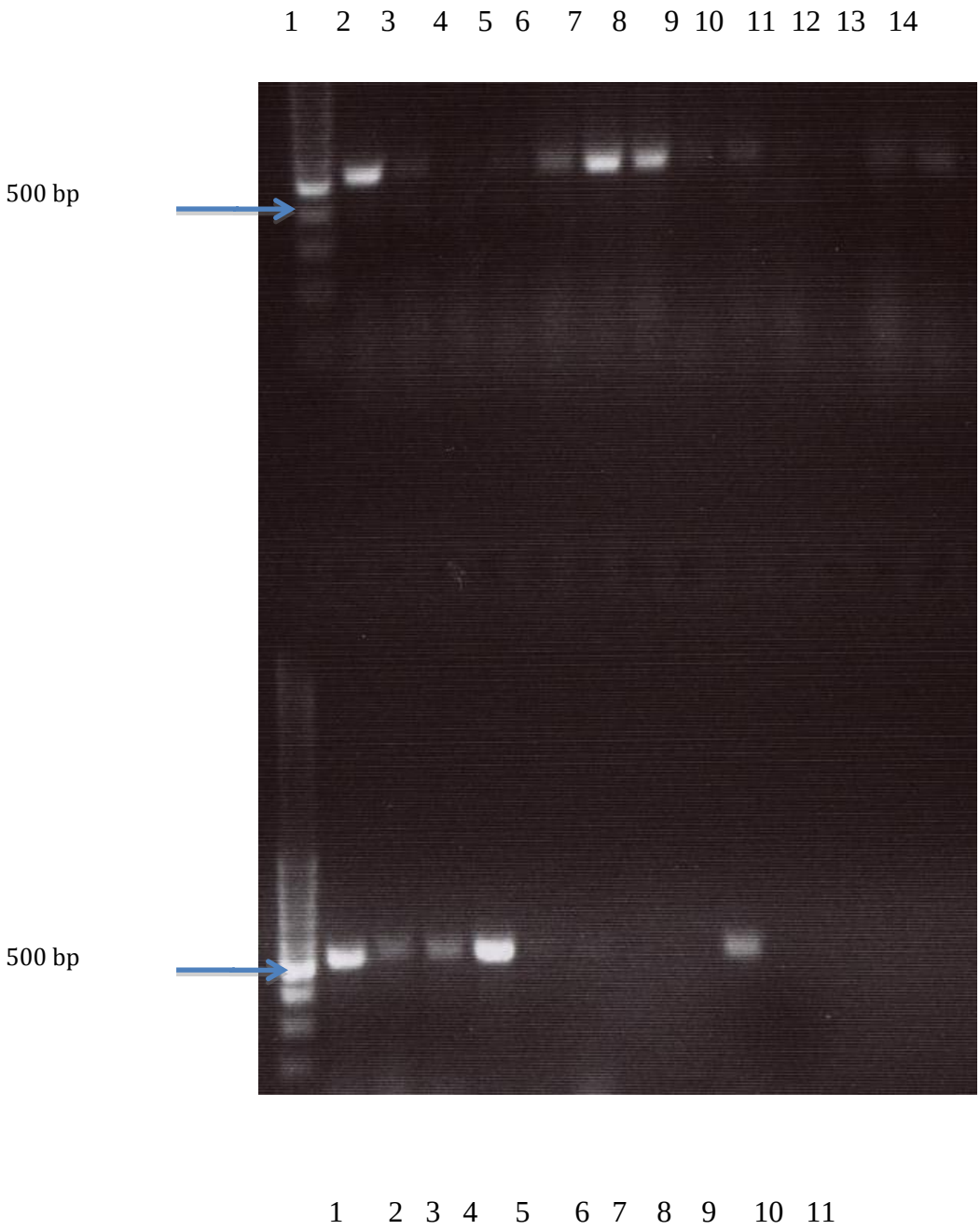

Figure 5 Representative ethidium bromide stained agarose gel showing DNA from mosquito blood meal amplified with avian *Haemosporidiae* primers. Row 1: lane 1(DNA marker), lane 2 to lane 14 (DNA extracted from blood meals of *Anopheles* mosquitoes collected in 2009 amplified with primers for avian *haemosporidiae* expected product size 580 bp), Row 2: lane 1(DNA marker), lane 2 to lane 9 (DNA extracted from blood meals of *Anopheles* mosquitoes collected in 2009 amplified with primers for avian *haemosporidiae* expected product size 580 bp), lane 10 (Positive control bird blood), lane 11 (Negative control).

