PRESENCE OF *Wolbachia*, A BIOCONTROL AGENT: SCREENING FOR VERTEBRATE BLOOD MEAL SOURCE AND WEST NILE VIRUS IN MOSQUITOES IN THE NORTH TEXAS REGION

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West Nile virus (WNV) is a geographically endemic mosquito-borne flavivirus that has spread across the United States infecting birds, mosquitos, humans, horses and other mammals. The wide spread nature of this virus is due to the ability of the mosquito vector to persist in broad, ecological diverse environments across the United States. In this study, mosquito populations in North Texas region were sampled for detection of *Wolbachia*, blood meal source, and WNV. The ultimate goal of this study was to examine the potential of a biocontrol agent, *Wolbachia* sp. that colonizes the hindgut of various insects, including mosquitos, as a natural means to interrupt virus transmission from mosquitos to other hosts, including humans. In Australia, *Wolbachia* sp. from fruit flies (*Drosophila melanogaster*) have been successfully used to block transmission of a similar pathogenic virus from mosquitos responsible for transmission of Dengue fever. Here, mosquitoes were collected using CDC style Gravid Traps in Denton, Texas, from October 2012 through September 2014. Collected mosquitoes were identified, sexed, and categorized as to the amount of host blood in their alimentary system using a Zeiss Axio Zoom microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY)**.** *Culex quinquefaciatus* was the dominant blood engorged species collected. Smaller populations of *Culex tarsalis* and *Aedes albopictus*, another known vector for WNV were also collected. Mosquito larva were also collected from the UNT water research field station and reared to adults. *Cx. tarsalis* was the dominant mosquito taken from this habitat. Samples of *Cx. quinquefasciatus*, *Cx. tarsalis* and *A. albopictus* were analyzed for *Wolbachia sp.* and to identify host blood in the mosquito

alimentary system. Total DNA extraction from the pool of mosquito samples was by both commercially available DNA extraction kits (Qiagen, Valencia, CA) and salt extraction technique. Polymerase chain reaction (PCR) was used to amplify and identify *Wolbachia* sp. 16SrDNA and mitochondrial DNA from vertebrate blood. The maternally inherited endosymbiont, *Wolbachia*, were found to be uniformly distributed across the mosquitoes sampled in this study. Blood meal analysis by PCR showed that *Cx. quinquefaciatus* fed more on birds than on mammalian blood sources based on the previously developed primers used in this study.

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CHAPTER 1

INTRODUCTION

West Nile virus (WNV), like other members of the genus *Flavivirus* within the family *Flaviviridae*, including, dengue, yellow fever virus, Japanese encephalitis virus, and tick-borne encephalitis virus, cause substantial human diseases (Burke and Monath 2001). WNV is a mosquito-borne flavivirus that is indigenous to Africa, Asia, Europe, and Australia, and subsequently caused large epidemics in Romania, Russia, and Israel (Campbell *et al.* 2002). The virus was first isolated in 1937 from the blood of a febrile patient in the West Nile district of northern Uganda (Smithburn *et al.* 1940). In the 1950's WNV was linked to epidemics of fever and encephalitis in the Middle East (Taylor *et al.* 1956; Paz 2006). Subsequently, sporadic outbreaks of human disease across regions of Africa, the Middle East, India, Europe and Asia have been attributed to WNV (Hubálek and Halouzka 1999). WNV outbreaks continued to spread across different regions of the world, spreading into the Mediterranean Basin, Romania and the Volga delta in southern Russia during the mid to late 1990's. The virus eventually drifted towards the western hemisphere (Hayes *et al.* 2005).

In 1999, the first locally acquired WNV disease in humans was detected in Queens, New York City, United State (U.S) (Nash *et al.* 2001). Following the first incursion of the virus into the U.S, WNV rapidly spread across the country, and became established with sustained transmission by 2005. The overall distribution of WNV during this time has extended from central Canada to southern Argentina (Gubler 2007). As of 2012, WNV has been detected in 65 different mosquito species in the U.S. (CDC 2012a) (http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm). In the summer of

2012, there was a historical WNV outbreak in the Dallas/Fort Worth area of North Texas. A total of 1,868 cases were reported for the entire state of Texas, 844 (45%) were neuroinvasive diseases, mostly among immuno-compromised individuals, usually adult males. There were 89 deaths (5% fatality rate) recorded. Forty-eight percent of case-patients resided in 4 counties around the Dallas/Fort Worth area (396 cases in Dallas, 259 cases in Tarrant, 64 cases in Collin and 183 cases in Denton counties) (Murray *et al.* 2013).

Important mosquito vectors of WNV appear to be the *Culex* species, the mosquito species that drives epizootic and epidemic transmission. The distribution of vectors of WNV varies across the different regions in the U.S. *Cx. pipiens* is found mostly in the northern half of the country, *Cx. quinquefasciatus* in the southern states, and *Cx. tarsalis* in the western states where it overlaps with the *Cx. pipiens* and *Cx. quinquefasciatus* (Andreadis *et al*. 2004; Kilpatrick *et al.* 2006a; Godsey *et al.* 2012).

Interestingly, maternally inherited endosymbiotic bacteria of the genus *Wolbachia* have been found in almost all groups of insects, including mosquitoes serving as vector for WNV. Given that these bacteria induce severe alterations in insect reproductive systems (Werren, 1997), many researchers have been attracted to investigating the theoretical mechanisms of host–bacteria interactions, as well as in applying this novel biocontrol strategy to insect borne diseases. For instance, one *Wolbachia* strain (wMelPop) has been used as a biocontrol agent to reduce the longevity of adult mosquitos, cutting the life span of the infected mosquitos in half, and resulting in the eventual decline of mosquitoes capable of transmitting pathogens (Sinkins and O'Neill 2000). Another *Wolbachia* strain known as *w*MelPop-CLA is capable of blocking viral

replication in *Aedes aegypti*. *w*MelPop-CLA virulence is not limited to mosquitoes carrying dengue virus, but also appears to help control other arboviruses such as Chikungunya virus (Moreira et al. 2009) by its action in mosquitoes. The introduced *Wolbachia* strains, which reduce the vector capacity of mosquitoes, are maintained from one mosquito generation to another through a phenomenon known as cytoplasmic incompatibility.

Cytoplasmic incompatibility is induced by *Wolbachia* in several insect species, including the mosquito *Culex pipiens*. Cytoplasmic incompatibility gives rise to a sterile mosquito progeny when mating occurs between a male mosquito infected with *Wolbachia* and female mosquitos that do not have *Wolbachia*. However, a viable progeny results when a *Wolbachia*-infected female mosquito mates with either a *Wolbachia*-infected male or uninfected male mosquito. Based on this phenomenon, a successful genetic approach to control transmission of some viruses such as dengue is being used around the world. (Moreira *et al*. 2009, Laven 1967).

1.1 *Wolbachia* Bacteria as Endosymbionts

The *Wolbachia* genus was first identified as rickettsia-like bacteria from the species of mosquito *Culex pipiens.* Therefore, the first group of *Wolbachia* identified was referred to as *Wolbachia pipiens* (Hertig and Wolbach 1924; Hertig 1936). Since then a large phylogenetic diversity has been found in *Wolbachia* that consists of deeply diverging supergroups (Bandi *et al.* 1998). The distribution of these supergroups is asymmetrical across different hosts such that: 1) Supergroups A and B are usually

detected in arthropods; 2) supergroups C and D are limited to filarial nematodes; 3) supergroup E is exclusively found in springtails; 4) supergroup F is often detected in both arthropods and nematodes; 5) only a single genus of termites has supergroup H; and 6) supergroup K is found in one spider mite species. The clustering of supergroup G is still unclear as there is controversy as to whether it should be designated to another supergroup of *Wolbachia* strains ((Lo *et al.* 2007; Lo and Evans 2007; Ros *et al*. 2009). Grouping of other strains of *Wolbachia* that are symbionts of filarial nematodes responsible for river blindness and elephantiasis in humans, is under debate as to be classified as a separate species because their biology is reasonably distinct from *Wolbachia* strains that infect insects (Bandi *et al.* 1998; Pfarr and Hoerauf, 2007). Recently, intracellular *Wolbachia* has been considered to be the most abundant endosymbiont bacteria in arthropods since they show significant global geographic distribution (Werren and Windsor 2000; Werren *et al.* 2008), and over 65% of insect species are estimated to harbor members of the *Wolbachia* groups (Hilgenboecker *et al.* 2008).

Maternal inheritance of *Wolbachia* endosymbiont bacteria infecting many arthropods, including insects, confer the capacity of manipulating the insect reproductive system thereby providing a means for *Wolbachia* intergenerational transmission and wide distribution in insect population (Werren *et al.* 2008). Their maternal inheritance is via vertical transmission from one generation to the next through eggs. The transmission mode is also linked to a number of advantageous reproductive phenotypes in an infected female, which includes cytoplasmic incompatibility (Yen and Barr 1971), feminization (Rousset *et al.* 1992), male killing (Hurst *et al.* 2000) or parthenogenesis induction and

feminization of genetic males (Werren *et al.* 2008). Cytoplasmic incompatibility is the generation of unviable progeny when uninfected females mate with a *Wolbachia* infected male (McGraw *et al*. 2002). However, viable progeny will only result from mating occurring between *Wolbachia* infected female mosquitoes and either infected or uninfected male mosquitoes, thus resulting in a selective reproductive advantage over uninfected females (Turelli and Hoffmann, 1995; Hoffmann and Turelli 1997). Cytoplasmic incompatibility phenotype is the driving mechanism through which maternally transmitted *Wolbachia* can efficiently invade host populations without being infectious or for their horizontal crossing between individuals (Turelli and Hoffmann, 1991; Hoffmann and Turelli 1997). Therefore, cytoplasmic incompatibility brought on by *Wolbachia* colonization of female insects offers a positive potential for applications to control pests and/or disease insect vectors of various human, animal and plant pathogens (Kambris *et al.* 2009; Moreira *et al.* 2009).

1.2 The Use of *Wolbachia* as a Biocontrol Agent against Mosquito-borne Diseases

Many pathogens transmitted by mosquitoes, such as dengue viruses, chikungunya viruses, malaria parasites, as well as WNV require a relatively long period of time to develop in the vector, referred to as the extrinsic incubation period (EIP), prior to transmission to a new host such as vertebrates (Watts *et al.* 1986; Rohani et al 2009). Fortunately, the EIP is quite long relative to the lifespan of an average mosquito; therefore, older mosquitos usually transmit the majority of the pathogens. Several groups have proposed the use of a *Wolbachia* strain (wMelPop) as a biocontrol agent to reduce the longevity of adult mosquitos for eventual decline of mosquitoes capable of

transmitting pathogens (Sinkins and O'Neill 2000; Brownstein *et al.* 2003; Cook *et al.* 2008). The *Wolbachia* strain wMelPop, isolated in 1997 from *Drosophila melanogaster*, was found to have a high level of virulence on its natural host, resulting in the halving of the lifespan of the fly. (Min and Benzer 1997)

Successful introduction of wMelPop into *Ae. aegypti*, a dengue vector, has been shown to reduce the vector lifespan by about 50%. At the same time, the mosquito maternally transmitted the wMelPop to its progeny with near 100% transmission efficiency (McMeniman *et al.* 2009).

Interestingly, data from various independent *Wolbachia* research groups from around the world reveal that different strains of *Wolbachia* are capable of eliciting pathogen control. wMelPop achieved this by shortening the life span of potential vectors preventing the pathogen from completing its EIP. By contrast, yhe *w*MelPop-CLA strain interrupts viral replication in the host insect, so that, viruses and other human pathogens, including dengue and chikungunya viruses, cannot be transmitted. (Hedges *et al.* 2008; Kambris *et al.* 2009, 2010; Moreira *et al.* 2009; Glaser and Meola 2010). The *w*MelPop-CLA strain seems to be the most potent *Wolbachia* isolate, so far, that interferes with viral replication. Its virulence is not limited to dengue virus in *Ae. aegypti*, but also appears to confer resistance to other arboviruses such as Chikungunya virus (Moreira *et al*. 2009). Further evidence indicates that the *w*MelPop-CLA strain inhibits the development of filarial nematodes in mosquitoes (Kambris *et al.* 2009) and avian malaria parasites (Moreira et al. 2009), implying that some *Wolbachia* strains may inhibit a broad range of human pathogens.

The interruption of pathogen life cycle, observed in natural *Drosophila* host and

in the *Ae. aegypti* artificially inoculated with *Wolbachia* strains, results in lifespan reduction of mosquitos capable of transmitting pathogens as well as completely blocking the replication of pathogens within the mosquito system, making *Wolbachia* introduction a viable bioconrol strategy (Moreira et al. 2009). It was recently observed that the native *w*Pip strain of *Wolbachia pipensis* in *Culex quinquefasciatus* has some effect on WNV (Glaser and Meola 2010). Although, this effect was much less pronounced when compared to that elicited by *Wolbachia* strains introduced from fruit fly to inhibit dengue virus in *Ae. aegypti* (Walker *et al*., unpublished observations). The mechanism through which *Wolbachia* confer resistance against pathogens, is not well understood, as viral interference is not common among all *Wolbachia* strains (Moreira *et al*. 2009; Osborne *et al.* 2009). Vectors of human pathogens such as dengue and malaria (*Ae. aegypti* and *Anopheles* mosquitoes respectively), are not naturally colonized by *Wolbachia* strains despite the fact that about 65% of insects, including different species of mosquitoes, are colonized by the endosymbiont. (Kittayapong *et al.* 2000; Ricci *et al*. 2002). This implies that many of the mosquito species that lack the ability to serve as vectors for disease agent transmission could be the result of the natural presence of *Wolbachia* endosymbiont strains capable of eliciting pest and disease control phenotypes.

Many medically important disease vectors are not naturally infected with *Wolbachia* endosymbionts or lack the presence of *Wolbachia* strains capable of expressing the desirable traits of disease and pest control, therefore there is potential to transfer strains of *Wolbachia* into new hosts in a process referred to as transinfection for the purposes of disease control (Hughes and Rasgon 2014).

Generally, two methods of transinfection have been successfully used to create arthropods that have *Wolbachia* not naturally found in their hind guts. They are:

(1) Embryo Microinjection: In this case, *Wolbachia* are injected into the posterior pole of preblastoderm embryos using a fine needle and micromanipulator or a nanoinjector after which the embryos are left to develop to adulthood, and the subsequent progeny are screened to determine if germline infection and transmission has taken place. This method is the most widespread technique used to develop transinfected lines of arthropods that are medically important; and

(2) Adult Microinjection: Adult microinjection differs in that the recipient is at the adult stage rather than the embryonic stage.

The two commonly used *Wolbachia* strains for transinfection (wMel and wMelPOP) for viral disruption in *Ae. aegypti* were originally transferred from *Drosophila melanogaster* by embryonic microinjection. Advances have been made in using *Wolbachia* as a biocontrol strategy in *Ae. aegypti*, a vector that transmit dengue virus. This achievement has been staged in open field-testing. A milestone was established by artificially creating two lines (wMel & wMelPop) of *Wolbachia pipiensis* infected *Ae. aegypti* (Hilgenboecker *et al*. 2008; McMeniman *et al*. 2009), both of which were native to *Drosophila melanogaster*. Inhibition of dengue transmission using this technique has been successfully accomplished in both laboratory and field based cages and research has now moved to open field release (Walker *et al.* 2011; Hoffmann *et al.* 2011). The biocontrol mechanism is different for each of the two *Wolbachia* strains (*wMel & wMelpop*) in terms of how they manipulate their hosts to limit disease transmission,

however, both strains induce cytoplasmic incompatibility (McMeniman *et al.* 2009; Walker and Moreira, 2011).

The spread of the introduced *Wolbachia* is enhanced by the reproductive competitive edge of *Wolbachia* infected (*Wolbachia* +) females over uninfected females (*Wolbachia* -) in natural populations (McMeniman *et al* 2009). The driving mechanism through which endosymbionts naturally spread in mosquito population involves a novel means known as cytoplasmic incompatibility. In cytoplasmic incompatibility, *Wolbachia* infected females (*Wolbachia* +) mate with either *Wolbachia* infected (*Wolbachia* +) or uninfected males (*Wolbachia* -) to produce viable progeny that maternally inherit the endosymbiont. On the contrary, non-viable progeny will arise from mating occurring between *Wolbachia* free-females (*Wolbachia* -) and *Wolbachia* infected males (*Wolbachia* +). This implies that, a *Wolbachia* infected female host can successfully pass on the endosymbiont to the proceeding generation, irrespective of *Wolbachia* status of the mating male whereas female mosquitoes lacking the endosymbiont will not survive subsequent generations to act as vector. This trait is one of the strongest arguments in support of using *Wolbachia* as a biocontrol strategy (Cook *et al*. 2008). In addition to cytoplasmic incompatibility, *Wolbachia* strains *wMel & wMelpop* have also been found to impair replication of dengue virus within the mosquitoes (Walker *et al*. 2011; Moreira *et al*. 2009; Bian *et al*. 2010).

This same ability to block dengue transmission has also been shown to be potent against other viruses (Moreira *et al*. 2009; Bian *et al.* 2010). Comparison of native *Wolbachia* induced resistance against WNV in the *Wolbachia-Culex quinquefasciatus* system to the tetracycline-cured lines and showed 2 to 3-fold lower rates of virus

transmission (Glaser and Meola 2010). In addition, the ability of *Wolbachia* to inhibit malaria parasite replication (*Plasmodium gallinaceum*) has been shown (Moreira *et al*. 2009). Several studies have also attributed some host characteristics to *Wolbachia* infection, for instance, *Wolbachia* infection can affect host fecundity (Evans *et al*. 2009), host dispersal (Caragata *et al*. 2011), host mating behaviors (De Crespigny *et al*. 2006b), host fertility (De Crespigny *et al*. 2006a), host locomotion (Peng *et al*. 2008), host immunity, and foraging in various insect species (Hedges *et al.* 2008). This *Wolbachia*-host interaction outcome can result in reduction or increase in host fitness, (Peng *et al.* 2008; Sasaki *et al*. 2002).

