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https://dx.doi.org/10.4314/nvj.v39i4.1 ORIGINAL ARTICLE

Molecular Identification and Phylogenetic Analysis of Acetylcholinesterase-2 Gene From *Culex pipiens* And *Culex quinquefasciatus* (Diptera: Culicine) in Nigeria

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SUMMARY

West Nile virus (WNV) is maintained in an enzootic cycle between ornithophilic mosquitoes and birds. There is paucity of information on population density of these vectors and the molecular details in Nigeria. We determined relative abundance and employed molecular methods to detect *Culex species*. Mosquitoes were caught from April 2013 to February 2014. Genomic DNA extraction was carried out and acetylcholinesterase 2 gene amplified by Polymerase Chain Reaction in different *Culex species*. *Culex pipiens quinquefasciatus* and *Cx. p. pipiens* were identified in different locations, while some other *Culex species* could not be identified. Culex p. quinquefasciatus were sequenced by Sanger's method. Vector density was 100 *Culex species* per man hour. Identification of *Culex pipiens and Cx. quinquefasciatus* from different locations in Ibadan, Nigeria reaffirms the presence of vectors and highlights possibility of enzootic transmission of WNV. Surveillance should be sustained in equine population and bird reservoirs.

Key word index: *Culex pipiens; Culex quinquefasciatus*; Nigeria.

INTRODUCTION

West Nile virus (WNV) infection is distributed worldwide (CDC, 2008). It is primarily maintained in an enzootic cycle involving ornithophilic mosquitoes and birds. Mammals are infected following increased transmission, as climatic and environmental conditions become favorable. Human and equids are infected when bitten by infected mosquitoes (CDC, 2010). Culex mosquitoes are vectors of wildlife disease and their distribution is limited to habitats heavily modified by human (Eastwood *et al.,* 2011). Human are dead-end hosts because viremia occurs at a level where mosquito infection cannot take place (Zeller and Schuffnecker, 2004). Symptomatic infections often manifest as febrile illness in 20% of infected individuals, with severe neuro-

invasive signs in less than 1% individuals (Pierson *et al.,* 2013). In 2013, a total of 2,469 cases of WNV infections with 51% neuroinvasive cases were reported in the United States of America, occurring more in early September (CDC, 2014). In Europe, WNV infection spread in south-eastern states most noticeably central Macedonian region of Greece with neurological symptoms and deaths (Danis *et al.,* 2011). In sub-Saharan Africa, horses have been diagnosed with WNV in South Africa (Venter *et al.,* 2011), Cote d'Ivoire, Senegal, Chad, Gabon, Djibouti and Democratic Republic of Congo (Cabre *et al.,* 2006). Anti-WNV complement fixing antibodies were detected in 72% adult horses tested from two stables in Lagos, southwest Nigeria (Olaleye *et al.,* 1989). Recently, 90.3% WNV neutralizing antibodies were found in a larger study among equines in Ibadan and Lagos, southwest Nigeria (Sule *et al.,* 2015) thereby raising concern about the possibility of human infections. However, the vector density in Ibadan has not been established and there is paucity of information on *Culex species* which is required for WNV surveillance. Therefore, we set out to determine relative abundance and identify some *Culex species* in some parts of Ibadan, Nigeria.

MATERIALS AND METHODS

Study Design

Culex species were spasmodically caught using CDC light trap and direct landing method in four sampling sites (two each) in Agodi and University of Ibadan environs. This was for one hour, on a monthly basis, from April 2013 to February 2014. In a separate catch for molecular identification, Culex mosquitoes were caught once from 6 different locations (Agodi, Awo Hall U.I., Yemetu, Department of Microbiology U.I., Botanical garden U.I., Apata) in Ibadan Oyo State, Nigeria and sorted morphologically into 10 pools.