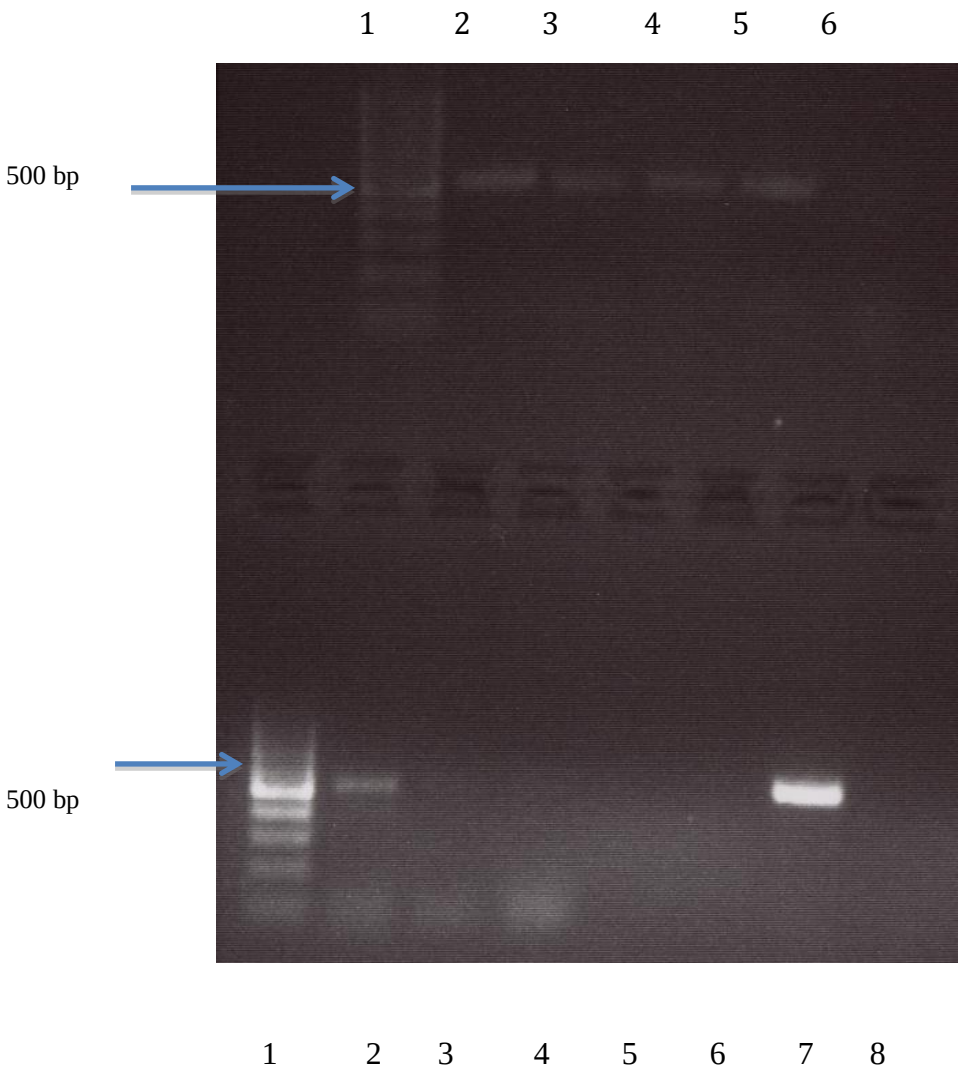

Figure 6. Representative ethidium bromide stained agarose gel showing DNA from mosquito blood meal amplified with avian *Haemoproteus* primers. Row 1: lane 1(DNA marker), lane 2 to lane 6 (DNA extracted from blood meals of *Anopheles* mosquitoes collected in 2009 amplified with primers for avian *heamoproteus* expected product size 524 bp), Row 2: lane 1(DNA marker), lane 2 to lane 4 (DNA extracted from blood meals of *Anopheles* mosquitoes collected in 2009 amplified with primers for avian *heamoproteus* expected product size 524 bp), lane 7 (Positive control bird blood), lane 8 (Negative control).

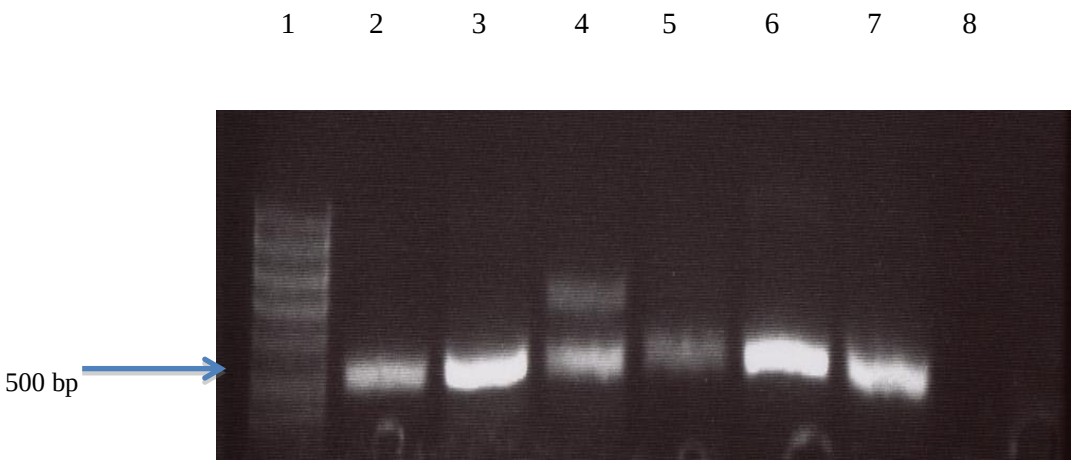

Figure 7. Representative ethidium bromide stained agarose gel showing DNA from mosquito blood meal amplified with avian *Plasmodium* primers. Lane 1 (DNA marker), lane 2 to lane 6 (DNA extracted from blood meals of *Anopheles* mosquitoes collected in 2009 amplified with primers for avian *Plasmodium*, expected product size 524 bp), lane 7 (Positive control bird blood), lane 8 (Negative control).

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VITA

Priya Krishnan was born in India and completed her high school studies in 1993 from D.T.E.A School. She then enrolled for Undergraduate program in Biological Sciences at the University of Delhi with a goal to pursue a career in institutions and organizations specializing in areas related to Biological Sciences. She was conferred the Bachelor of Science degree in 1996 after which, she worked on a research project on Vesicular Arbuscular Mycorrhizal (VAM) fungi as a lab technician in the School of Life Sciences, Jawaharlal Nehru University, New Delhi until 1997. With a keen desire to get an exposure on Business Administration and Management, she registered for Chartered Accountancy, which is equivalent of US CPA (Certified Professional Accountancy) in Delhi in 2000 and learnt several dimensions of Accounting and Finance Management in the 3 years of internship that followed in the program. She enrolled for MS program in Biological Sciences at Virginia Commonwealth University (VCU) in January 2010 and was awarded a Graduate Teaching Assistantship by the Department of Biology at VCU for the duration of the program. Priya Krishnan graduated from Virginia Commonwealth University with a Master of Science degree in Biology in Fall 2011.