Only female mosquitos feed on blood and transmit disease, hence the majority of the studies on the control of disease agents are directed towards female mosquitoes. Egg viability, fecundity and ability to blood feed have been considerably reduced due to wMelPop infections in the *Ae. aegypti* model. (McMeniman and O'Neill 2010; Turley *et al* 2009). In studying the influence of *Wolbachia* infection on reproductive success in *Ae. aegypti*, Turley *et al*. (2013) found that neither the *Wolbachia* infected males or the male larva had an effect on the quantity or the viability of the sperm, whereas a significant decline was observed in *Wolbachia* infected female fecundity (Turley *et al.* 2013). When the effects of diverse blood types of vertebrates (both humans and non-humans) on the fecundity of *Wolbachia* infected mosquito were investigated, a 27% reduction in fecundity was observed (McMeniman *et al.* 2011) and a subsequent study showed 23% reduction in fecundity (Turley *et al*. 2013). To date, the effect of *Wolbachia* inoculation on the size of the blood meal has not been investigated for wMel infected mosquitos. However, for wMelPop infected mosquitoes, a smaller blood meal was taken than for

uninfected ones. This may be a reason for the decline in fecundity of wMelPop infected female mosquitos, since a smaller blood meal yields less energy for egg synthesis (Turley *et al.* 2009).

Efforts to control mosquito borne diseases are not only directed at viral infection but also to sporozoan infections such as malaria, one of the most devastating diseases worldwide, particularly in the highly endemic regions of the globe. For instance, in Africa over a million deaths are recorded annually as a result of *Plasmodium* infection transmitted by mosquitoes. The high vulnerability of people in regions endemic to plasmodium infection has resulted in research efforts being focused on several novel techniques, including interference with *Plasmodium* development and/or infection of vector mosquito by initiating an effector molecule active against the *Plasmodium* parasite. Among the strategies that could potentially replace insecticide based mosquito control are 'transgenesis', which is the direct manipulation of the vector mosquito itself and 'paratrangenesis', which is the modification of mosquito symbionts in order to deliver antipathogenic effector molecules within the mosquito (Sibao and Jacobs-Lorena 2013).

Studies that have shown success in producing anti-pathogenic effector molecules include a report of a laboratory *Escherichia coli* recombinant strain that produced a single chain immuno-toxin that is active against the malaria parasite (Yoshida *et al*. 1999). The formation of plasmodium oocyst was considerably repressed by the activated immune system of somatic *Wolbachia*-infected *Anopheles* mosquitoes (Hughes *et al*. 2011), although, naturally *Wolbachia* infected *Anopheles* mosquitoes have not been found (Walker & Moreira 2011). *Wickerhamomyces anomalus* (a yeast) was reportedly found in the reproductive organs of the primary vectors of malaria, *Anopheles gambiae* and

Anopheles stephensi, thus raising the hope of using other symbionts of the malaria vector, such as yeast, to deliver anti-malaria effector molecules (Ricci *et al*. 2002, Hurwitz *et al*. 2011).

The use of *Wolbachia* as a biocontrol agent is not only limited to pathogen control in vectors transmitting viral and sporozoan disease, but the technology has also been attempted in nematode parasite control. Lymphatic filariasis (LF) commonly known as elephantiasis, is a globally significant disease with about 1.4 billion people at risk around the world. The disease is caused by at least three different filarial round worms: 1) *Wuchereria bancrofti* responstible for about 90% of cases of LF; 2) *Brugia malyi*; and 3) *Brugia timori*. *Aedes polynesiensis* is the vector for LF. Application of *Wolbachia* as a biocontrol agent was specifically targeted to Lymphatic filariasis (LF). *Wolbachia* has been used as a biocontrol agent in both laboratory and field based experiments to control the Polynesian tiger mosquito, (*Aedes polynesiensis)* that transmit LF. (WHO Fact Sheet $N^0$102). This biocontrol approach is based upon *Wolbachia*-induced conditional sterility in males, known as cytoplasmic incompatibility. The results from repeated release of incompatible males, *Wolbachia* infected males, which mates with uninfected females to produce unviable progeny, therefor suppressing the population of nematodes (O'Connor *et al.* 2012).

1.3 Ethical concerns on the applications of trangenesis and paratransgenesis in the control of disease vectors.

The first field trial of *Wolbachia* infected *Ae. aegypti* mosquitoes was carried out in the state of Queensland, Australia in Cairns and subsequently in Yorkeys Knob and

Gordonvale, also both in North Queensland, during the 2011 wet season. The adapted mosquitoes became established in wild mosquito populations. Trials have also been set up in other locations, while additional field trials have been repeated in Cairns (Frentiu *et al*. 2014). A number of questions and concerns were raised about the release of *Wolbachia* infected mosquitos; one of the main concerns raised by members of the community was whether the approach would endanger humans and other animals in the community. People also expressed concerns, about whether or not *Wolbachia* could be introduced to humans if bitten by *Wolbachia* infected mosquitoes (McNaugton unpublished observations). Interestingly, no report of *Wolbachia* infection has ever been reported in humans or any other mammal, birds, reptiles or fish (Popovici *et al*. 2010). *Wolbachia pipientis* was suspected of potentially being a human rickettsial pathogen and was tested when the organism was first discovered in the1930's. It was, however, observed that *Wolbachia pipientis* did not pose a medical threat to humans or animals. Even though humans are naturally exposed to *Wolbachia*, no evidence of *Wolbachia* infecting either humans or animals has been recorded to date. However, some other scientific questions concerning the safety of releasing *Wolbachia* infected mosquitos have been raised, including virus evolution in response to the presence of *Wolbachia* (McMeniman and O'Neill 2010). This is a reasonable concern considering the mechanism of resistance conferred by the *Wolbachia* endosymbiont to virus infection is yet to be fully understood. The resolution of transgenesis and paratransgenesis tolerance will depend on consideration of risks versus benefits. Arguments in favor of transgenesis and paratransgenesis will ultimately be substantiated on the benefits of saving lives. Thus, over time the transgenesis and paratransgenesis approach may eventually gain ground

owing to its overwhelming advantages (Wang, and Jacobs-Lorena 2013). Overall, this subject should be treated with fairness with justification that is based on science and whether there is solid evidence of overwhelming associated benefits.

1.4 West Nile Virus (WNV)

West Nile Virus (WNV), a member of the Flaviridae family, was discovered in the Nile district of Uganda, Africa in 1937 (Smithburn *et al.* 1940). However, it was overlooked and not regarded an important health issue until the 1950's when it was connected to Japanese-like encephalitis in the Middle East (Taylor *et al*. 1956). Subsequently, WNV was implicated in human disease outbreaks in various countries in Africa, the Middle East, Asia and Europe (Hubálek and Halouzka 1999). The outbreaks increased through the latter half of the 1990's, and the occurrence of WNV moved to the Western Hemisphere (Hayes *et al.* 2005). In 1999, the first locally acquired WNV disease in humans was detected in Queens, New York City, U.S.A (Nash *et al.* 2001). Although the mode of WNV introduction to the U.S. was not clear, it was presumed that its' importation could be associated with infected mosquitos, birds or other infected animals who were incubating the virus (Lanciotti *et al.* 1999). The spread of WNV after the initial outbreak was rapid in the Western Hemisphere peaking when WNV became endemic in many parts of the America from central Canada to South America and the Caribbean (Gubler 2007).

WNV gained recognition as the most widely spread arthropod born virus (arbovirus) worldwide due to its persistence across a wide geographical expanse (Kramer

et al. 2008). According to the Center for Disease Control and Prevention, approximately 96% of the U.S counties have reported transmission of the WNV in infected mosquitoes, humans, birds, horses, or other mammals since it came to the U.S. Despite the geographical and climatic diversity present across the states in the U.S, WNV distribution is quite extensive. This is probably due to its broad host range and because of the virus's ability to adapt, and persist in a large variety of ecosystems. In addition, WNV outbreak generally happen during late summer to early fall, the season that coincides with the arrival of large population of migratory birds and the booming of ornithophilic mosquitoes (Hubálek and Halouzka 1999). Thus, it is possible that infected migratory birds could carry the virus and from which the local mosquito population could pick up the virus and spread it to other animals and humans. It is thought that this phenomenon has enhanced the spread of WNV across the U.S.

1.5 Transmission of West Nile Virus

The transmission of WNV is believed to naturally oscillate from avian hosts that serve as a reservoir for the virus, through the mosquitoes, especially the *Culex sp*, that serve as the vectors to transmit the virus to other vertebrates, including humans and horses, which are regarded as dead-end hosts (Ilkal *et al.* 1997; Wilson *et al*. 2008). Studies corroborate that natural cycling of WNV is maintained in birds and *Culex* spp. mosquitoes (Sardelis *et al.* 2001; Goddard *et al* 2002). However, many wild vertebrates, including wolves, bears, crocodiles, and alligators, (Lichtensteiger *et al.* 2003; Farajollahi *et al.* 2003; Klenk *et al.* 2004) as well as domestic animals such as horses, cats, and dogs

can be naturally infected (Komar 2003; Read *et al.* 2005). The primary route of transmission of WNV to humans is through the bite of infected mosquitoes (Campbell *et al.* 2002) although WNV transmission through transfusion of infected blood products or solid organ transplantation is also possible (Pealer *et al.* 2003; Iwamoto *et al.* 2003). *Culex* species are often seen as the major culprit in the transmission of WNV, however, the virus has been detected in other mosquito species such as *Aedes*, *Anopheles* and many other species in Europe and Africa (Hubálek and Halouzka 1999; Hubálek 2008), and in North America (Hurlbut 1956; Bernard *et al*. 2001; Bernard and Kramer 2001). In the U.S. 65 different mosquito species have been shown to carry WNV although only a few *Culex species* convey epizootic and epidemic transmission (CDC 2012b, http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm). Different parts of the U.S tend to be associated with different species of mosquito that transmit WNV. For instance, the most prevailing vector for WNV in the northern portion of the U.S is *Culex pipiens,* while in the southern states WNV transmission is linked to *Culex quinquefasciatus.* In western states, WNV is spread by *Culex tarsalis* overlaping with *Culex pipiens* and *Culex quinquefasciatus* (Andreadis *et al*. 2004; Kilpatrick *et al*. 2006a; Godsey *et al*. 2012). In addition, it has been demonstrated that WNV can be replicated and transmitted by ticks and other blood-sucking arthropods under experimental conditions (Hutcheson *et al*. 2005; Oesterle *et al.* 2010). These potential vectors have not been reported to transmit WNV naturally (Anderson *et al.* 2001).

Since the first incursion of WNV in the U.S, in 1999, the virus has become well established and successfully spread across the U.S. The establishment and increase in WNV in North America may be due to varying ecological factors such as availability of

bird population that serve as the reservoir for viral amplification resulting in an increase in the numbers of viremic birds from which mosquito vectors, such as *Cx. quinquefasciatus* and *Cx. tarsalis,* acquire and spread the virus (Murray *et al.* 2010a). Following the introduction of WNV into the U.S. in 1999, the virus was reported to have spread through Canada (Pepperell *et al*. 2002), Mexico (Fernandez-Salas *et al*. 2003), Central America, the Caribbean, (Cruz *et al.* 2005; Komar and Clark 2006; Barrera *et al*. 2008), and South America (Blitvich 2008; Adrian *et al.* 2008). Since WNV establishment in the U.S. reports from 48 states, including Texas, show human cases of WNV disease were on the rise (CDC 2010). Like many zoonotic arboviruses found in temperate climates, the time and spatial distribution of WNV was shown to be related to peak transmission recorded during the summer and early fall; i.e., July through September. Within this time frame, two-thirds of reported cases are found in the six-week period between mid-July to the end of August (CDC 2010a). A variety of biotic and abiotic factors have been shown to be associated with WNV spread, including precipitation and drainage patterns (Ruiz *et al.* 2007), local irrigated landscapes (DeGroote and Sugumaran 2012), abandoned swimming pools (Reisen *et al.* 2008; Harrigan *et al.* 2010) and increased temperature (Hartley *et al.* 2012). Temperature has been correlated with a rise in human cases of WNV infection, both at the regional and national levels (Soverow *et al.* 2009). The identification of WNV, epidemiology, is usually by foci and occasionally by intense outbreak (CDC 2010a). A major WNV outbreak in the U.S. occurred in the state of Texas was in 2012, with the North Texas area serving as the epicenter. (http://www.dshs.state.tx.us/news/releases/20120817.aspx). Statewide analysis showed that a total of 1,868 cases were reported, including 844 (45%) WNV neuroinvasive

diseases cases and 89 deaths (5% fatality rate) with 48% of case-patients residing in four counties around the Dallas/Fort Worth, Texas area. A 2012 WNV seasonal outbreak from May 1, 2012 through December 6, 2012 resulted in economic cost greater than $47.6 million (Murray *et al*. 2010b; Murray *et al.* 2013).

1.6 Clinical Presentation of West Nile Virus

West Nile virus has been shown to be responsible for over 12,000 cases of meningitis and encephalitis and over 1,100 fatalities in the U.S, since the first recorded case in 1999 (Murray *et al*. 2010a). In many human cases, WNV infection manifests asymptomatically, however, long-term West Nile virus infection complications can result in neurological disorders (Nash *et al.* 2001). Symptomatic patients can experience limited febrile illness with fatigue, nausea, vomiting, eye pain, headache, myalgia, arthralgia, lymphadenopathy and rash (Hrnjakovic-Cvjetkovic *et al*. 2009). During WNV infection, neurons of the affected victims were observed to secrete an antiviral pro-inflammatory cytokine (Klein *et al*. 2005). WNV RNA has been found in the urine of 20% of convalescing patients who have had persisting infections for up to seven years resulting in persistent infection of the kidneys with accompanying renal pathology (Murray *et al*. 2010a). Diagnosis of WNV is based on serological testing, virus isolation or detection of viral antigen, or viral RNA from samples obtained from infected individuals. Although studies have suggested that the use of ribavirin and interferon alfa-2b may be useful in the treatment of WNV diseases, the most important control measure still lies in the elimination of mosquito vector (Hrnjakovic-Cvjetkovic *et al*. 2009

1.7 Blood meal identification of vector mosquitoes on avian and mammalian hosts

Studies have shown that blood meal patterns of arthropods that are involved in transmitting vector-borne disease, such as lyme disease caused by *Borrelia* (Pichon *et al*. 2005), *Leishmania* parasites transmitted by sand flies (Haouas *et al*. 2007), trypanosomes transmitted by tsetse flies (Aksoy 2003), human malaria parasites transmitted by *Anopheles* mosquitoes (Cohuet *et al*. 2006; Kent et al. 2007), and WNV transmitted by Culex mosquitoes (Apperson *et al.*, 2004; Kilpatrick *et al*. 2006b), rely on temporal and spatial convergence between vectors, vertebrate hosts and pathogens. Thus, blood-feeding patterns of arthropods could play an important role in both understanding outbreaks and transmission of vector-borne pathogens. Mosquito-feeding patterns on individuals has been studied using DNA profiling of unique host genetic markers (Gokool *et al*. 1993).

Mitochondria are maternally inherited organelles with genomes roughly 16.5kb in size and can range in copy number from hundreds to thousands per single cell (Crozier, 2012). Cytochrome b (Cyt b), a well-described protein from complex III of the electron transport chain of the mitochondrial oxidative phosphorylation system, has been used to resolve vertebrate evolutionary relationships based on the DNA sequence; therefore it is a prime candidate for identification of the host in arthropod blood meal studies (Hatefi 1985; Irwin and Árnason 1994). Another selected molecular target for DNA barcoding is the cytochrome c oxidase I (COI) subunit. Although it is an underutilized tool for arthropod blood meal identification, COI is still useful in the study of blood meal patterns in arthropods, including mosquito hosts (Hebert *et al.* 2003). The use of DNA sequencing

for COI and Cyt b can enhance the identification of the specific host, and a specific result obtained with one approach can confirm the other. Even though both COI and Cyt b sequences have been widely used in vertebrate blood typing, the sequence representation in databases of the COI barcode is more complete than for Cyt b (Molaei *et al.* 2006). Studies involving identification of blood meal sources in *Culex spp*. utilize only Cyt b group-specific avian and mammalian primers to identify the vertebrate host, the result obtained, gave a blood meal identification rate greater than 90%, (Molaei and Andreadis 2006). However, in another study of Anopheles spp. blood meal patterns, the proportion of identified blood meal was as low as 32% (Molaei *et al.* 2008). To investigate the vector capability of mosquito species in the transmission of WNV, understanding the feeding habits of mosquito populations will enhance the understanding of how horizontal transmission of the virus from one vertebrate host to another can occur. Generally, the feeding behavior of *Cx. pipiens* complex mosquitoes (i.e. *Cx. p. pipiens* and *Cx. p. quinquefasciatus*) had implicated the mosquitoes in this complex as endemic vectors of WNV due to their mammalophilic activity (feeding on mammals) (Murphey *et al.* 1967; Means 1968; Buescher and Bickley 1979), as well as ornithophilic feeding habits (feeding on avians) in the Southern part of the U.S (Magnarelli 1977).

Isolation of WNV from some *Culex spp*. and the voraciousness of this group of mosquitoes for avian and mammalian blood meals, have implicated them to be the major player in the distribution of WNV. This enzootic vector feature of *Culex spp* for WNV transmission has been corroborated by laboratory demonstration of the vector's competence for WNV (Turrell *et al.* 2001, Turrell *et al.* 2005).

Culex species is mostly implicated for WNV, especially in the southern region of

the U.S, is *C. quinquefasciatus.* Reports have shown different preferences for vertebrate blood meals. For instance, early reports suggested that in North America *C. quinquefasciatus* feed predominantly on birds and less than 1% of the time on human (Bohart and Washino 1978; Reisen and Reeves 1990). Whereas, a more recent report from California demonstrated that *C. quinquefasciatus* feeds uniformly on both mammals and birds (Nielsen *et al.* 2008). The presence of *C. quinquefasciatus* is not limited to just North America but is present in many areas of the world. However, *C. quinquefasciatus* is seen as the major driver of WNV in the southern region of the U.S, including the State of Texas, (Molaei *et al*.2007) making it the major vector focus of this study.