Polymerase Chain Reaction

Six pools of morphologically identical mosquitoes were macerated in separate mortars after addition of 500µl Phosphate Buffered Saline and stored at -20^{o}C until tested. DNA extraction was carried out using Zymo Research ™ kit. Isolated mosquito DNA was re-suspended in 50µl Tris-EDTA (TE) buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). PCR was performed using Applied Biosystems GeneAmp 9700 thermalcycler with 0.20 µM corresponding sense and antisense primers, 5×PCR buffer, 250 µM dNTP, 2 mM $MgCl_2$, 2.5 units of Taq polymerase, and approximately 6ng of genomic DNA. Cycling conditions were 94^{o}C 3mins, 35cycles of 94^{o}C 30secs, 56^{o}C 30secs, 72^{o}C 1min, and final extension 72^{o}C 10mins. Primers ACEquin 5'-CCTTCTTGAATGGCTGTGGCA-3' and B1246s 5'-TGGAGCCTCCTCT TCACGGC-3' were used to amplify 274bp of acetylcholinesterase 2 gene of *Cx. p. quinquefasciatus* as described previously by Smith and Fonseca (2004). A different primer set ACEtorr 5'-TGCCTGTGCTACCAGTGATGTT-3', ACEpip 5'-GGAAACAACGACGT ATGTACT-3' and B1246s 5'-TGGAGCCTCCTCTTCACGG-3' were used to detect Ace-2 gene of *Cx. p. pipiens* (610bp) and *Cx. torrentium* (416bp) according to methods employed by Manley et al. (2015). Cycling conditions were 94°C 3mins, 35cylces of 94°C 30secs, 50°C 30secs, 72°C 1min, and final extension 72°C 10 mins. Amplicon was visualized on Biorad™ transilluminator using 1.5% agarose gel containing Syber safe. Although the study was done according to same protocol used by two independent studies, (Smith and Fonseca, 2004; Manley et al., 2015).

Phylogenetic Analysis

Amplified products corresponding to expected band size were sequenced commercially by Sanger's method using Big dye terminator at Macrogen®. Nucleotide sequences for Forward and reverse primer amplification were aligned using Staden®. Phylogenetic analyses was carried out based on aligned partial nucleotide sequences of Culex pipiens and Cx. quinquefasciatus species which was generated using neighbor-joining method in MEGA7.0.26, using amino acid p-distances. Also, tree-building methods such as maximum parsimony and maximum likelihood, were used to confirm topology of neighbor-joining tree.

RESULTS

A total of 3,055 *Culex species* were caught in Ibadan from April 2013 to February 2014. First primer set identified Ace-2 gene in *Cx. p. quinquefasciatus* from two pools in Yemetu and Apata areas. Accession numbers for *Cx. p. quinquefasciatus* submitted to the USA National Centre for Biotechnology Information are MH998597 and MH998598. On the other hand, second primer set identified *Cx. p. pipiens* in two locations within University of Ibadan.

