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Survey, DNA barcoding and phylogenetic analysis of mosquito vectors of Japanese encephalitis from Mananthavady Taluk of Wayanad district, Kerala

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Abstract
Mosquito species survey in the Mananthavady Taluk of Wayanad district in Kerala state in southern India revealed the presence of 12 species of mosquito vectors transmitting Japanese encephalitis. DNA barcoding and molecular phylogeny assessment was done for the collected species of vector mosquitoes and the results are discussed.

Keywords: mosquito, vector borne disease, Japanese encephalitis, Kerala, DNA barcoding

Introduction

Mosquitoes (Diptera: Culicidae) are important medical and veterinary insect vectors. Globally, there are around 3500 mosquito species in 112 genera (Harbach and Howard, 2007; Wang *et al*., 2012; Harbach, 2014) [9, 10, 28]. The mosquito population in India is diverse, with 393 species divided into 49 genera and 41 subgenera, of which 31 species are now recognised as potential vectors spreading various human and animal diseases (Bhattacharyya *et al*., 2014; Benelli, 2016; Benelli and Beier, 2017) [3, 4, 5]. Malaria, dengue, Japanese encephalitis, West Nile viral disease, yellow fever, chikungunya, lymphatic filariasis and Zika are the diseases of major concern spread by mosquitoes, of which the first five raise panic among people as it may become fatal (WHO, 2014) [29].

Japanese encephalitis (JE), a disease caused by mosquito borne Flavivirus (Family Flaviviridae) spread by mosquito vector, is of major public health importance due to its high epidemic potential and high case fatality rate (CFR). About one third of the infection results in fatality and almost half of the survivors suffer from mild to severe neuropsychological sequelae (Solomon *et al*., 2000) [26]. The first outbreak of encephalitis linked to Japanese encephalitis virus (JEV) was recorded in Japan (1871) and hence the disease was named Japanese encephalitis (Solomon *et al*., 2000) [26]. JE epidemics have been documented in several places of India, and it is considered a major paediatric problem as serological surveys shows population infected with JE are mostly children and those in early adulthood (Hoke *et al*., 1988) [11]; In 1955, Tamil Nadu was the first state to recognise JE through serological surveys (Namachivayam *et al*., 1982) [19]. Later, outbreaks of JE have occurred in many Indian states and the first epidemic occurred in 1973 in Bengal (Sengupta *et al*., 1974; Rodrigues, 1984; Kumar, 2014) [17, 22, 25]. Approximately half of India's population lives in JE-endemic areas, with 1,500 to 4,000 cases reported each year (Kabilan, 2004) [14]. Mosquito-borne diseases constitute one of the major public health concerns in the state of Kerala too. In Kerala, the first outbreaks of JE occurred in 1996 in Kottayam and Alappuzha districts ((Dhanda *et al*., 1997; John, 2006) [8] and the highest number of cases and deaths were reported in 2011 (Vanaja and Sumodan, 2019) [27]. Even though, *Culex* (*Cx.*) mosquitoes are the major culprits in the transmission of JE, some *Anopheles* (*An.*) and *Mansonia* (*Ma.*) species are also suspected to spread the disease (Kumar, 2014; Pearce *et al*., 2018) [14, 20]. The major JE vector in Southern Asia, Eastern Asia, and South-eastern Asia is Cx. *tritaeniorhynchus* and in India *Cx. vishnui* and *Cx. pseudovishnui* are also identified as major vectors spreading the disease (Innis, 1995; Solomon *et al*., 2000) [12, 26]. During the present survey of mosquitoes in the Mananthavady Taluk of Wayanad district, surprisingly enough, many suspected JE vectors (8 species of *Culex*, 2 species of *Mansonia* and 2 species of *Anopheles*) were collected and identified. DNA barcoding and molecular phylogeny studies were performed for the collected species to assess their unambiguous identification and evolutionary status.

Methodology

Sampling and taxonomic identification: Mosquito sampling was carried out during three different seasons – pre monsoon, monsoon and post monsoon during 2019 – 2021. A total of 12 species of JE vectors belonging to three genera were collected from different places in Mananthavady Taluk of Wayanad district using various methods like light traps, human landing catches, resting collection, larval collection and sweep net collection. Taxonomic keys (Christophers, 1933; Barraud, 1934) [7, 2] were used to identify the species level and confirmed with the help of experts from ICMR – Vector Control Research Centre, Pondicherry, India.

DNA barcoding and molecular phylogeny: Individual samples' whole genomic DNA was isolated from leg tissue using a DNA extraction kit according to the manufacturer's instructions and amplified using a polymerase chain reaction using the forward (5′ – GGA TTT GGA AAT TGA TTA GTT CCT T – 3′) and reverse (5′ – AAA AAT TTT AAT TCC AGT TGG AAC AGC – 3′) primers for their mitochondrial cytochrome oxidase subunit I (COI) gene (Kumar *et al*., 2007) [16]. PCR reaction was performed in a 50µl reaction volume containing 5µl of template DNA, 5µl

of 10X reaction buffer (100mM Tris, 500mM KCl, 15mM $MgCl_2$ and 0.1% Gelatin at pH 9.0), 1µl of 10mM dNTPs, 1µl of each primer, 0.5µl Taq polymerase (2.5 units) and nuclease free water. The dideoxy chain termination approach (Sanger and Coulson, 1975) [24] was used to sequence the purified PCR products using an ABI 3730XL automated sequencer. To locate similar sequences in the NCBI (www.ncbi.gov) database, BLAST tool (Altschul *et al*., 1990) [1] was used. Additional sequences were retrieved from the NCBI database. The forward and reverse strands were aligned using ClustalW in BioEdit software to ensure that the sequences are clear without any mismatches. MEGA X was used to create phylogenetic trees utilising several aligned partial COI gene sequences (Kumar *et al*., 2018). Best-fit nucleotide substitution model was selected from 24 models available in MEGA X based on the minimum Akaike Information Criterion (AIC) value (Posada and Buckley, 2004) [21] and Bayesian Information Criterion (BIC) value. The maximum likelihood (ML) phylogenetic tree's reliability was calculated using bootstrap values after 1000 iterations.