Studies of the blood meal pattern of *C. quinquefasciatus* in different parts of the world indicate widespread variation in *C. quinquefasciatus* host choice, probably due to tempo-spatial availability and accessibility of the vertebrate host for blood feeding. An early blood meal analysis for host preference in *C. quinquefasciatus* carried out in different regions in Australia gave varying results per region. A study from Southwestern Queensland showed that the majority of host meals were birds (~80%) irrespective of habitat under study; 12% were found to be humans when urban areas were studied but in wooded areas, just 1% were human (Kay *et al*.1985). Another study executed in Northern Queensland revealed that 54% of blood meals identified were from dogs, while humans accounted for 8.9%, and birds 29.7% (Kay *et al.* 1979). In yet another study from Southern Australia, it was found that human blood meal amounted to 19% of the vertebrate blood meals identified, while fowl was 70%, and dogs had a lower percentage of only 5% (Lee *et al.* 1962). A Bangladesh study of three different respective locations found 78%, 97% and 72% of the identified blood meals were human; overall,

approximately 93% identified meals were human. These studies indicate a significant variation in host choice of *C. quinquefasciatus*. When a natural habitat near farms in North Carolina was studied for *C. quinquefasciatus* blood meal behavior, no human feeding was found and there was 91% bird feeding (Irby and Apperson 1988). In a contrasting situation, analysis of blood meal sources from *C. quinquefasciatus* in two urban sites and one wooded site in Louisiana, demonstrated that the mosquitoes were opportunistic feeders on humans or birds (Niebylski and Meek 1992). Study in sites that were adjacent to a dog kennels resulted in greater than 96% dog blood meals. But more typical residential areas produced a blood meal range of 65–70% dog, 9–15% human, and 6–30% bird blood, while a wooded area had 23–33% dog, 13–23% human, and 43–53% bird blood (Niebylski and Meek 1992). With respect to the available literature on the avian and mammalian blood meal pattern of *C. quinquefasciatus*, it appears that there is a considerable level of variation as far as the choice of host to be fed upon is concerned. The knowledge of blood meal pattern of local mosquito populations will go a long way in determining the degree of vector competency of *C. quinquefasciatus* with respect to WNV distribution as well as deciphering the horizontal bridging of WNV from avian or other mammals to humans.

This study seeks to address the following questions:

1. Do the local mosquito species in the study area (Denton) have natural-occuring *Wolbachia* infections, and if so, what species of *Wolbachia* bacteria are present; and
2. What animals are mosquitos feeding on to determine the potential source of mosquito infection and/or viral transmission?

Thus, this study was driven by the following hypothesis:

1. If the local mosquito species in the study area naturally have *Wolbachia*, then screening for *Wolbachia* from mosquito samples collected in the area should give positive PCR reactions using *Wolbachia*-specific primers sets; and

2. If the mosquito species collected in the study area have fed on avian or mammalian blood then PCR screening should give positive PCR reactions when corresponding primers sets are used.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mosquitoes Collection Location

At the commencement of this study in the fall of 2012 through spring 2013, Mosquitoes larvae were initially collected from open water ponds located at the University of North Texas (UNT) water Research field station, Denton, Texas. Mosquitoes were transported into the lab and allowed to emerge into adults under laboratory conditions. The collected mosquitos were screened for natural *Wolbachia* infection using a PCR technique.

In the summer season of 2013, (July-October, 2013) adult mosquitoes (both blood engorged and non-engorged mosquitos) were collected with the aid of CDC gravid traps (John W. Hock company) from the Denton area. Selected blood engorged female mosquitos, mostly *Culex quinquefasciatus* were tested for the presence of avian and mammalian mitochondrial DNA fragments, as well as for the presence of West Nile virus. In the 2014 summer season, adult mosquitoes were collected around Denton, using CDC gravid traps, while blood engorged mosquitoes were also routinely received from the Dallas mosquitoes control unit, in order to extend mosquito sampling size to the Dallas County area.

2.2 Mosquitos Collection

During the Winter/spring season of late 2012 to early 2013, the mosquito larvae were collected from the open water ponds located at the University of North Texas

(UNT) water Research field station, Denton, Texas, while the adult mosquito (both blood engorged and non-engorged mosquitos) were collected with the aid of CDC gravid traps from the Denton area, in the summer season of 2013, (July-October). Mosquito larvae samples were routinely collected from UNT water research field station open ponds in jars, the larvae samples in the jar were transported into the laboratory and placed in mosquito cages (Figure 2.1) where the mosquito larvae were allowed to emerge into adult after 1-3 days of larva collection. The adult mosquitos inside the mosquito cages were maintained at the temperature condition of the laboratory (20-25^{0}C) and the relative humidity of the Laboratory room. The mosquito feeding was accomplished using 10% sucrose as an energy source. After the mosquitos matured to adult stage, they were aspirated out of the cages and frozen in a -80^{0}C Freezer, subsequently the mosquitos were identified under a Zeiss Axio zoom microscope. The identified mosquitos were placed in a pool of 50 mosquitos or less in screw capped tubes, which were stored in a -80^{0}C for future use, while a portion of the identified mosquitos were prepared for DNA extraction.

DNA extraction was by both DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) and DNA extraction buffer (2% Hexadecyltrimethyl Ammonium Bromide, 1.4 M NaCl, 0.02M EDTA, 0.1M Tris pH 8, 0.2% 2-β mercaptoethanol) (Zouache, *et al*. 2009).

Collection of mosquito larvae

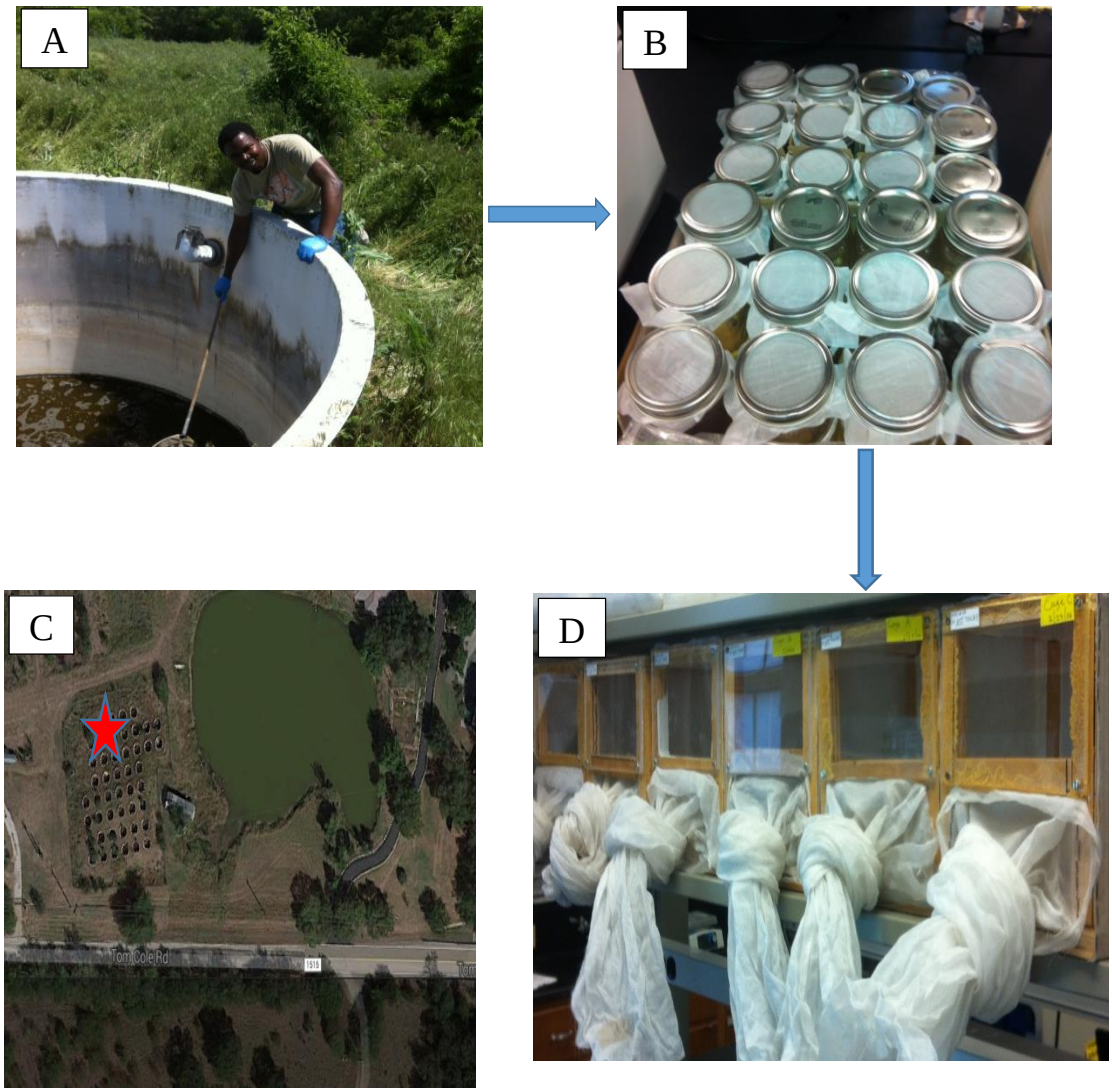

Figure 2.1: Mosquito larvae collection

Mosquitoes Larvae collection from an open water pond located at the University of North Texas water Research field station, Denton, TX. The mosquito larvae were collected using netting (A) and placed in the jars screwed with insect netting-cover (B) for onward transportation to the lab. The collected mosquito larvae were allowed to emerge into adult mosquitoes in the mosquito cages that we designed and built in our lab (C). The area marked with a star in the google map picture of the UNT Water Research Field Station (D) indicates the location of the open 10,000 gallon water tank where mosquito larvae were collected.

2.3 Field Collection of both Blood Engorged and Non-Engorged Adult Mosquitos

CDC gravid traps (John W. Hock Company) were set at different locations around Denton County (UNT Campus areas and the UNT Water Research Field Station) to collect both non-engorged and blood engorged mosquitos (Fig 2.2). The mosquitoes collected were identified as to species, gender as well as feeding condition, (Figure 2.3, 2.4) using Zeiss Axio zoom microscope. The identified mosquitos were placed in a pool of not more than 50 mosquitos and stored at -80℃, until DNA extraction.

Figure 2.2: CDC gravid trap set up

A CDC gravid trap set up in a location around UNT, the black bow on which the gravid trap stood, containing infusion solution that was made by adding 0.5kg of hay to about 114L of tap water and allowed to incubate outside of the lab for 5-7 days. The infusion solution attracts mosquitoes and the battery-powered fan in the pipe forced the mosquito into the net on top (A). The captured mosquitoes in the netting were transported to the lab to freeze kill the mosquito for identification (B). The area marked X in the google map picture is the location where we collected most of the mosquito samples (C).

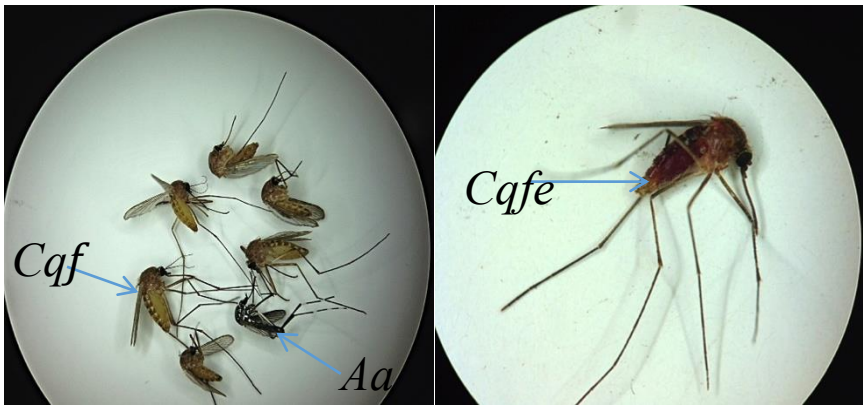

Figure 2.3 Mosquito species captured

Identification of different mosquito samples as pointed by the arrow. The arrow shows the identity of mosquitos by genus and species initials such that; Aa indicate *Aedes albopictus, Cqf* indicate *Culex quinquefasciatus* female and *Cqfe indicate Culex quinquefasciatus* female engorged, as visualized under the Zeiss Axio zoom microscope (Zeiss). Magnification: X 35

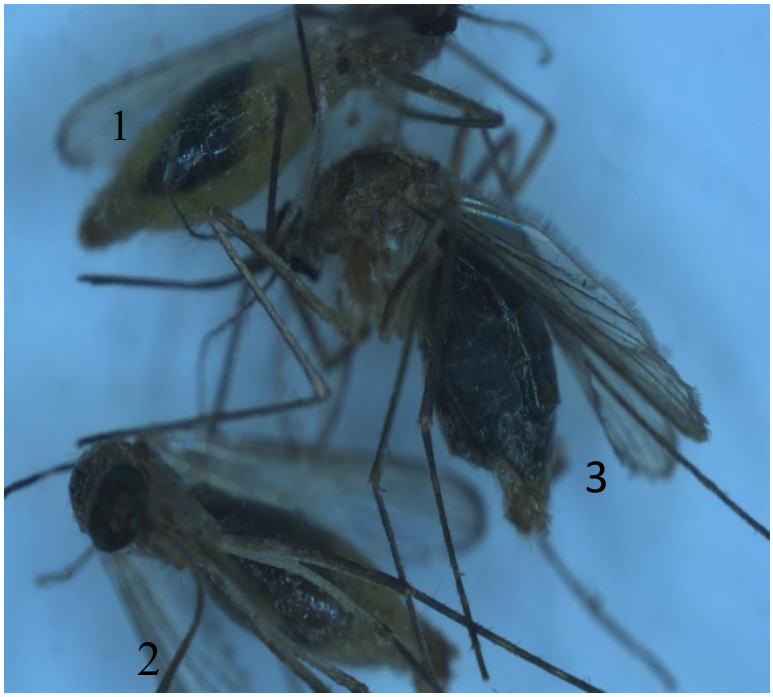

Figure 2.4: Identification of mosquito species

Degree of engorgement of *C. quinquefasciatus (Cqfe)* by visual sighting under Zeiss Axio zoom microscope (Zeiss) 1. Engorged *Cqfe* 2. More Engorged *Cqfe* 3. Most Engorged *Cqfe*. Magnification: X 50

2.4 DNA Extraction

Extraction of DNA was carried out from a pool of 3-5 mosquitos using both a salt extraction method (Zouache *et al*. 2009) and commercially available DNA extraction kits (Qiagen, Valencia, CA.) following the manufacturer protocol. For the salt extraction method, 3-5 whole pooled insect samples were crushed in 250μL of DNA extraction buffer (2% hexadecyltrimethyl ammonium bromide, 1.4M NaCl, 0.02M EDTA, 0.1M Tris, pH 8, 0.2% β-mercaptoethanol) pre-warmed to 60℃ using a thermo cell cooling and heating block, (Bioer Technology, Hangzhou, China). Homogenates were incubated for 15 minutes at 60℃ in the heating block. Proteins were removed in one volume of chloroform/isoamyl alcohol (24/1) and the DNA solution was carefully transferred from the upper layer of chloroform/isoamyl alcohol interphase into a freshly labeled eppendorf tube, after centrifugation at 8000 rpm for 3 minutes. The DNA collected was precipitated at room temperature for 10 minutes with one volume of isopropyl alcohol. The DNA pellet was washed once with 70% ethanol, air dried, and then dissolved in 100 μL of TE Buffer. The DNA was extracted, checked for purity as well as quantified spectrophotometrically by reading at 260/280-ratio wavelength in a Synergy H4 Hybrid Reader spectrophotometer (BioTek, Winooski, VT). The DNA was subsequently resolved on 0.8% agarose gel in TAE buffer for 35 to 45 minutes at 90 Volt, stained with ethidium bromide, and viewed under the UV light, aided by Molecular Imager, ChemiDoc XRS (BIO RAD, Hercules, California).

2.5 Polymerase chain Reaction (PCR)

PCR was performed to identify the presence of *Wolbachia* endosymbionts and the vertebrate blood meal type. Previously published primers were used to target the 16S rDNA of *Wolbachia* and cytochrome B and cytochrome C oxidase genes of vertebrate mitochondria DNA, targeted for blood meal typing. All PCRs were performed in 25µL reaction volumes containing the 1X standard taq reaction buffer, 1X Cresol Red, (loading dye) 1.5mM MgCl2 (used with MgCl2 free reaction buffer), 200 µM dNTPs (1 mM for multiplexing blood meal samples), 0.2 µM (50 pM for multiplexing blood meal samples) of each primer set (forward and reverse), 1.5 µL of template DNA and 0.625U of Taq DNA polymerase.

2.6 *Wolbachia* 16S rDNA amplification

The PCR conditions for *Wolbachia* 16S rDNA amplification were (Table 2.1 and Table 2.4): initial denaturation 95℃ for 6 minutes, followed by 40 cycles of 95℃ for 15seconds, 52°C for 45seconds and 68°C for 1minutes and a final extension of 68℃ for 7minutes.

2.7 PCR conditions for avian and mammalian blood meal amplification

Using avian forward/revers primer set were (Table 2.2 and Table 2.4):
The PCR conditions for amplifying mitochondrial DNA were: initial denaturation 95℃ for 5minutes, 36 cycles of 95℃ for 30seconds, 57℃ for 50 seconds, 72℃ for 40seconds

and a final extension of 72℃ for 7minutes. (Sorenson *et al.* 1999; Kent and Norris 2005); while the PCR conditions for amplifying mammalian mitochondrial DNA were: initial denaturation 95℃ for 10minutes, 36 cycles of 95℃ for 30 seconds, 55℃ for 45seconds, 72℃ for 1.5minutes and a final extension of 72℃ for 7minutes (Ngo and Kramer, 2003; Molaei *et al*. 2006).