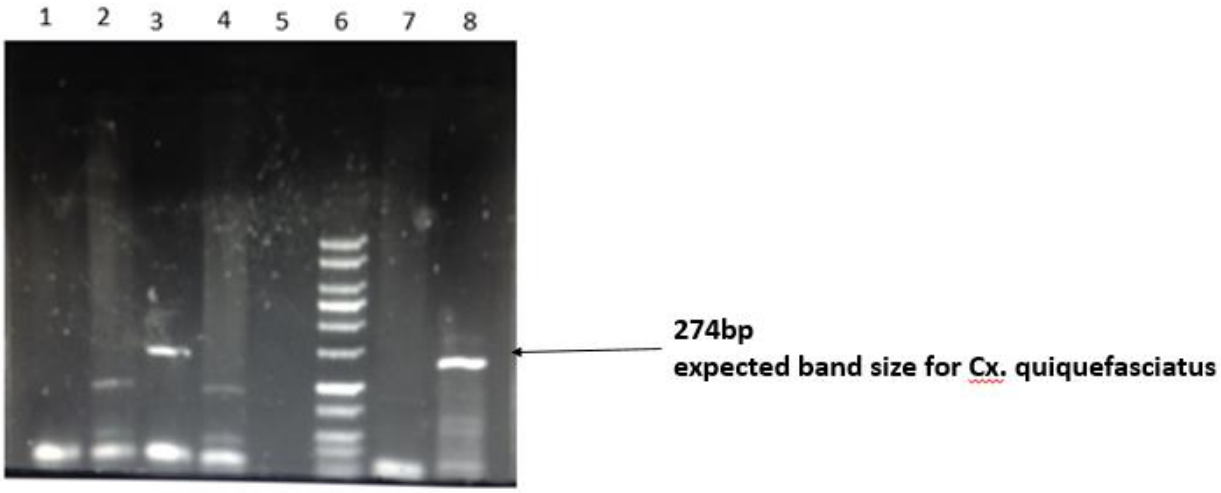

Fig. 1. Molecular identification of *Cx. p. quinquefasciatus* in lanes 3 and 8

Fig. 1: *Culex species* were obtained from the following locations Lane 1 –Agodi, Lane 2 –Awo Hall, Lane 3 –Yemetu, Lane 4 – Department of Microbiology UI environs, Lane 5 – Negative control, Lane 6 – DNA Ladder, Lane 7 –Botanical garden UI, Lane 8 – Apata.

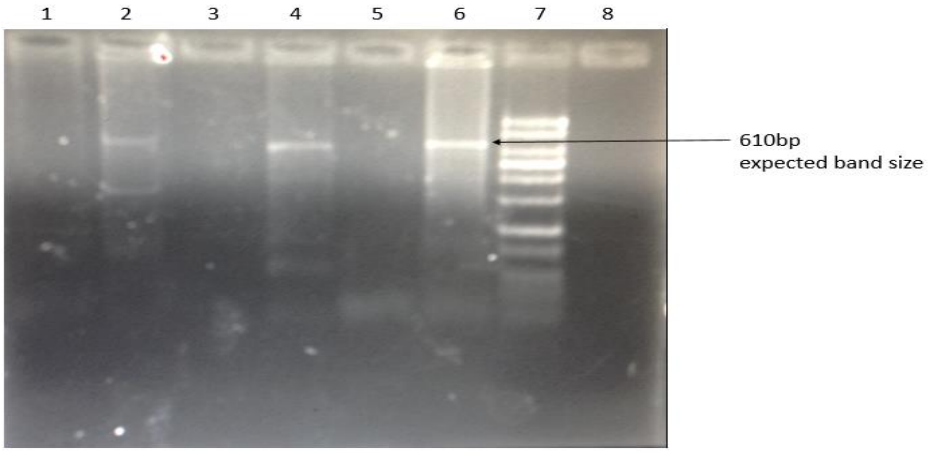

Fig 2. Molecular identification of Ace 2 gene of *Cx. p. pipiens*.

Fig. 2: Culex speices were obtained from the following locations: Lane 1= Yemetu, Lane 2 = Awo hall, Lane 3 = Apata, Lane 4 = Botanical garden, Lane 5 = negative control, Lane 6 = Department of Microbiology environs in University of Ibadan, Lane 7 = 100bp DNA ladder and Apata.