Multiplex for Pig573F, Human741F, Goat894F, Dog368F, Cow121F, UNREV102B: The PCR conditions for amplifying mitochondrial DNA were: initial denaturation 95℃ for 5 minutes, 35 cycles of 95℃ for 1 minutes, 58℃ for 1minute, 72℃ for 1 minute and a final extension of 72℃ for 7minutes (Kent and Norris, 20

Table 2.1: *Wolbachia* primer sets: Primers set used to amplify *Wolbachia* DNA from the Mosquito sample

Gene Name	Primers	PCR Product Size (bp)	Sequence (5'-3')	Reference
16S	99F	895	TTGTAGCCTGCTATGGTATAACT	(O'Neill *et al.* 1992)
	994R		GAATAGGTATGATTTTCATGT	
16S	Wspec f	438	CATACCTATTCGAAGGGATAG	(Werren *et al.* 2000)
	Wspec r		AGCTTCGAGTGAAACCAATTC	
Wsp	81F	610	TGG TCC AAT AAG TGT ATG AAG AAA C	(Zhou *et al.* 1998)
	691R		AAA GGG GAC TGA TGA TGT	

2.8 Cloning of *Wolbachia* amplicon into *Escherichia coli* strain DH5∝

The amplicons for *Wolbachia* 16SrDNA with the expected band size of 438bp using a primer set specific for the *Wolbachia* 16SrDNA gene was cloned into bacteria, specifically *E. coli* DH5∝ strain. The amplicon was purified using the ZR DNA Sequencing Clean-up kit (Zymogen, Orlando, FL). The clean-up was to get rid of the PCR reagent in order to obtain a pure DNA for downstream application. Therefore, the PCR amplicon showing the expected band size on 2% agarose gel, was ligated into pCR8⁄GW⁄TOPO TA Cloning topo vector (Invitrogen, Grand Island, NY) and subsequently transformed into *E. coli* DH5α. The plasmid carrying the amplicon alongside the primer set (forward and reverse primers) specific for the amplicon (insert) were sent for sequencing. The cloning strategy used was topo vector recombination based and the steps involved in the cloning include:

- PCR product clean up: To remove reagents that inhibit downstream enzymatic activity, ZR DNA Sequencing Clean-up kit (Zymogen, Orlando, FL) was used to clean the PCR products to be cloned following the manufacturer protocol.
- Recombination of the PCR product with the Vector: TOPO Vector was used for cloning the PCR product into pCR8⁄GW⁄TOPO TA cloning vector (Invitrogen, Grand Island, NY) according to the manufacturer's protocol.
- Transformation of bacteria cells: DH5α *E. coli* cells were transformed with the recombinant topo vector-amplicon by a heat shock method of transformation of One Shot max efficiency Chemically Competent E. coli DH5α (Invitrogen, Grand Island, NY). The TOPO Cloning reaction (2 µl) (recombinant topo vector-amplicon) was added to a vial of one shot chemically competent *E. coli* DH5α

cells and was gently mixed. The cells were incubated on ice for 30 minutes, after which the cells were heat-shocked for 30 seconds at 42°C without shaking, using thermo cell cooling & heating block (Bioer Technology, Hangzhou, China). The tubes containing the cells were immediately transferred to ice and incubated for 2 minutes after which 950µl of S.O.C. media, previously warm up to room temperature, was added. The tubes were tightly capped, and placed horizontally in a 37°C incubator with constant shaking at 225 rpm for 1 hour so that the transformed bacteria cells could recover. Bacteria cells (50µl – 250µl) from each transformant were separately plated on pre-warmed LB selection plates, containing 50µg/ml spectinomycin plate, and incubated overnight at 37°C. Distinct colonies from the overnight cultures were picked and inoculated into LB broth with the 50µg/ml spectinomycin and were incubated overnight with agitation of 200 rpm. Aliquots of broth culture (3ml) from the overnight culture were centrifuged at 13000rpm for 30sec. The supernatant was removed and the plasmid was extracted from the pellet using a ZR plasmid extraction kit (Zymogen) following the manufacturer recommendation. Gel electrophoresis on an 0.8% (w/v) agarose gel (1X TAE buffer) was used to analyze the undigested plasmid DNA run at 90 volt for 45 to 60 minutes. The gel was stained in ethidium bromide and visualized under the UV light, aided by Molecular Imager, ChemiDoc XRS (BIO RAD, Hercules, California). An approximate band size of the vector carrying the PCR fragment was obtained (Fig. 3.3.1 - Fig. 3.3.4).

- Confirmation of the amplicon (insert) in the plasmid: The primer sets specific for the PCR amplicon inserted into the TOPO vector were used to amplify the

Wolbachia 16S rDNA amplicon cloned into the TOPO vector, directly from the plasmid extracted from the transformed *E. coli* strain (Fig. 3.3.5). In addition, the plasmid (*Wolbachia* 16S rDNA amplicon in the entry vector) was digested with EcoRI (Fig. 3.3.6)

- Sequencing: The plasmid carrying the amplicon to be sequenced was prepared by diluting to a concentration range of 100 to 200 ng/µl. The primers set was also diluted to 2 µM, and the samples for sequencing were prepared by mixing 8 µl of template (100 – 200 ng/µl plasmid) and 4µl of a single 2 µM primer stock such that only one of either the forward or reverse primers were added to the template DNA to be sequenced in a single reaction tube. The prepared samples were sent to Europhin MWG Operon (Huntsville, AL) for sequencing.

2.9 Avian and Mammalian blood meal identification

Multiplex for Pig573F, Human741F, Goat894F, Dog368F, Cow121F, UNREV102B. The PCR conditions for mitochondrial DNA were: initial denaturation 95℃ for 5mins, 35 cycles of 95℃ for 1mins, 58℃ for 1min, 72℃ for 1 minute and a final extension of 72℃ for 7minutes (Kent and Norris, 2005).

- For mammalian amplification

The PCR conditions for mitochondrial DNA were: initial denaturation 95℃ for 10minutess, 36 cycles of 95℃ for 30 seconds, 55℃ for 45 seconds, 72℃ for 1.5 minutes and a final extension of 72℃ for 7 minutes (Ngo and Kramer 2003; Molaei *et al*. 2006).

- For avian amplification

The PCR conditions for mitochondrial DNA were: initial denaturation at 95℃ for 5mins, 36 cycles of 95℃ for 30sec, 57℃ for 50 seconds, 72℃ for 40 seconds and a final extension of 72℃ for 7 minutes. (Sorenson et al. 1999; Kent and Norris 2

Table 2.2: Avian and Mammalian primers: Primers sets used to amplify Vertebrate blood meal (avian and mammal) for Cytochrome b & c oxidase

Gene Name	Primers	PCR Product Size (bp)	Sequence (5'-3')	Reference
Cytb	L14841	358	CCATCCAACATCTCAGCATGATGAAA	(Kocher *et al.* 1989)
	H15149		GCCCCTCAGAATGATATTTGTCCTCA	
Cytb	Avian for	508	GACTGTGACAAAATCCCNTTCCA	Cicero and Johnson (2001)
	Avian rev		GGTCTTCATCTYHGGYTTACAAGAC	
Cytb	Avian b for	515	CCCTCAGAATGATATTTGTCCTCA	Sorenson *et al.* (1999)
	Avian b rev		CCTCAGAAKGATATYTGNCCTCAKGG	
Cytb	Pig573F	453	CCTCGCAGCCGTACATCTC	
	Human741F	334	GGCTTACTTCTCTTCATTCTCTCCT	
	Goat894F	132	CCTAATCTTAGTACTTGTACCCTTCCTC	
	Dog368F	680	GGAATTGTACTATTATTCGCAACCAT	
	Cow121F	561	CATCGGCACAAATTTAGTCG	
	UNREV102B		GGTTGKCCTCCAATTCATGTTA	Kent and Norris (2005)
Cytb	Mammal for	772	CGAAGCTTGATATGAAAAACCATCGTTG	Ngo and Kramer (2003)

	Mammal rev		TGTAGTTRTCWGGGTCHCCTA	
Cytb	cytb1	638	CCATGAGGACAAATATCATTCTG	Kirstein and Gray (1996)
	cytb3		GGGTGTTCDACTGGYTGBCCTCC	
Cytb	Columbiforme F	333	CTMACMGGMYTACTACTMGCCG	Ngo and Kramer (2003)
	Columbiforme R		GGTTTGGCCAATGTAGGGGAC	
	Falconiforme F	282	TCCCCTACATYGGRCAAACCA	
	Falconiforme R		GGGGAGAATAGKGCTAGGGTTG	
	Passeriforme F	165	GGGGAGAAATAGKGCTAGGGTTG	
	Passeriforme R		GGGGAGAATAGKGCTAGGGTTG	
	Galliforme F	210	ATTTCGGCTCCCTATTAGCAG	
	Galliforme R		GTCCGATGTGAAGGAAGATACAGATGA AGAAGAA	Ngo and Kramer (2003)

2.10 West Nile Virus RNA Detection

Pools of blood-engorged mosquitos were processed for RNA extraction using Tri reagent (Sigma-aldrich, St Louise MO). The mosquito pool was homogenized in TRI Reagent (1 ml/50 - 100 mg tissue) (Carolina Biological Supply Company, Burlington, NC) using a microfuge tube pestle. (USA Scientific, Inc. Ocala, FL). The homogenate was left for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. Chloroform at 0.2 ml per 1 ml of TRI Reagent was added, samples were tightly covered and shaken vigorously by vortexing for 15 seconds. The resulting mixture was stored at room temperature for 2 to15 minutes and centrifuged at 12,000 g for 15 minutes at 4^0C. Following centrifugation. The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interphase and organic phase. The aqueous phase was transferred to a fresh tube while the interphase and organic phase were saved at 4^0C for subsequent isolation of DNA and proteins. RNA was precipitated from the aqueous phase using isopropanol (0.5 ml of isopropanol per 1ml of TRI Reagent). The samples were stored at room temperature for 5 to10 minutes and centrifuge at 12,000g for 8 minutes at 4^0C. The supernatant was discarded and the RNA pellet washed (by vortexing) with 1 ml 75% ethanol and subsequent centrifugation at 7,500g for 5 minutes at 4^0C. The ethanol wash was removed and the RNA pellet briefly air-dried for 3 to 5 minutes. The RNA pellet was dissolved in RNase-free water treated with diethyl pyrocarbonate (DEPC) and incubated for 10 to15 minutes at 55 to 60^0C for the RNA sample to be completely dissolved. The final

preparation of total RNA is essentially free of DNA and proteins and has an OD 260/280 ratio 1.6 to 1.9. The RNA bands were resolved in agarose gel.

2.11 RT-PCR (First Strand Synthesis)

Reverse transcription was carried out on the RNA extracted using the random and Poly A primers. 2 µg of each RNA sample were mixed with 0.5µg of primers (1 µl Random or Poly T at 0.5µg/µl) and the volume was made up to 15µl with water. The tubes were heated to 70℃ for 5 minutes and cooled on ice for 2 to 3 minutes. M-MLV 5x reaction buffer (5µl), 1.25µl dNTP, 1µl M-MLV RT and 2.5µl of water were added, bringing the volume to 25µl. Tubes with the poly-A primer were incubated at 37℃ for 1 hour while tubes with random primers were incubated at 42℃. 1µl of the reaction was used for the PCR and the rest was stored at -20℃.

2.12 PCR for WNV amplification

For West Nile virus amplification, PCR conditions were (Table 2.3, Table 2.4): 50℃ for 2mins, 95℃, for 2mins and 40 cycles of 95℃ for 15 seconds, 58℃ for 30 seconds and 72℃ for 15 seconds and a final extension of 72℃ for 9 minutes. A WNV RNA positive control was supplied by Dr. Bethany Bolling (University of Texas Medical Branch, Galveston, Texas). The virus was originally isolated from the brain of a dead Blue Jay found on August 13, 2013 near 8607 Joggers Lane, Humble TX, 77346. The Viral RNA was isolate from Virus that was passaged twice in Vero cells (Dr. Bethany

Bolling, University of Texas Medical Branch, Department of Pathology, Galveston, Texas). The viral RNA was extracted using TRIzol reagent by Dr. Bethany Bolling. A WNV cDNA was obtained by carrying out reverse transcription using both random primers as well as the polyA tail primers on WNV genomic RNA sent to us. First strand synthesis of cDNA was by reverse transcription PCR (as described in the RT-PCR First Strand Synthesis section above).

2.13 Primer sets used to amplify West Nile Virus from the Mosquito sample (Zhang *et al.* 2013).

Table 2.3 list the two primer set that were tested on total RNA isolated from pool of 2-5 mosquitoes sample

Table 2.3: WNV primer sets

Gene name	Primers	Sequence (5'-3')
Genomic RNA (gRNA) F	WNVNY99 F	GCGGCGGCAATATTCATG
	WNVNY99 R	ACGTTGTAGGCAAAGGGCAA
	WNVKUN&WNVNSW2011F	GCGGCGGCAATATTCATG
	WNVKUN&WNVNSW2011R	CCGTGAACCTAAAAAACGCC

Table 2.4: PCR reagent and conditions: Concentrations of Reagent and Thermocycler Conditions used for the PCR with *Wolbachia*, vertebrate (avian and mammal) and WNV-specific Primer Sets.

Primer set	PCR Reagents	Concentration	Thermocycle
Wolbachia	Buffer	1x	95℃ for 6min Go for 1 cycle
	Cresol red loading dye	1x	95℃ for 15 seconds 52℃ for 40sec 68℃ for 1min Go for 36 cycle
	dNTP	0.2mM	
	Each primer (F and R)	0.2mM	
	MgCl2 (in MgCl2 free buffer)	2mM	68℃ for 7 minute for 1 cycle
	Template DNA	1µl	
	Taq Polymerase	1unit/reaction	4℃ for ever

	Buffer	1x	95℃ for 5min Go for 1 cycle
	Cresol red loading dye	1x	95℃ for 30sec
	dNTP	0.2mM	

Avian cytb	Each primer (F and R)	0.2mM	57℃ for 50sec 72℃ for 40sec Go for 36 cycle
	MgCl2 (in MgCl2 free buffer)	2mM	72℃ for 7min Go for 1cycle
	Template DNA	1µl	
	Taq Polymerase	1unit/reaction	4℃ for ever

Mammal all cytb	Buffer	1x	95℃ for 10min Go for 1 cycle
	Cresol red loading dye	1x	95℃ for 30sec
	dNTP	0.2mM	55℃ for 45seconds 72℃ for 1.5min Go for 36 cycle
	Each primer (F and R)	0.2mM	
	MgCl2 (in MgCl2 free buffer)	2mM	72℃ for 7min Go for 1cycle
	Template DNA	1µl	
	Taq Polymerase	1unit/reaction	4℃ for ever
	Buffer	1x	95℃ for 5min Go for 1 cycle

Mamal Univ cytb	Cresol red loading dye	1x	95℃ for 1min 58℃ for 1min 72℃ for 1min Go for 35 cycle
	dNTP	0.2mM	
	Each primer (F and R)	0.2mM	
	MgCl2 (in MgCl2 free buffer)	2mM	72℃ for 7min Go for 1cycle
	Template DNA	1µl	
	Taq Polymerase	1unit/reaction	4℃ for ever

COI	Buffer	1x	94℃ for 1min Go for 1 cycle
	Cresol red loading dye	1x	94℃ for 30sec 50℃ for 40sec 72℃ for 1min
	dNTP	0.2mM	
	Each primer (F and R)	0.2mM	go for 5cycle
	MgCl2 (in MgCl2 free buffer)	2mM	94℃ for 30sec 54℃ for 40sec 72℃ for 1 min 72℃ for 10min Go for 35cycle
	Template DNA	1µl	
	Taq Polymerase	1unit/reaction	4℃ for ever
Cytb L14841	Buffer	1x	95℃ for 3.5min Go for 1 cycle

	Crezol red loading dye	1x	95℃ for 30sec
	dNTP	0.2mM	57℃ for 50sec
	Each primer (F and R)	0.2mM	72℃ for 40sec
			Go for 36 cycle
	MgCl2 (in MgCl2 free buffer)	2mM	72℃ for 5min
			Go for 1cycle
	Template DNA	1µl	
	Taq Polymerase	1unit/reaction	4℃ for ever

WNV	Buffer	1x	50℃ for 2min
	Cresol red loading dye	1x	95℃ for 2min
			Go for 1 cycle
	dNTP	0.2mM	95℃ for 15sec
	Each primer (F and R)	0.2mM	60℃ for 30sec
	MgCl2 (in MgCl2 free buffer)	2mM	72℃ for 15sec
			Go for 40 cycle
	Template DNA	1µl	
	Taq Polymerase	1unit/reaction	4℃ for ever

CHAPTER 3

RESULTS

3.1 Mosquito Collecting

Mosquito larvae were collected beginning in the fall of 2012. At the time of collection, approximately noon, there were not many adult mosquitoes observed because of the time of day and since it was not mosquito season. However, mosquito larvae were found around the edges of 10,000 gallon, in-ground fiberglass tanks. These larvae were collected into glass jars by using mosquito net scrap (Figure 2.1). The mosquito larvae were transported to the laboratory and placed in purpose-built mosquito cages (Figure 2.1) and adult mosquitoes were seen to emerge the following day. Most of the emerging mosquito species were *Culex spp,* mainly the *Culex tarsalis* and *Culex quinquefasciatus.*

Mosquito species collected by CDC gravid traps during the summer of 2013 and 2014 were mostly *Culex quinquefasciatus* with a few *Aedes albopictus* while *Culex tarsalis* were rarely captured. Among the mosquito species captured, the primary blood engorged species were the *Culex quinquefasciatus* (Fig. 2.2 – Fig 2.3).

Mosquito collections using the CDC gravid traps were routinely carried out in the study location. When traps were set out, birds were sighted in the vicinity of the trapping location and the following morning when mosquito traps were collected, feathers of birds were sometime seen in the trapping net or in the bowl containing the infusion solution. This observation suggests easy accessibility of mosquito to the avian host for blood meals. The gradient of mosquito populations captured through the gravid trap increased from the beginning of mosquito season (summer, May/June) through to the end of summer (August/September). The peak of mosquito collection was in September 2013

(Figure 3.1). There were variations in the number and proportion of different mosquito species collected at different collection times however, statistical analysis of this difference was not performed.

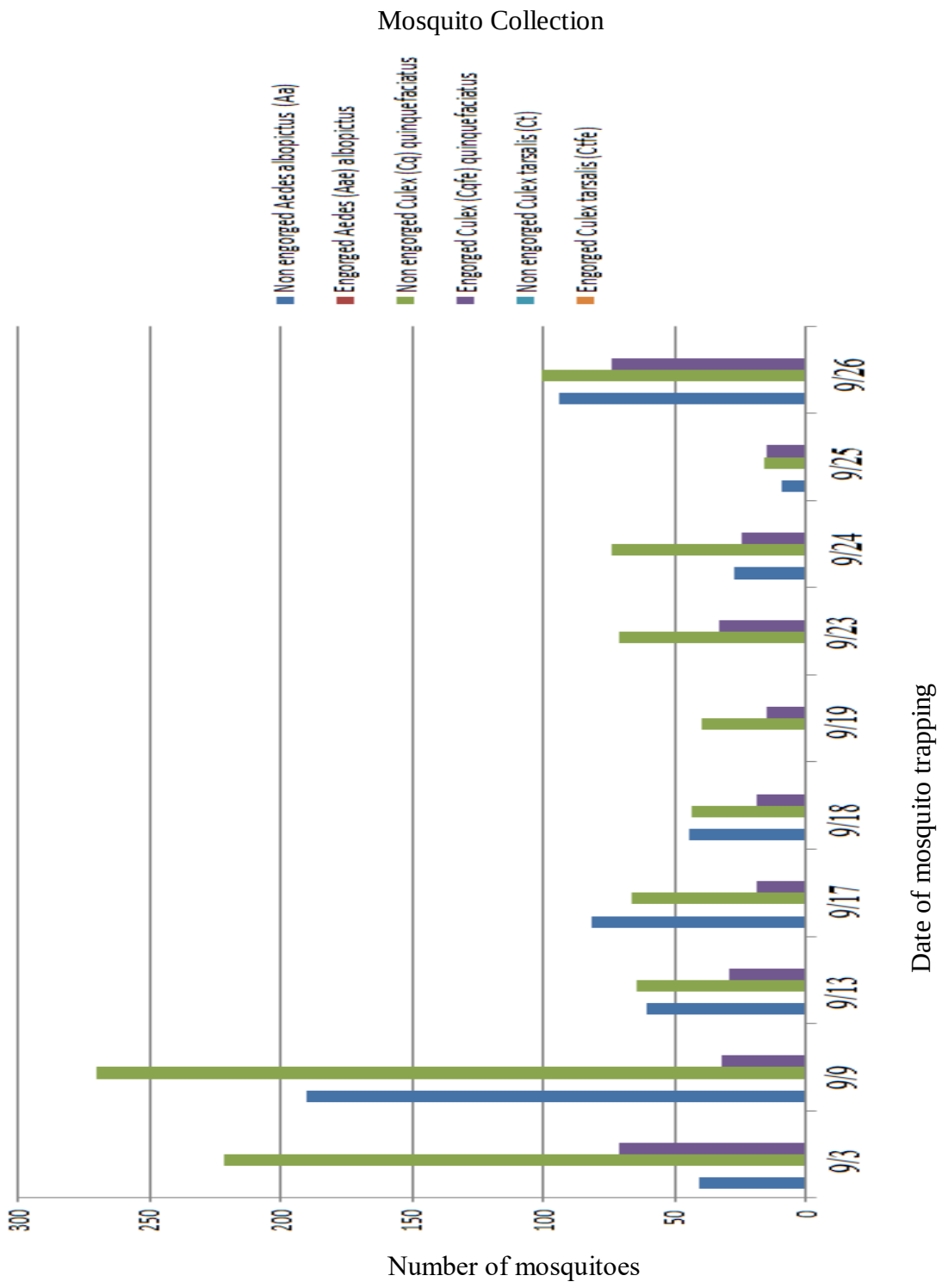

Figure 3.1: Mosquito collected in summer

Total mosquitos collected by date in September 2013, identified to the species and the blood engorgement status in a single location at the University of North Texas, Denton campus, Denton, TX.

3.2 *Wolbachia* Screening

When primer sets that amplify *Wolbachia* 16SrDNA genes were tested on the total mosquito DNA for the presence of *Wolbachia*, the 'wsp' primer that amplify the region of *Wolbachia* 16SrDNA gave an expected band size of about 438bp across the mosquito sample tested for *Wolbachia*. The PCR testing for the presence of 16SrDNA of *Wolbachia* using 'wsp' primer showed that *Wolbachia* infection is uniform across the mosquito population tested in this study (Fig. 3.3.1 to Fig. 3.3.6). Sequencing of selected *Wolbachia* amplicons with the correct-sized band cloned in topo vector, (Fig 3.3.4) showed up to 99% homology to *Wolbachia* endosymbionts of *Culex quinquefasciatus* 16S ribosomal RNA, when entered into the GeneBank database (BLAST)(Appendix F).

3.3 Amplification with wsp *Wolbachia*-specific primer set tested on mosquito samples

Figure 3.3.1

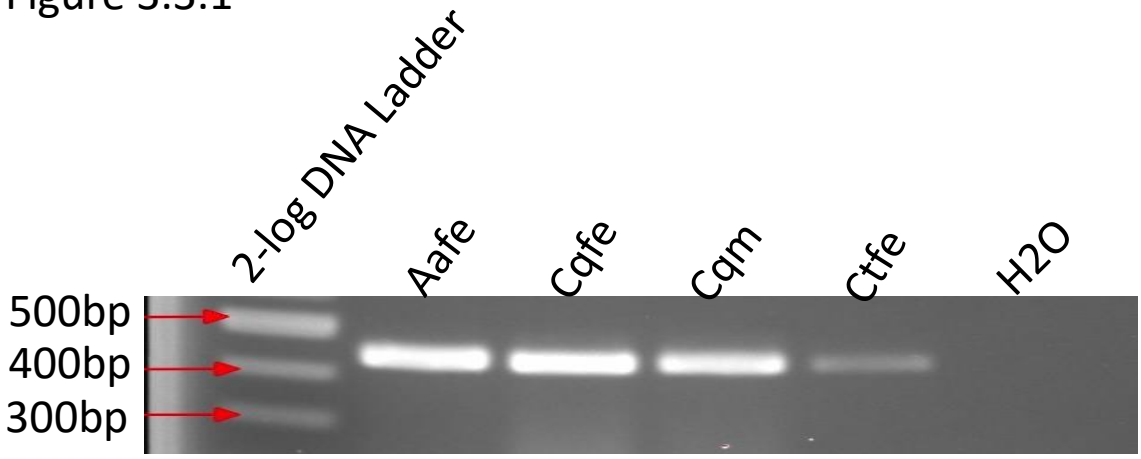

Figure 3.3.1: *Wolbachia* PCR amplicon

Gel electrophoresis showing the PCR product from amplification of mosquito samples with *Wolbachia*-specific primers set wsp that amplify 16SrDNA (438bp) of the *Wolbachia* endosymbiont. Samples notation: Aafe- *Aedes albopictus* female engorged, Cqfe- *Culex quinquefasciatus* female engorged, Cqm- *Culex quinquefasciatus* male, Ctfe- *Culex tarsalis* female engorged. A 2-log DNA ladder (New England Biolabs, Ipswich, MA) was used as the molecular weight standard (first lane).

Figure 3.3.2

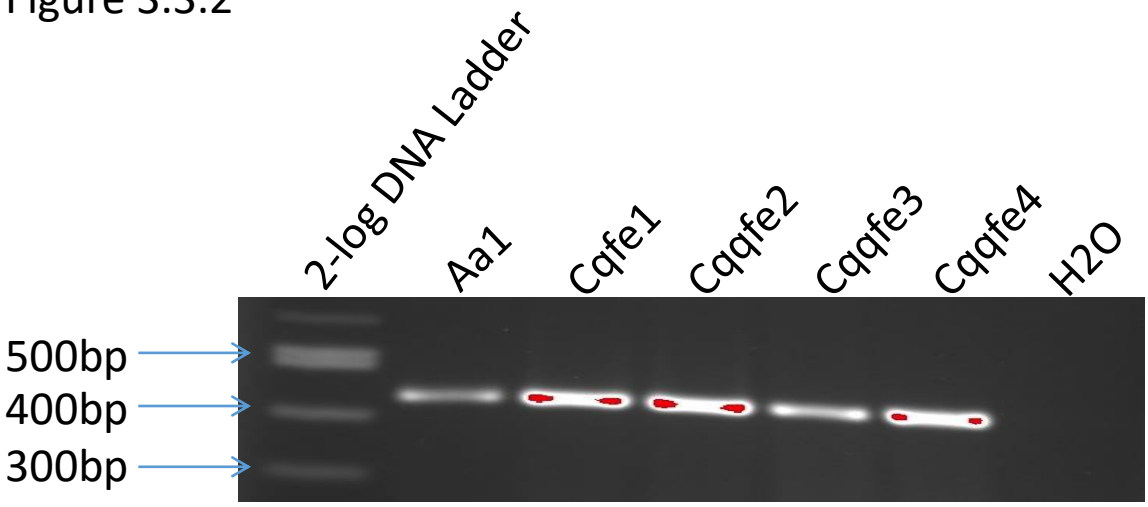

Figure 3.3.2: *Wolbachia* PCR amplicon

Gel electrophoresis showing the PCR product from amplification of mosquito samples with *Wolbachia*-specific primers set (wsp) that amplify 16SrDNA (438bp) of *Wolbachia* endosymbiont. Samples notation: Aa1- *Aedes albopictus*, Cqfe1 to Cqfe4- *Culex quinquefasciatus* female engorged. 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane).
The mosquito samples tested were collected from the UNT main campus.

Figure 3.3.3

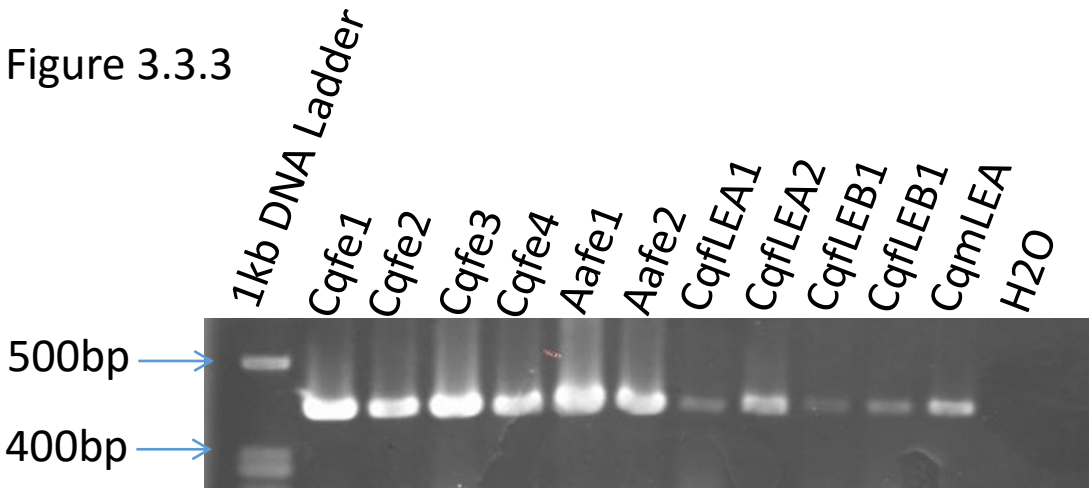

Figure 3.3.3: *Wolbachia* PCR amplicon

Gel electrophoresis showing the PCR product from amplification of mosquito samples with *Wolbachia*-specific primer set (wsp) that amplify 16SrDNA (438bp) of *Wolbachia* endosymbiont. Samples notation: Cqfe1 to Cqfe4-*Culex quinquefasciatus* female engorged, Aafe1 and Aafe2- *Aedes albopictus* female engorged, CqfLEA1 and CqfLEA2- *Culex quinquefasciatus* female lab emerged in cage A, CqfLEB1- *Culex quinquefasciatus* female lab emerged in cage B, CqmLEA- *Culex quinquefasciatus* male lab emerged in cage A. 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane). The mosquito samples tested were collected from UNT water research field station (Lab emerged) and UNT campus (field collected by gravid trap).

Cloning of *Wolbachia* 16SrDNA gene fragment amplicon

Gel showing the extracted Plasmid DNA carrying *Wolbachia* amplicon fragment from transformed DH5α cell (DH5α cell + topo + wolb amp)

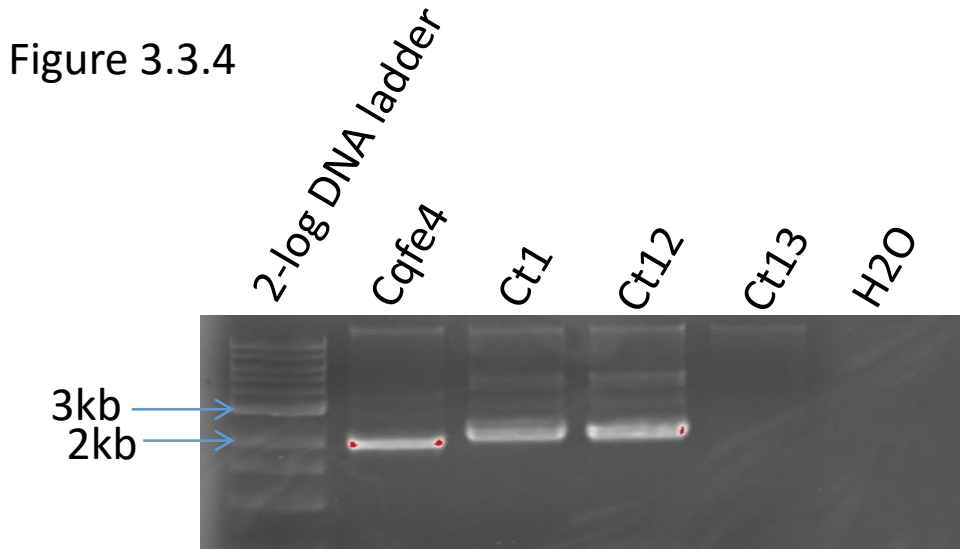

Figure 3.3.4: Cloning *Wolbachia* 16SrDNA

Gel electrophoresis showing the cloning of *Wolbachia* 16SrDNA gene fragment amplicon. Gel showing the extracted plasmid DNA carrying wolbachia amplicon fragment from transformed DH5α cell (DH5α cell + topo + wolb amplicon). Cqfe4 - *Culex quinquefasciatus* female engorged. Ct1, Ct12 & Ct13 - *Culex tarsalis.* A 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane).

Figure 3.3.5

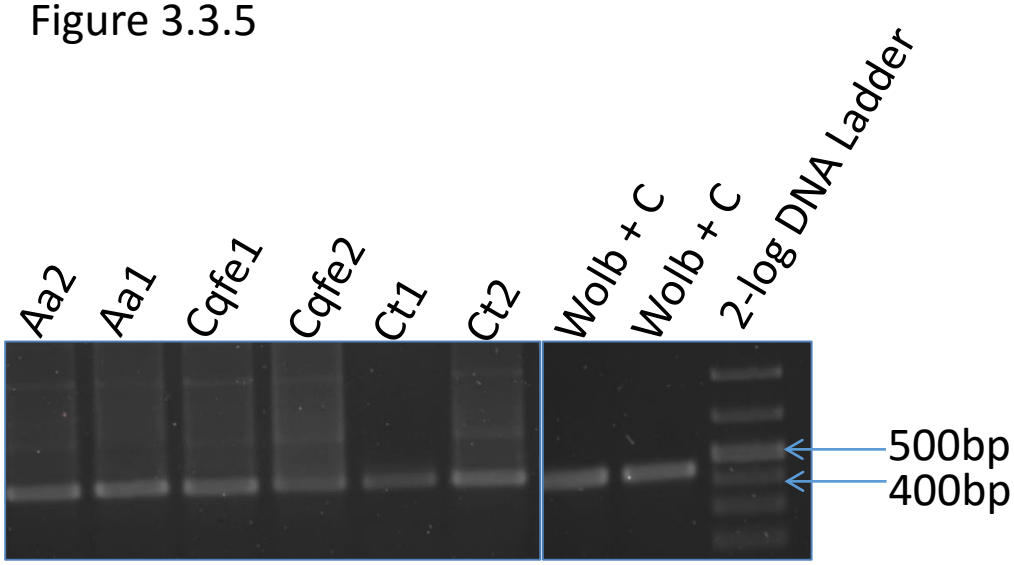

Figure 3.3.5: PCR amplicon of cloned Wol

Gel electrophoresis showing PCR amplification products of *Wolbachia* 16S rDNA fragment cloned into a topo vector. Samples notation: Aa1and Aa2- *Aedes albopictus* Cqfe1 and Cqfe2- *Culex quinquefasciatus* female engorged, and Ct1, Ct2 *Culex tarsalis* and Wolb +C- *Wolbachia* positive control. 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane).

Figure 3.3.6

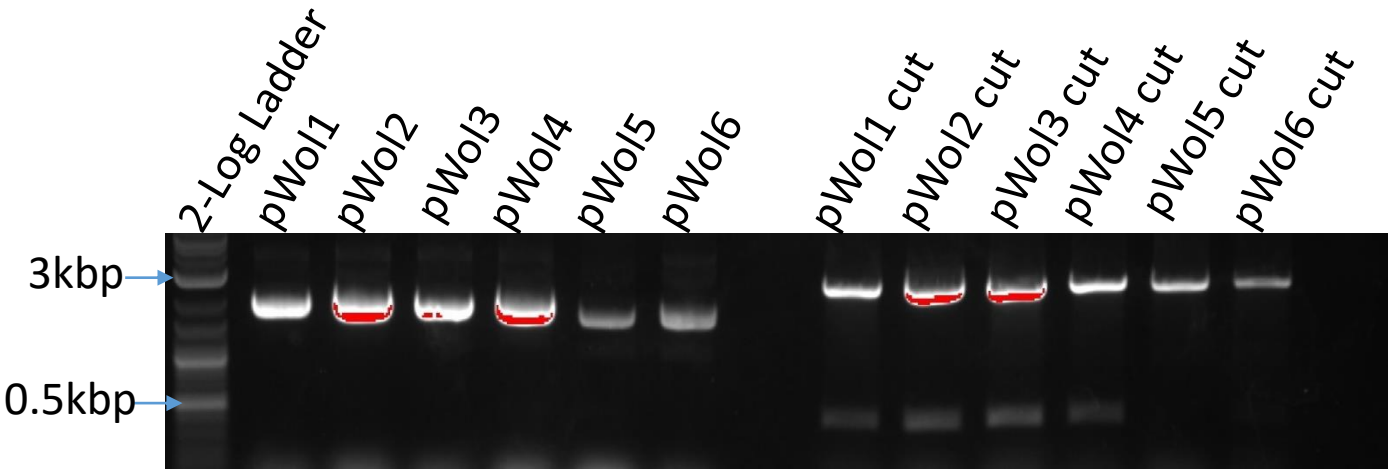

Figure 3.3.6: Digested clone of Wol

Gel electrophoresis showing the cloning of *Wolbachia* 16SrDNA gene fragment amplicon (438bp) into pCR8⁄GW⁄TOPO vector (2817bp).
The first lane show a 2-log DNA ladder (New England Biolab Ipswich, MA) used as the molecular weight standard.
The next lanes (pWol1 to pWol6) show the circularized plasmid (*Wolbachia* 16SrDNA gene fragment amplicon (438bp) plus pCR8⁄GW⁄TOPO vector (2817bp clone).
The last set of lanes in the gel (pWol1 cut to pWol6 cut) show the corresponding plasmids that were digested by EcoRI restriction enzyme. EcoRI cut out the insert (*Wolbachia* 16SrDNA gene fragment amplicon (438bp)) as seen in the lower band leaving the vector backbone.

3.4 Blood Meal Identification

It is believed that natural cycling of WNV is maintained in birds that act as a reservoir of the virus and *Culex spp*. mosquitoes serve as the vector that transmit the virus to incidental host (Ilkal *et al.* 1997; Wilson *et al*. 2008). This was further corroborated that many wild vertebrates, including wolves, bears, crocodiles, and alligators, (Lichtensteiger *et al.* 2003; Farajollahi *et al.* 2003; Klenk *et al.* 2004) as well as domestic animals such as horses, cats, dogs etc. can be naturally infected through the bite of an infected mosquito (Komar 2003; Read *et al*. 2005). Therefore, blood from selected female mosquitos from the field-captured mosquitos was used to identify the animals that the mosquitos were feeding on to potentially identify where the viruses are obtained or transmitted to.