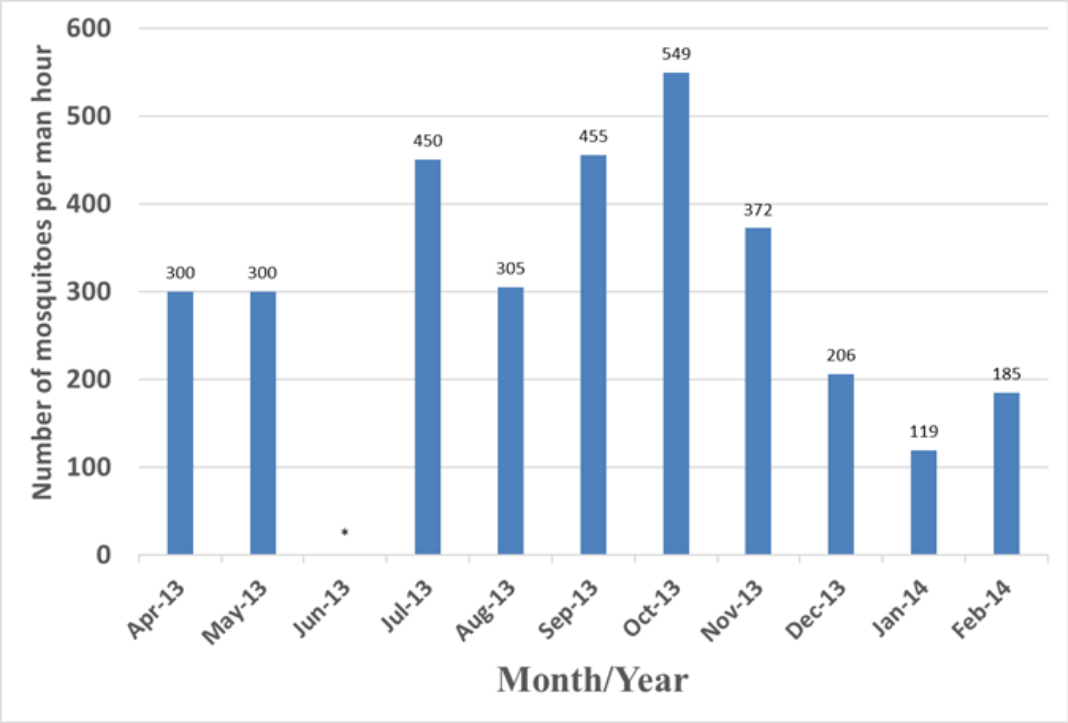

Fig. 3. Distribution pattern of *Culex species* in parts of Ibadan from April 2013 to February 2014