Primers sets previously developed to specifically amplify the cytochrome b (cyt b) and cytochrome c oxidase I (COI) subunit of mitochondria DNA for avian and mammals without amplifying the mosquito DNA, were used to test for the blood type. Since *Culex quinquefasciatus* are the predominant blood engorged mosquito species captured during this study, (Figure 3.1) the blood typing tests were restricted to the *Culex quinquefasciatus* tagged "*Culex quinquefasciatus* female engorged" (Cqfe).

Among the primers sets used for blood meal identification, the avian primer sets and avian b primer set gave PCR amplification with the expected band size while the primer sets specific for mammalian blood identification showed only few PCR amplifications with the correct-size band (Fig. 3.3.1 – Fig. 3.3.4). This potentially indicates that the blood meal host among the Cqfe samples collected on the University of North Texas Denton Campus, have a higher proportion of avians than mammals.

The following gel electrophoresis show the detection of Mosquito (*C. quinquefasciatus*) blood meal source DNA PCR amplified with primers specific for avian, 'Avian' were tested (Band size 508bp) (Cicero and Johnson 2001).

Figure 3.4.1

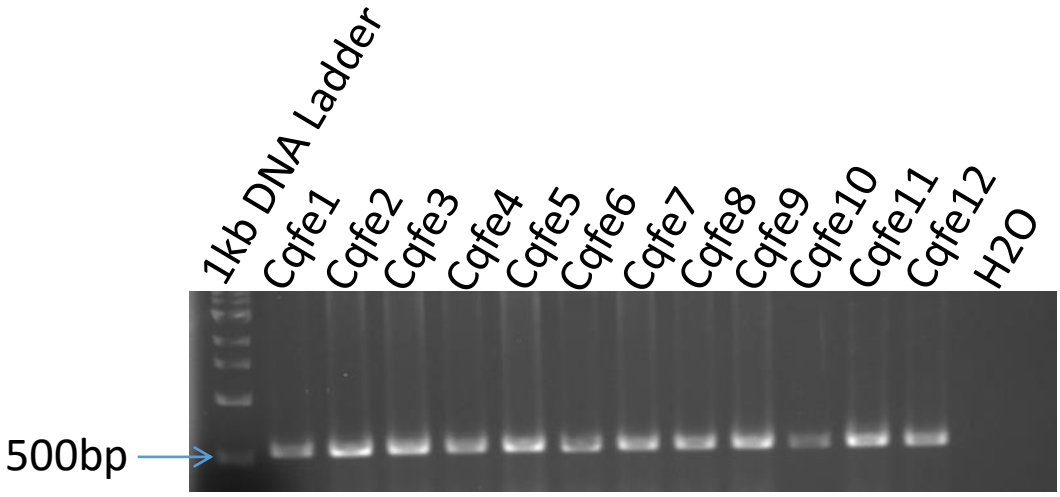

Figure 3.4.1: Avian primer-spec amplicon

Gel electrophoresis (2% agarose) showing uniform avian amplicon for avian blood meal identification from Cqfe mosquito samples collected at a single location at the University of North Texas Denton Campus. Quick load 1 kb DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane) while mosquito samples. Cqfe1- Cqfe12, *Culex quinquefasciatus* female engorged were tested.

The following gel electrophoresis shows the detection of blood meal source of mosquitoes samples collected (*C. quinquefasciatus)* by PCR when primers specific for avian 'Avian b' were tested (band size 515bp) on mosquito samples collected from UNT Campus Denton.

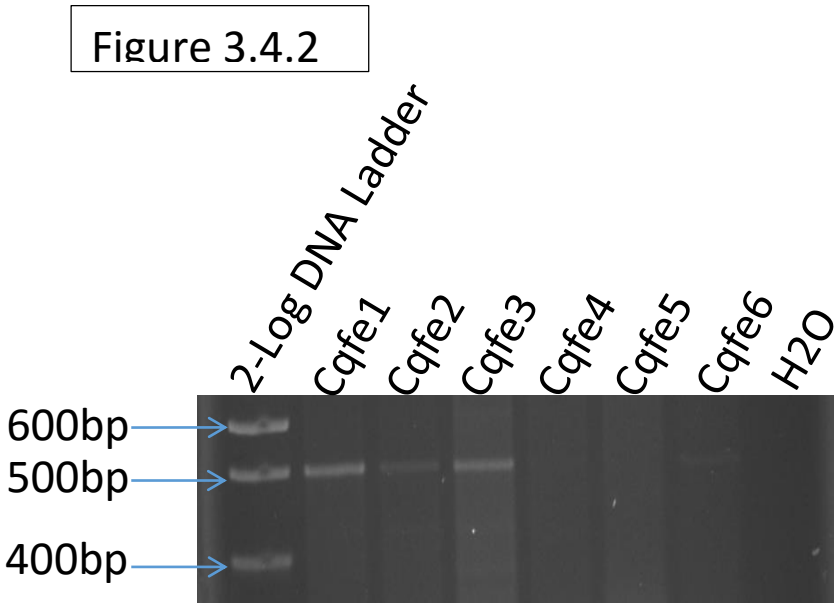

Figure 3.4.2: Avian primer-spec amplicon

Gel electrophoresis (2% agarose gel) showing PCR amplified avian blood sample using the 'Avian b' primer set tested on mosquito samples collected in May-September, 2013. Some samples (Cqfe1-Cqfe3) yielded the expected band size, while others (Cqfe4-Cqfe6) gave a faint or no clear band. These mosquito samples were collected from UNT Campus Denton. Cqfe1 to Cqfe6- *Culex quinquefasciatus* female engorged. A 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard.

Figure 3.4.3

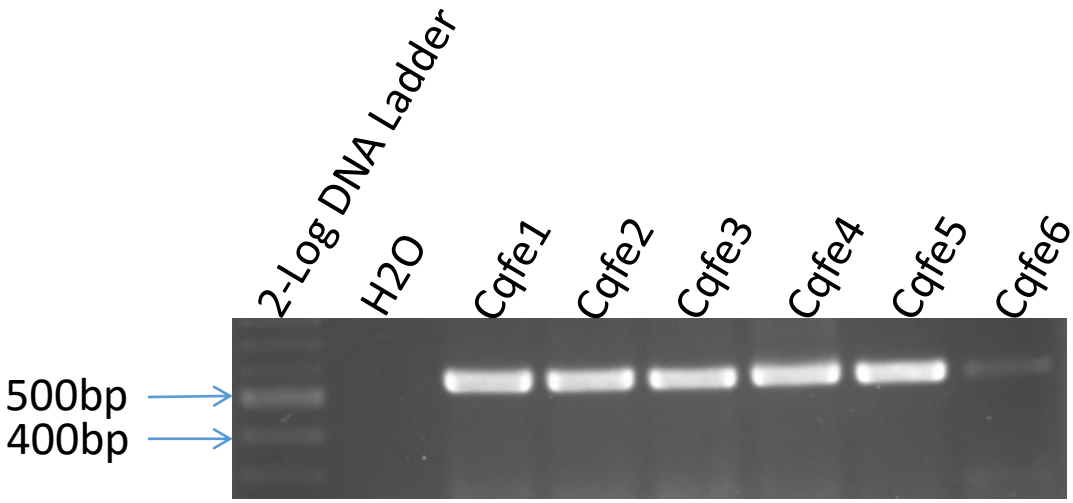

Figure 3.4.3: Avian primer-spec amplicon

Gel electrophoresis (2% agarose gel) showing uniform amplification of avian blood when 'Avian b' primer was tested on blood engorged mosquito samples (Cqfe) collected. These mosquito samples were collected from UNT Campus Denton. Cqfe1 to Cqfe6- *Culex quinquefasciatus* female engorged. A 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane).

Figure 3.4.4

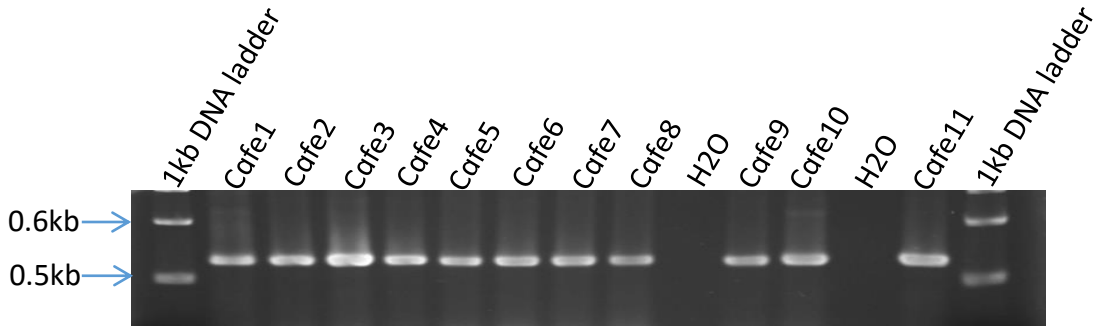

Figure 3.4.4: Avian primer-spec amplicon

Gel electrophoresis (2% agarose gel) showing uniform amplification of avian blood when 'Avian b' primer was tested on mosquito sample (Cqfe) collected. Cqfe1 to Cqfe11-*Culex quinquefasciatus* female engorged. A 1kb DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard.

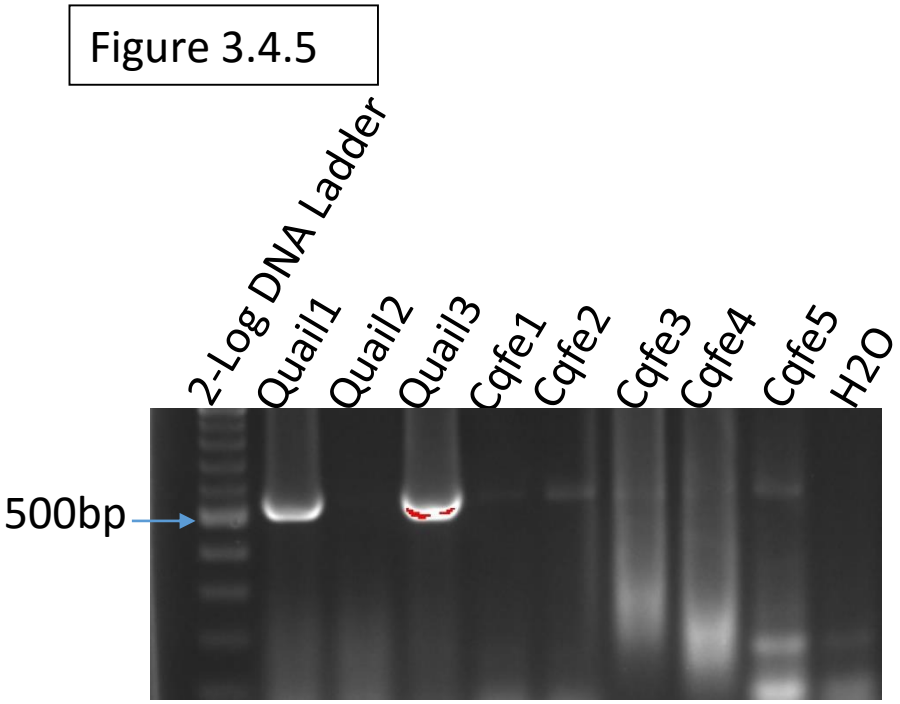

Figure 3.4.5: Avian primer-spec on quail

Gel electrophoresis (2% agarose) showing amplicon when 'avian' primer sets was used to test for quail (positive) and avian blood meal identification from Cqfe mosquito samples collected at a single location at the University of North Texas Denton Campus. 2-Log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane) while the next three lanes were for quail DNA sample, and mosquito samples, Cqfe1- Cqfe5, (*Culex quinquefasciatus* female engorged) occupy rest of the lanes.

3.5 Mammalian blood meal

PCR analysis of blood meal sources from mosquito samples collected from the UNT campus indicates that feeding on mammalian blood hosts was lower compared to avian blood host. During the DNA extraction, 1 to 3 engorged mosquitos, usually *C. quinquefasciatus female engorged* (Cqfe), collected at the same period of time from the same location using a single trap, were crushed in a test tube, and the DNA were extracted. The extracted DNA was then used as template for subsequent PCR reaction. Different primer sets listed in table 2.2, were tested on mosquito samples, especially *C. quinquefasciatus*, the species that were mostly engorged among our collection in 2013. The number of samples tested by PCR varies for individual primer set used. When the mammal primers set specific for the amplification of cytochrome b were tested on 192 samples, only 7 of the samples gave the expected band size of the cytochrome b fragment. Out of 157 samples tested using 'UNREV102B' reverse primers with individual forward primer for Pig573F, Human741F, Goat894F, Dog368F, Cow121F, (Table 2.2) three gave band sizes that match the expected size of 772bp for dog (Figure 3.5.3); Out of 28 samples tested for 'L14841' and 'H15149' primer set, four gave expected band size of 358bp. However, PCR reaction with avian specific primer sets yielded more positive reaction compared to those that are specific for mammals (Figure 3.4.1 to Figure 3.4.5). When 'Avian' primer sets were tested on a total of 217 sample, 33 of the samples show positive PCR reaction as the PCR gave an expected band size of 508bp, from amplifying avian cytochrome b (Figure3.4.1). When another avian primer set (Avian b) was tested on the samples, 22 out of 192 samples gave an expected band size of 515bp from the PCR reaction (Figure 3.4.2 to Figure 3.4.5).

The following gel electrophoresis (2% agarose) show the detection of blood meal source by PCR when primers specific for vertebrate 'Vert. H14841' were tested (band size 358 bp) on Mosquito samples (*C. quinquefasciatus*) collected from the UNT campus, Denton.

Figure 3.5.1

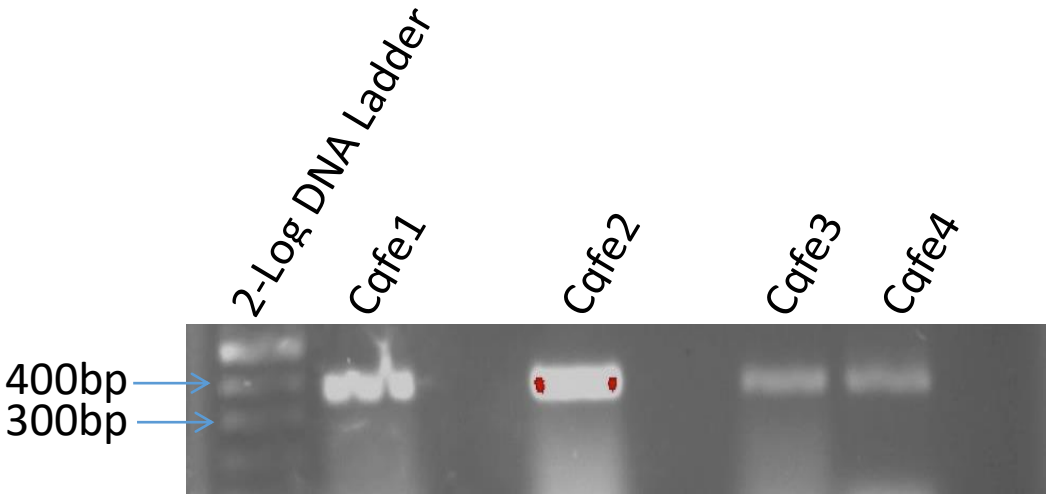

Figure 3.5.1: Vert primer-spec amplicon

Agarose gel electrophoresis (2%) showing the amplification of cytochrome b universal to the vertebrates (band size 358 bp) using H14841 and H15149 on Cqfe samples. Cqfe1- 4 *Culex quequinfaciatus* female engorged. A 2-log DNA ladder (New England Biolabs, Ipswich, MA) was used as the molecular weight standard (first lane).

The following gel electrophoresis shows the detection of Mosquito (*C. quinquefasciatus*) blood meal source by PCR when primers specific for mammal 'Mammal all' were tested.

Figure 3.5.2

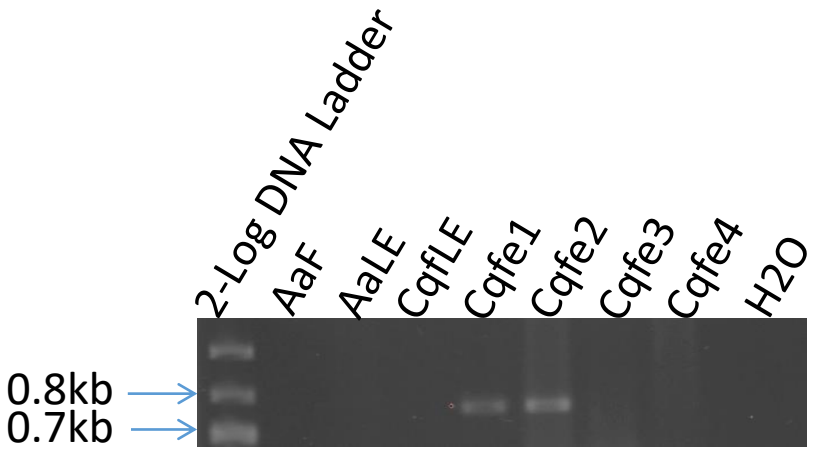

Figure 3.5.2: Mammal primer-spec amplicon

Agarose gel electrophoresis (2%) of PCR products showing the amplification of Cytochrome b when 'mammal all' primers were tested on the mosquito samples, an approximate expected band size 772 bp, that is common to all mammals cytochrome b was seen for two samples (Cqfe1 and Cqfe2). AaF- *Aedes albopictus* field collected, AaLE- *Aedes albopictus* lab emerged, CqfLE- *Culex quinquefasciatus* female and Cqfe1-Cqfe4 - *Culex quinquefasciatus* female engorged. A 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane).

The following gel electrophoresis show the detection of Mosquito (*C. quinquefasciatus*) blood meal source by PCR when primers specific for mammal 'Mammal Universal' (Forward primers for Pig, Humans, Goat, Dog, Cat and Universal Reverse primer) were tested.

Figure 3.5.3

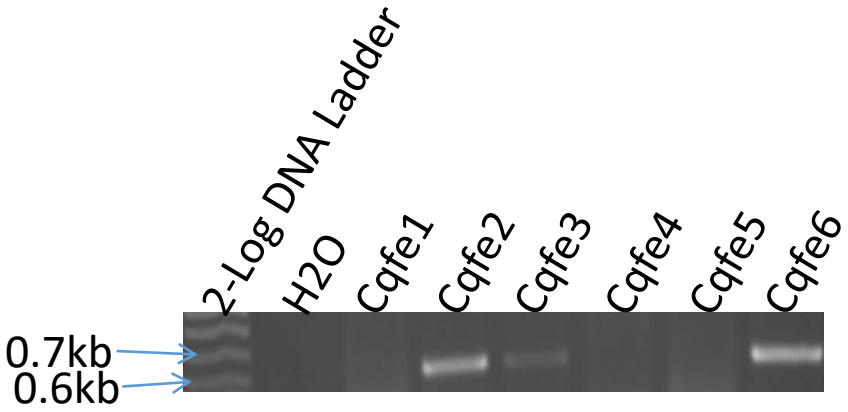

Figure 3.5.3: Mammal univ primer amplicon

Gel electrophoresis (2% agarose) of PCR products from the amplification of cytochrome b using universal reverse primers 'UNREV102B' and individual forward primers for human741, Goat894F, Dog368F, Cow121F were used to amplify 680bp band size fragment from cytochrome b. Band shown for Dog amplification in lane Cqfe2, Cqfe3 and Cqfe6 (Band size 680 bp). Cqfe1- *Culex quinquefasciatus* female engorged. A 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane).

3.6 Mosquito screening for West Nile Virus Detection

The virus RNA detection was attempted by testing the NY99 and 'WNVKUN' primer set on the mosquito samples collected from the UNT campus. The total RNA extraction from the mosquito samples was carried out using TRI Reagent following the manufacturer recommendation (appendix F) and the total RNA was checked in 1% agarose gel (Fig 3.6.1). Reverse Transcription-PCR was used for first strand synthesis of the complementary DNA (cDNA) 1ul of RT-PCR product (cDNA) was then used as the template for PCR with the NY99 primers set. WNV genomic RNA, (kindly supplied by Bethany Bolling) was used as the positive control for the WNV testing. When the PCR products were resolved in 2% agarose gel electrophoresis, virtually all the samples tested seem to be negative for WNV, however, a few samples appear to show a corresponding band size with the positive control (Fig 3.6.2- Fig 3.6.4). This result was not confirmed by sequencing.

The following gel electrophoresis (1%) show the Total RNA extracted, using TRI Reagent from mosquito sample (*C. quinquefasciatus female engorged*) from UNT campus, Denton in 2013

Figure 3.6.1

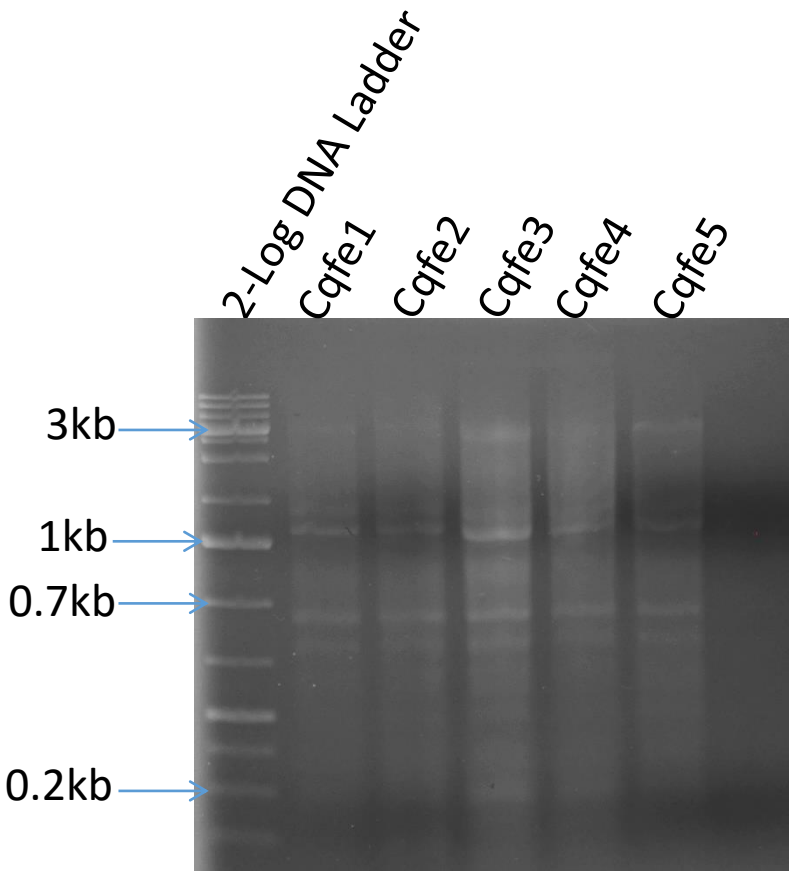

Figure 3.6.1: Total RNA from mosquito

Gel electrophoresis (1% agarose) of total RNA extracted from 5 mosquito samples (Cqfe) collected from the UNT campus. The total RNA extracted was used for reverse transcription PCR reactions before WNV primers were tested on them. Cqfe1 to Cqfe5- *Culex quinquefasciatus* female engorged. A 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard.

The following gel electrophoresis show the PCR product when primers set 'NY99' specific for WNV capsid gene region, were tested on the Mosquito sample C. *quinquefasciatus female engorged* (Cqfe) collected from the UNT Denton main campus in 2013.

Figure 3.6.2

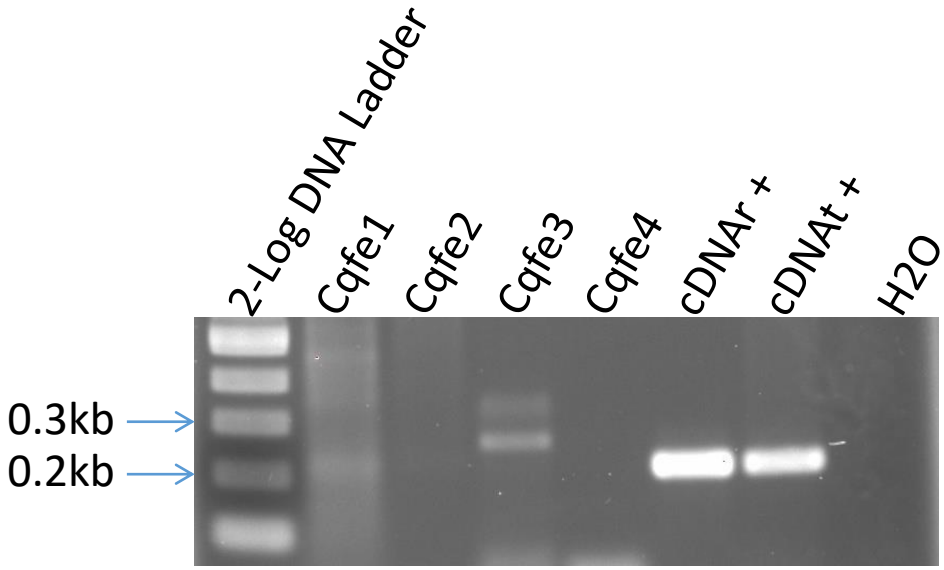

Figure 3.6.2: RT-PCR screening for WNV

Gel electrophoresis (2% agarose) of PCR products obtained from the amplification of capsid region of WNV using 'NY99' primers on mosquito samples, *Culex quinquefasciatus* female engorged (Cqfe) collected from the UNT Denton main campus. cDNAr + represent positive control resulting from random primer synthesized cDNA that was obtained from WNV while cDNAt + depict the positive control resulting from poly-A tail primer synthesized cDNA that was obtained from WNV. 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard.

Figure 3.6.3

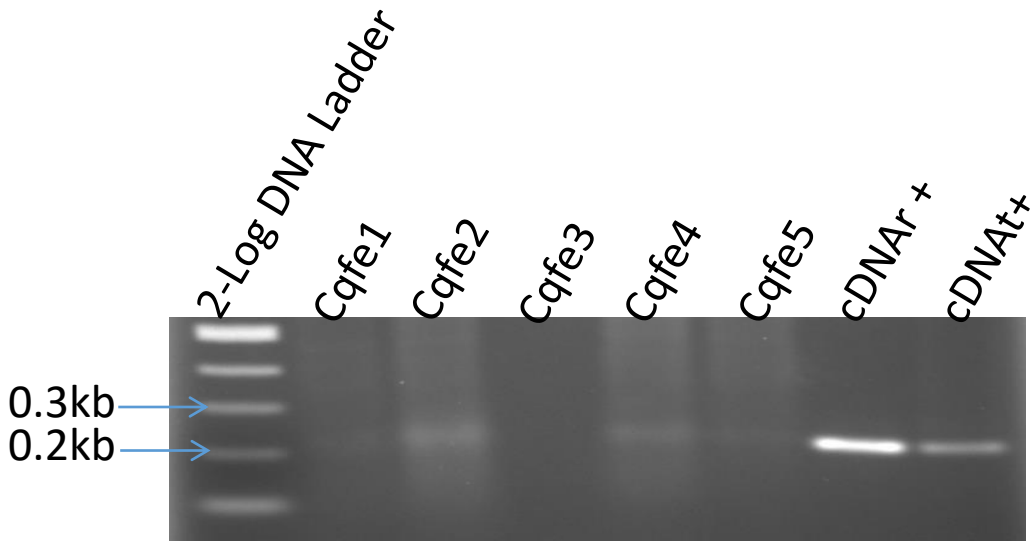

Figure 3.6.3: RT-PCR screening for WNV

Gel electrophoresis (agarose 2%) of PCR products obtained from the amplification of capsid region of WNV using 'NY99' primers on mosquito samples, *Culex quinquefasciatus female engorged* (Cqfe) collected from UNT campus. cDNAr + represent positive control resulting from random primer synthesized cDNA that was obtained from WNV while cDNAt + depict positive control resulting from poly-A tail primer synthesized-cDNA that was obtained from WNV. 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane)

The following gel electrophoresis show the PCR product when primers set 'WNVKUN' specific for WNV were used for the amplification of WNV gene, from the Mosquito sample (*C quinquefasciatus female engorged*) collected from UNT campus in 2013.

Figure 3.6.4

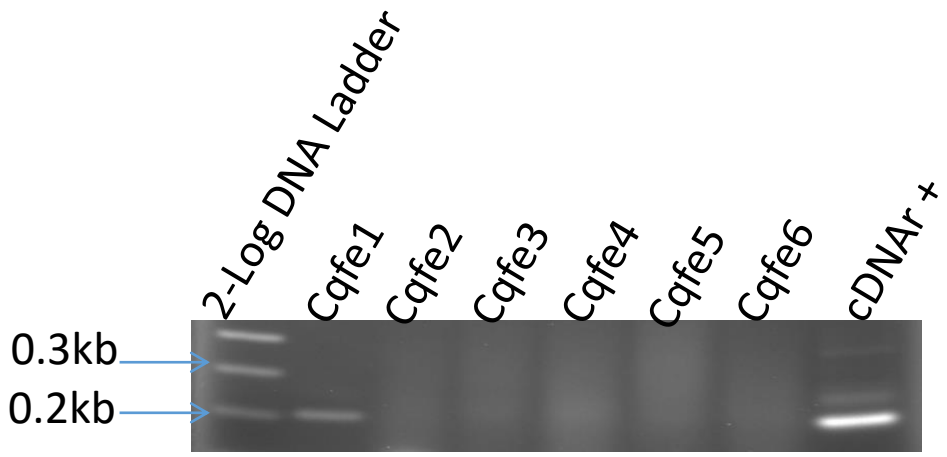

Figure 3.6.4: RT-PCR screening for WNV

Gel electrophoresis (agarose 2%) of PCR products obtained from the amplification of WNV gene using 'WNVKUN' primers on mosquito samples, *Culex quinquefasciatus* female engorged (Cqfe) collected from UNT campus. Cqfe1 to Cqfe6 - *Culex quinquefasciatus* female engorged, +cDNAr represent positive control resulting from random primer synthesized cDNA obtained from a WNV infected bird. A 2-log DNA ladder (New England Biolab Ipswich, MA) was used as the molecular weight standard (first lane).

CHAPTER 4

DISCUSSION

4.1 *Wolbachia* endosymbiont

It has been shown that *Wolbachia* strains *w*MelPop and *w*MelPop-CLA reduce vector competence in *Cx. quinquefasciatus*, as well as in other mosquito species that are vectors of human and animal pathogens such as dengue virus, chikungunya virus as well as West Nile Virus. Several reports have indicated that *Wolbachia* strains native to *Drosophila* such as *w*MelPop and *w*MelPop-CLA could increase resistance against arbovirus infection within the mosquito thereby resulting in decreased virus transmission in a naturally *Wolbachia* infected mosquito system when compared to mosquitoes free of *Wolbachia.* (Glaser and Meola 2010). Therefore, utilization of *Wolbachia,* which is an endosymbiont that is frequently found in many insects, could serve as a potential strategy to control many of the arthropod borne diseases. The *Wolbachia* endosymbionts are maternally inherited and not only show a wide distribution across many insects, including mosquitos, but also are spread rapidly across mosquito population if the endosymbiont is introduced into a new population of mosquito. This ability of mosquitoes to quickly spread their endosymbionts to a wide population is anchored on a phenomenon known as cytoplasmic incompatibility, where a viable mosquito progeny results from mating between *Wolbachia* positive female mosquito and male mosquito having/lacking *Wolbachia*. On this premises, a *Wolbachia* positive female mosquito will continuously produce a new generation of a *Wolbachia* positive mosquito, thus causing a proliferating dispersal of its endosymbiont among the mosquito population.

In 1997, *Wolbachia* strains isolated from *Drosophila melanogaster* were found to be virulent to their natural host, thereby halving the host life span (Min and Benzer 1997). This strategy was adopted in mosquitoes to reduce their vector capacity by introducing *Wolbachia strains* such as *w*MelPop. This organism can cut short the life span of mosquitos into half, thereby, preventing the mosquitoes from maturing to serve as vectors. In addition, *Wolbachia* strains such as *w*MelPop-CLA, capable of inhibiting pathogen development in the mosquito system, have also been utilized to accomplish viral control in the mosquito vector. (Sinkins and O'Neill 2000). In a recent study, it was shown in *Culex quinquefasciatus endosymbiont* that *Wolbachia,* reduced the ability of *Culex quinquefasciatus* to spread West Nile virus (Glaser and Meola 2010). However, Dodson *et al.* (2014) claim that *Wolbachia* (*w*AlbB strain) in *Culex tarsalis* enhanced WNV growth in the mosquito. Therefore, screening for *Wolbachia* presence in local mosquito population in the North Texas region, will serve as the initial step in delineating whether or not *Wolbachia* could serve as an effective potential biocontrol strategy of WNV in the area.

Among the primers sets tested for amplification of diagnostic genes of *Wolbachia*, (Table 2.4), it appeared that the 'wsp' primer set that amplifies the *Wolbachia* 16SrDNA region of the endosymbiont, was the most optimized for *Wolbachia* detection in this study. (Figure 3.3.1 – Figure 3.3.4). Therefore, PCR products from a 'wsp' primers set were cloned into a topo vector for sequencing. (See appendix G). When the sequencing results were blasted against database sequences, up to 99% homology to *Wolbachia* endosymbiont of *C. quinquefasciatus* was observed. Based on the PCR testing for *Wolbachia* in this study, the endosymbiont seems to be uniformly distributed across the

local mosquito population in the study area. Because *C. quinquefasciatus* and other *Culex sp.* have been implicated as the principal vector of WNV in the southern states of the U.S, including the North Texas area (Reisen *et al.* 2005; Andreadis 2004). It was not surprising to find that many of the mosquitoes collected belong to this species, especially the blood engorged ones, and interestingly, many of these test positive for *Wolbachia*. The screening for the presence of *Wolbachia* among the *Culex* mosquitos in this study was done using PCR technique. Results from mosquitoes allowed to emerge in lab or collected in the field demonstrated that the endosymbiont is widely present across the *Culex spp* mosquitoes allowed to emerge in the Lab and in field collected mosquitoes.

4.2 Blood Meal Identification

Understanding the outbreaks and transmission of vector-borne pathogens like WNV can be facilitated by determining that blood-feeding pattern. Determination of vertebrate species that the mosquito obtains its blood meal from, is a potential indicator of the WNV pool in the environment. Significant temporal and spatial proximity between vectors, vertebrate hosts and pathogens could be regarded as factors that ensure cycling the virus among vertebrates through mosquito serving the vector. It was believed that WNV was transmitted by *Culex* spp. (Apperson *et al.* 2004; Kilpatrick *et al*. 2006a). In order to identify the potential vertebrate host that the mosquito is feeding on, PCR amplification of a region of mitochondrial DNA was chosen since this is usually a reliable and well-known diagnostic marker for blood meal identification for both mammalian and avian sources of arthropod blood meals. In addition, there are

mitochondrial sequences for many organisms that have been published and made available in the GeneBank database. Therefore, published primers sets that are available for amplifying specific vertebrate host cytochrome b and cytochrome c oxidase but discriminate against the mosquito genes were used in this study.

Previous studies have shown that, the feeding behavior of *C. quinquefasciatus* had implicated the mosquito as epidemic vectors of WNV due to their mammalophilic (Murphy *et al.* 1968; Buescher and Bickley 1979), as well as their ornithophilic feeding habits, in the Southern part of the U.S (Magnarelli 1977). Mosquito collecting in this study confirms that *C. quinquefasciatus* is the most common mosquito species found to be engorged when compared to the rest of the mosquito species. Therefore, blood typing experiments were mainly conducted on the engorged *C. quinquefasciatus.* Several primers sets were tested to amplify cytochrome B or cytochrome C oxidase region of the mitochondria DNA of the vertebrate host on which the mosquito feed. (Table 2.2). It was realized, however, that only primers sets 'avian1' and 'avianb' seem optimized to yield the expected band size when the PCR product was resolved in 2% agarose gels by electrophoresis. Meanwhile, avian primers that are order specific, such as the Columbiforme, Falconiforme, Passeriforme, Galliforme did not give distinct band size for the PCR products. In general, the PCR testing based on the avian primer set used in this study show that, more avians are fed upon by *C. quinquefasciatus* than mammals, as there are more positive PCR product for avian blood meal host. A number of factors could have contributed to the reason why more positive results were found for avian versus the mammal blood source. One such factor is that the host DNA concentration may not be sufficient for amplification due to blood meal volume from mammalophilic

feeding contrary to ornithophilic feeding. Similarly, blood meal digestion may have denatured host DNA, thus giving a negative PCR result. In addition, as with other studies, the location of the sampling could impact what blood meals were found. These are rational assumption about influencing factors affecting the success of blood meal detection because the time interval between insect feeding and the collection was unknown. Another important factor resulting in the high success rate of avian amplification, is that, avian red blood cells are nucleated while many mammalian red blood cell are anucleated. This could affect the host DNA yield and in turn the PCR success.