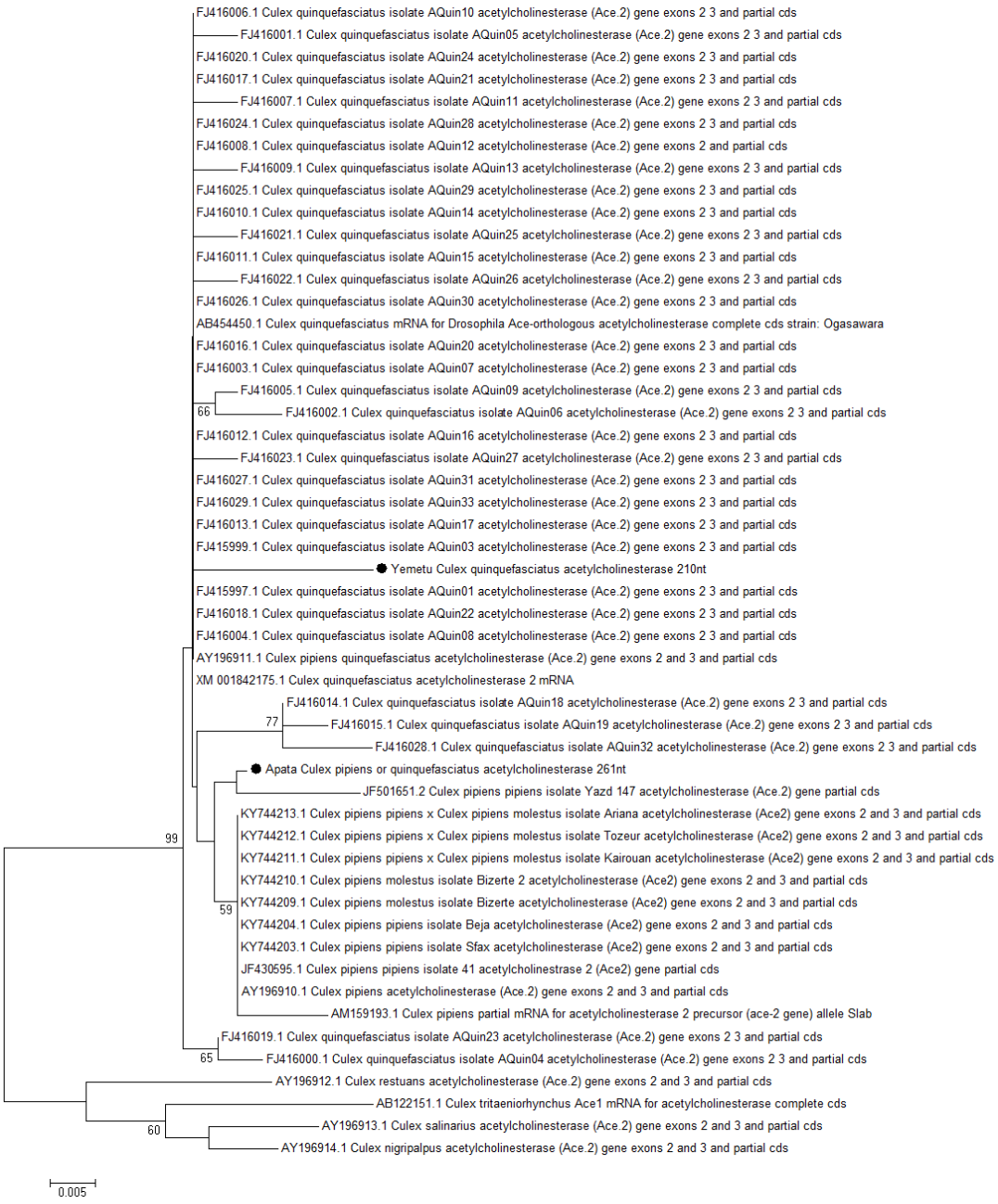

Fig 4. Phylogenetic tree showing sequences of Cx. p. quinquefasciatus from Apata and Yemetu in Ibadan, Nigeria.

DISCUSSION

Identification of *Cx. p. pipiens* and *Cx. p. quinquefasciatus* in this present study is an important step in investigating possibility of WNV enzootics and epidemics. Molecular detection of *Cx. p. quinquefasciatus* (Fig. 1) in two separate locations in Oyo State shows varying vector ecology which reinforces the need for intense mosquito control efforts. This is a significant finding because prior to this study, most researchers in Nigeria relied on morphometric identification of *Culex mosquitoes* (Adeleke *et al.*, 2008). This present study employed PCR technique to overcome the challenges of species misidentification which is very common because of the numerous species that exists. Indeed, there were other *Culex species* that could not be identified using the first set of Ace-2 primers designed by Smith and Fonseca (2004). Second primer set detected *Cx. p. pipiens* (Fig. 2) which shows that robustly designed primers will detect more *Culex* species. The presence of these two *Culex species* indicates that if a viremic horse, bird or other reservoirs is introduced into Ibadan, there is the possibility of an outbreak of west nile virus disease. It is common practice to move horses round the country during polo tournaments. Since WNV infection was reported among febrile patients in 2013 in Maiduguri, northeast Nigeria (Baba *et al.*, 2013), there is need for active WNV surveillance because of the presence of these vectors. Between 1999 and 2012 in the United States of America, WNV was detected in 65 species of mosquitoes including members of the genera *Culex, Aedes, Anopheles, Coquillettidia, Culiseta, Deinocerites, Mansonia, Orthopodomyia, Psorophora* and *Uranotaenia* (CDC, 2015). Several of the mosquitoes in these genera are present in the tropical rain forest region of Nigeria due to a favorably warm climate as corroborated in a separate report from Ibadan where morphometric means was used (Opayele *et al.*, 2017). Preponderance of *Culex species* in this study (Fig. 3) is an early warning of the possible risk of WNV transmission if viremia is high in any horse (or other bird reservoirs). Sule *et al.* (2015) reported 97.8% previous antibody to WNV among 45 horses in Ibadan which further supports the need for increased surveillance especially where horse are kept and around the Ibadan Polo club particularly among horse handlers and grooms, who are at higher risk of infection. To confirm molecular details of Culex species identified in this study, the Maximum Likelihood Neighbor-joining method was employed to determine close similarity. Indeed, our isolates from Apata and Yemetu clustered with *Cx. p. quinquefasciatus* from several parts of the world as shown in Fig. 4.

In conclusion, we have shown in this study that *Cx. p. pipiens* and *Cx. p. quinquefasciatus* which are competent vectors of WNV are circulating in different locations in Ibadan. With the preponderance of vectors, there is the likelihood of WNV transmission if birds and infected horses are in close proximity. This is a major risk factor for West Nile disease especially with the high anti-WNV antibodies against previous infection in horses in Ibadan. This study provides base-line information for initiating surveillance programs and vector control activities. These vector control efforts should be sustained and veterinary public health officials should consider vaccinating all equines to avoid enzootics or possible epidemics.

Conflict of Interest

We declare that we have no conflict of interest.

Authors' contributions

AOB conceived the idea, designed the study and caught *Culex species*; AOB, OAV, MMM carried out molecular detection; all authors read and approved the final manuscript before submission.

Acknowledgement

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