In order to account for variation arising from DNA yield, two methods of DNA extraction were used in this study; extraction using a DNeasy extraction kit (Qiagen Valencia CA) and the salt extraction method (Zouache *et al*. 2009). There was no significant difference in PCR success of host blood detection using either method. The finding in this study corroborates the previous studies where host preference for blood meal feeding among mosquitoes was determined. It has been previously suggested that many mosquito species have preferential hemophilic behavior towards certain host species, such that a feeding pattern is not necessarily based upon the abundance, availability and accessibility of a vertebrate host species alone. This factor has been studied for mosquitoes that feed on birds (Kilpatrick *et al.* 2006a; Apperson *et al.* 2004; Savage *et al.* 2007; Hess *et al*. 1968). In WNV epidemiology, as well as in other viruses, it is possible that avian species generally serve as the incubating reservoir of the viruses, however, it has been shown in previous studies that only few species actually function as primary amplifiers of the virus, and only a small portion of bird species play a significant

role in a local WNV transmission dynamics (Loss *et al.* 2009). Thus, it is possible to say that the local mosquito population in this study feed more on avian than mammal species, based on the results obtained.

CHAPTER 5

CONCLUSIONS, RECOMMENDATION AND AREAS FOR FURTHER STUDY

Based on the trend and number of both engorged and non-engorged mosquitoes collected from the field, the highest number of mosquitoes collected was during summer (June, July, August and September) with August and September being the peak collection periods for engorged and non-engorged *C. quinquefasciatus* and *C. tarsalis*. Both the salt extraction and Qiagen DNA extraction kits were optimized method for genomic DNA isolation from mosquito samples. The 'wsp' primer set gave a consistent *Wolbachia* 16SrDNA amplicon size over the mosquito species *C. tarsalis*, *C. quinquefasciatus* and *A. albopictus* (Fig.3.3.1 to Fig. 3.3.4). Sequencing of selected PCR product for *Wolbachia* gave a high degree of homology with the database endosymbiont of *Culex* mosquito with up to 99% homology. The *Wolbachia* was found to be uniformly spread across mosquito samples tested in this study.

The blood typing was based on the primers that have been previously used to target the cytochrome b and cytochrome c oxidases (Table 2.2). Based on the result obtained by PCR technique, the blood meal source testing was highly positive for avian compared to mammal as a source of blood meal (Fig. 3.4.1 – Fig. 3.4.4 and Fig. 3.5.1 – Fig. 3.5.3.) This indicates that the local mosquito population feed more on avian species. However, few positive tests were found for mamalophilic feeding, for example, in only one case out of 119 mosquito samples tested in 2013, using a multiplex PCR specific for pig, human, canine, goat and bovine forward primers along with their universal reverse primers, the one positive, matched the canine cytochrome b band size, linking the possibility of blood meal on dogs (Fig. 3.5.3). However, the results for blood meal host

shown in this study are from female *C. quinquefasciatus* mosquitos collected from a single location on the UNT-Denton campus. Additional samples from the Dallas area are yet to be extensively studied.

FUTURE PERSPECTIVE

The ultimate goal of this study is to be able to use selected *Wolbachia* endosymbiont bacterial strains to interrupt WNV life cycle within the mosquito system. Therefore, the establishment of natural *Wolbachia* infection of the local mosquito population in the study area is an indication of the feasibility of using *Wolbachia* strains capable of eliciting interruption or suppression of WNV in their competent vector. This will be accomplished by embryonic or adult transfection of potent *Wolbachia* strains into the WNV vector mosquitoes. This has been accomplished in *Aedes aegypti*, the vector for dengue virus (Van-den-Hurk *et al.* 2012).

In addition, new primer sets are being tested, in order to identify the blood meal host to the species level, especially for birds so the potential viral source or viral transmission destinations by mosquito can be pinpointed.

APPENDIX A

PROTOCOL FOR DNA EXTRACTION USING DNEASY BLOOD & TISSUE KIT (QIAGEN)

The protocol was followed based on the manufacturer recommendation (Qiagen) as follow

1. A pool of 1-5 mosquitoes was crushed in 180 µl of 1X PBS and 20µl of proteinase K in a 1.5ml microcentrifuge tubes using microcentrifuge pestle.
2. Add 200µl Buffer AL. Mix thoroughly by vortexing. Incubate blood sample at 56°C for 10 min.
3. Add 200µl ethanol (96–100%). Mix thoroughly by vortexing.
4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
5. Place the spin column in a new 2 ml collection tube. Add 500µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube, add 500µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
8. Elute the DNA by adding 200µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g.
9. Optional: Repeat step 8 for increased DNA yield.

Notes before starting

Perform all centrifugation steps at room temperature (15–25°C).

Redissolve any precipitates in Buffer AL and Buffer ATL.

Add ethanol to Buffer AW1 and Buffer AW2 concentrates.

Equilibrate frozen tissue or cell pellets to room temperature.

Preheat an incubator to 56°C.

(DNeasy Blood & Tissue Handbook, 2011; www.qiagen.com/handbooks)

APPENDIX B

PROTOCOL FOR DNA EXTRACTION USING DNA EXTRACTION BUFFER (2% Hexadecyltrimethyl Ammonium Bromide, 1.4M NaCl, 0.02M EDTA, 0.1M Tris, pH 8, 0.2% β-mercaptoethanol) (Zouache *et al*. 2009)

1. Crush a pool of 1-5 whole mosquitoes in 250μL of DNA extraction buffer (2% hexadecyltrimethyl ammonium bromide, 1.4M NaCl, 0.02M EDTA, 0.1M Tris, pH 8, 0.2% β-mercaptoethanol) pre-warmed to 60℃ in a heat block.
2. Incubated the Homogenates for 15 min at 60℃ in a heating block.
3. Remove Proteins in one volume of chloroform/isoamyl alcohol (24/1) by adding 250μL of chloroform/isoamyl alcohol (24/1).
4. Centrifuge at 8000rpm for 3 minutes at room temperature
5. Carefully transfer the DNA solution from the upper layer of chloroform/isoamyl alcohol interphase into a fresh-labeled 1.5ml eppendorf tube.
6. Add one volume of isopropyl alcohol to precipitate the DNA collected and allow the precipitation reaction at room temperature for 10 min.
7. Centrifuge at 11000 rpm for 10min to obtain the DNA pellet, and carefully decant the supernatant, and keep the DNA pellet.
8. Wash the DNA pellet once with 70% ethanol by adding 500 μL of 70% ethanol.
9. Centrifuge at 13000 rpm for 10min, and carefully decant the supernatant while keeping the DNA pellet.
10. Air dry the DNA pellet by inverting the microcentrifuge tube over Kimwipe paper, and then dissolve the DNA in 100 μL of 1X TE Buffer
11. Store the DNA at -20℃ till further use.

APPENDIX C

PROTOCOL FOR THE PURIFICATION OF THE PCR AMPLICON USING THE ZR DNA SEQUENCING CLEAN-UP KIT (Zymoresearch)

The protocol was used as follow based on the manufacturer recommendation (Zymoresearch)

1. Add 240µl of Sequencing Binding Buffer to a 5-20 µl sequencing reaction.
2. Transfer the mixture to a provided Zymo-Spin IB Column (Zymogen, Orlando, FL) in a Collection Tube.
3. Centrifuge at 13,000 rpm (15,000 - 16,000 x g) for 30 seconds.
4. Add 300µl Sequencing Wash Buffer to the column. Centrifuge at 13,000 rpm (15,000 - 16,000 x g) for 30 seconds.
5. Add 6-20µl water directly to the column matrix. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge at 13,000 rpm (15,000 - 16,000 x g) for 15 seconds to elute the DNA.

Ultra-pure DNA is now ready to be loaded into the sequencer.

APPENDIX D

PROTOCOL FOR CLONING PCR FRAGMENTS INTO TOPO VECTORS

The protocol was used based manufacturers recommendation, pCR®8⁄GW⁄TOPO® TA Cloning® Kit' (Invitrogen, Grand Island, NY), as follow;

1. PCR amplify the fragment.
2. Purify the PCR fragment, by Zymogen PCR purification column (See Appendix C).
3. Set up TOPO cloning reaction as follows:

   Fresh PCR product 0.5µl

   Supplied Salt solution 0.5µl

   TOPO vector 0.5µl

   Sterile ultra-pure water to a final volume of 3µl. The topo reaction kit 'pCR®8⁄GW⁄TOPO® TA Cloning® Kit' (Invitrogen, Grand Island, NY).
4. Incubate at room temperature for 30 minutes.
5. Transform the cloning reaction into DH5α competent cells by heat shock method. Place DH5α competent cells from -80℃ on ice to melt. Add 2-3uL of the topo reaction to DH5α competent cells, mix gently by tapping the tube containing the mixture 2-3 times. Allow the mixture to sit for 30 min, after which it was heat shocked at 42℃ for 30 second. Place on ice for 2minutes
6. Re-suspend in 950µl SOC medium). Incubate for an hour at 37°C. by shaking in the incubator at 225rpm
7. Plate on Spectinomycin+ plates (LB plates containing 50ug/ml spectinomycin) and incubate at 37°C overnight.
8. Select single colonies for analysis.

APPENDIX E

PROTOCOL FOR TRANSFORMING ONE SHOT DH5α

The protocol was followed based on the manufacturer recommendation (TOPO TA Cloning)

1. Add 2 µl of the TOPO Cloning reaction from the TOPO Clone into a vial of One Shot max efficiency Chemically Competent *E. coli* DH5α competent cells (Invitrogen, Grand Island, NY) and mix gently. Do not mix by pipetting up and down.
2. Incubate on ice for 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 950µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50µl from each transformation on a pre-warmed selective plate (Spectinomycin plate) and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20µl of S.O.C. medium to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis
   (Reference: TOPO TA Cloning Version R 8 April 2004 25-0184)

APPENDIX F

PROTOCOL FOR RNA EXTRACTION FROM MOSQUITO POOL USING

TRI REAGENT- RNA / DNA / PROTEIN ISOLATION REAGENT

(TRI Reagent was used according to manufacturer recommendation, Molecular Research Center, Inc., Cat. No. TR 118,)

The following procedure was performed at room temperature, except the otherwise stated.

1. HOMOGENIZATION: Homogenize 2 - 5 mosquito pool in TRI Reagent (Sigma-Aldrich, St Louise MO) (1 ml/50 - 100 mg tissue) using a microfuge tube pestle. (Sample volume ≤ 10% of the volume of TRI Reagent used for homogenization).

2. PHASE SEPARATION: Incubate the homogenate at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Add 0.2 ml chloroform to the homogenate that was made in 1 ml of TRI Reagent, in the previous step. Cover the samples tightly and vortex for 15 seconds. Incubate the mixture at room temperature for 2-15 minutes and centrifuge at 12,000 rpm for 15 minutes at 4°C.

3. RNA PRECIPITATION: Transfer the aqueous phase to a fresh tube while the rest (interphase and organic phase) are saved at 4°C DNA and proteins if need be. Precipitate RNA from the aqueous phase by mixing with isopropanol. Add 0.5 ml of isopropanol per 1 ml of starting TRI Reagent to precipitate RNA. Incubate samples at room temperature for 5-10 minutes and centrifuge at 12,000 rpm for 8 minutes at 4°C to pellet RNA on the side bottom of the tube.

4. RNA WASH: Decant the supernatant and wash the RNA pellet with 75% ethanol and subsequent centrifugation at 9000 rpm for 5 minutes at 4 C. Add ≥1 ml of 75% ethanol per 1 ml of starting TRI Reagent used for the initial homogenization.

5. RNA SOLUBILIZATION: Decant the ethanol wash and air-dry RNA pellet briefly for 3 - 5 minutes, without allowing the sample to completely air dried to avoid a decrease in RNA solubility.

Dissolve RNA in RNase-free diethyl pyrocarbonate (DEPC) treated water and incubate for 10-15 minutes at 55-60°C.

6. RESULTS: Expected yield and purity: A pool of 2-5 mosquitoes can yield 0.8 - 2 µg RNA and purity level of 1.6 - 1.9 at OD 260/280 ratio. Total RNA is isolated by TRI reagent including small (~2 kb) and large (~5 kb) ribosomal RNA, low molecular weight (0.1-0.3 kb) RNA, and discrete high molecular weight (7-15 kb) RNA whose band can be visualized in agarose gel stained with Ethidium bromide .

(Reference: Ausubel *et al.* 1990; Chomczynski and Sacchi 1987; Chomczynski, 1993, Chomczynski and Mackey, 1995a, b; Wu 1997)

APPENDIX G

SEQUENCES OF PCR PRODUCTS USED TO IDENTIFY *WOLBACHIA* ENDOSYMBIONT OF MOSQUITOES

Page: 1 / 3

1/30/2014

Samples:

Bases: 1aR 99% identity to *Wolbachia* endosymbiont of *Culex quinquefasciatus* Pel strain wPip 16S ribosomal RNA, complete sequence

Average spacing: 12970

NNNNNNNNNNNNNGTGTACAGACCCGAGAACGTATTCACCGTGGCATGCTG
ATCCACGATTACTAGCGATTCCAACTTCATGTACTCGAGTTGCAGAGTACAAT
CCGAACTGAGATGTCTTTTAGGGATTAGCTTAGGCTTGCGCACCTTGCAGCCC
ATTGTAGACACCATTGTAGCACGTGTGTAGCCCACTCCATAAAGGCCATGAT
GACTTGACATCATCCCCACCTTCCTCCAGCTTATCACTGGCAGTTTCCTTAAA
GTACTCAGCATTACCTGATGGCAACTAAGGATGAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAACACTTG
TGTAAAATCCGGCCGAACCGACCCTATCCCTTCGAATAGGTATGAAGGGCGA
ATTCGGAGCCTGCTTTTTTGTACAAAGTTGGCATTATAAAAAAGCATTGCTCA
TCAATTTGTTGCANCAAACAGGTCACTGTCANTCAAAATAAAATCATTATTTG
GGGCCCGAGCTTAAGACTGGCCGTCGTTTTACAACGTCNNGACTGGGAAAAC
ATCCATGCTAGCTTTAAACGCGAGA

1/30/2014

Samples:

Bases: 2bR 99% identity to *Wolbachia* endosymbiont of *Culex quinquefasciatus* Pel strain wPip 16S ribosomal RNA, complete sequence

Average spacing: 12973 317 41

NNNNNNNNNNNNNNGTGNNNNNACCCGAGAACGTATTCACCGTGGCATGCT
GATCCACGATTACTAGCGATTCCAACTTCATGTACTCGAGTTGCAGAGTACAA
TCCGAACTGAGATGTCTTTTAGGGATTAGCTTAGGCTTGCGCACCTTGCAGCC
CATTGTAGACACCATTGTAGCACGTGTGTAGCCCACTCCATAAAGGCCATGA
TGACTTGACATCATCCCCACCTTCCTCCAGCTTATCACTGGCAGTTTCCTTAA
AGTACTCAGCATTNCCTGATGGCAAATAAGGATGAGGGTTNNGCTNGNGNGA
CNN

1/30/2014

Samples:

Bases: 3aR 100% *Wolbachia* endosymbiont of *Culex quinquefasciatus* Pel strain wPip 16S ribosomal RNA, complete sequence

Average spacing: 12971 520 25

NNNNNNNNNNGNNNGTGNNNNNACCCGAGAACGTATTCACCGTGGCATGCT
GATCCACGATTACTAGCGATTCCAACTTCATGTACTCGAGTTGCAGAGTACAA
TCCGAACTGAGATGTCTTTTAGGGATTAGCTTAGGCTTGCGCACCTTGCAGCC
CATTGTAGACACCATTGTAGCACGTGTGTAGCCCACTCCATAAAGGCCATGA
TGACTTGACATCATCCCCACCTTCCTCCAGCTTATCACTGGCAGTTTCCTTAA
AGTACTCAGCATTACCTGATGGCAACTAAGGATGAGGGTTGCGCTCGTTGCG
GGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAACACTT
GTGTAAAATCCGGCCGAACCGACCCTATCCCTTCGAATAGGTATGAAGGGCG
AATTCNAANCCNGTTTTTTGTACCAANTTTGCNTTANAAAAAAAAAATGCTCC
ACAAATTGGTGCAACGAAACAGGCATTTGTTGNCGGAATTAAATTTATT

1/30/2014

Samples:

Bases: 3bF 100% identity *Wolbachia pipientis* strain wAurGLB7 16S ribosomal RNA gene, partial sequence

Average spacing: 12973 440 30

NNNNNNNNNCNNNNNNNCNGTGTTGCATGGCTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCATCCTTAGTTGCCATCA
GGTAATGCTGAGTACTTTAAGGAAACTGCCAGTGATAAGCTGGAGGAAGGTG
GGGATGATGTCAAGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTA
CAATGGTGTCTACAATGGGTTGCAAGGTGCGCAAGCCTAAGCTAATCCCTAA
AAGACATCTCAGTTCGGATTGTACTCTGCAACTCGAGTACATGAAGTTGGAA
TCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTCTTGT
ACACACTGCCCGTCACGCCATGGGAATTGGTTTCACTCTTANCTANNAANNA
ATTTNTTAATAACTTGTTAATN

1/30/2014

Samples:

Bases: 3bR 99% identity to *Wolbachia* sp. 16S ribosomal RNA gene, partial sequence

Average spacing: 12973 643 21

NNNNNNNNNNGNNNGTGTACAGACCCGAGAACGTATTCACCGTGGCATGCT
GATCCACGATTACTAGCGATTCCAACTTCATGTACTCGAGTTGCAGAGTACAA
TCCGAACTGAGATGTCTTTTAGGGATTAGCTTAGGCTTGCGCACCTTGCAACC
CATTGTAGACACCATTGTAGCACGTGTGTAGCCCACTCCATAAAGGCCATGA
TGACTTGACATCATCCCCACCTTCCTCCAGCTTATCACTGGCAGTTTCCTTAA
AGTACTCAGCATTACCTGATGGCAACTAAGGATGAGGGTTGCGCTCGTTGCG
GGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAACACTT
GTGTGAAATCCGGCCGAACCGACCCTATCCCTTCGAATAGGTATGAAGGGCG
AATTCGGAGCCTGCTTTTTTGTACAAAGTTGGCATTATAAAAAAGCATTGCTC
ATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTT
GGGGCCCGAGCTTAANACTGGCCGTCGTTTTTACAACGTCCTGACTGGGAAA
AACATCCATGCTAGCGTTAAACGCGAGAGTANGGAACTGCCAGGNNTCANAT
AAAACGAANGGNTCT

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