Results

Around the world, the main vector of JE is *Cx. tritaeniorhynchus*, whereas in India, the *Cx. vishnui* subgroups (*Cx. tritaeniorhynchus*, *Cx. vishnui* and *Cx. pseudovishnui*) are the major vectors of JE, followed by *Mansonia* and *Anopheles*. Japanese encephalitis virus (JEV) has been so far isolated from 15 species of mosquitoes from India, comprising 10 species of *Culex* and three species each of *Anopheles* and *Mansonia* (Samuel *et al*., 2000; Kanojia, 2007) [23,15]. Out of these, we could collect 12 species belonging to 3 genera (*Culex*, *Mansonia* and *Anopheles*) from Mananthavady during the present study, namely *Cx. tritaeniorhynchus*, *Cx. vishnui*, *Cx. pseudovishnui*, *Cx. gelidus*, *Cx. infula*, *Cx. bitaeniorhyncus*, *Cx. fuscocephala*, *Cx. quenquefasciatus*, *Ma. indiana*, *Ma. uniformis*, *An. barbirostris* and *An. peditaeniatus*. The *Cx. vishnui* subgroups (*Cx. tritaeniorhynchus*, *Cx. vishnui* and *Cx. pseudovishnui*) were the major vectors collected during this study period (Fig. 1). The graphical representation of the abundance of JE vectors collected were illustrated as clustered column (Fig. 2).

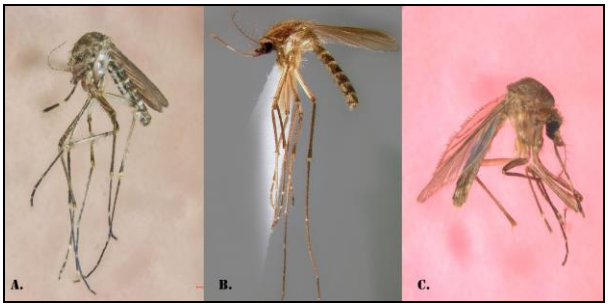

Fig 1: The major Japanese encephalitis vectors of India collected during the present study: A) *Cx. vishnui*, B) *Cx. tritaeniorhyncus*, C) *Cx. pseudovishnui*

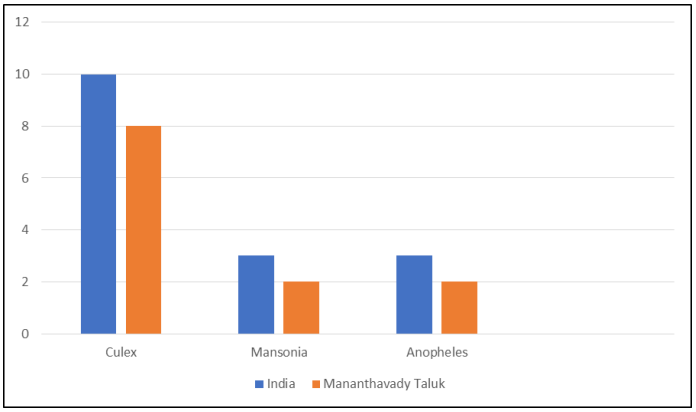

Fig 2: Clustered column showing number of Japanese encephalitis vectors in India and Manathavady Taluk of Wayanad, Kerala.

Table 1: List of JE vector mosquito species collected and barcoded during the present study with the NCBI GenBank accession numbers

SI. No.	Species	Place of collection	GenBank Accession No.
1.	*Culex tritaeniorhyncus*	Mananthavady, Wayanad	MW922794.1
2.	*Culex vishnui*	Mananthavady, Wayanad	MW549044.1
3.	*Culex pseudovishnui*	Mananthavady, Wayanad	MW922745.1
4.	*Culex quenquefasciatus*	Makkiyad, Wayanad	MW926770.1
5.	*Culex gelidus*	Nadakkal, Wayanad	MW542314.1
6.	*Culex infula*	Mananthavady, Wayanad	MW922750.1
7.	*Culex bitaeniorhyncus*	Mananthavady, Wayanad	MW555571.1
8.	*Culex fuscocephala*	Nadakkal, Wayanad	MW535377.1
9.	*Mansonia indiana*	Mananthavady, Wayanad	MW922742.1
10.	*Mansonia uniformis*	Makkiyad, Wayanad	MW542318.1
11.	*Anopheles barbirostris*	Makkiyad, Wayanad	MW922751.1

DNA barcoding of 11 species (except *An. peditaeniatus*) of the collected mosquito species using COI gene sequences were done and the sequence data was deposited in the NCBI GenBank (Table 1). The already reported partial COI sequences of the other four mosquito species found in India (namely *An. peditaeniatus*, *An. subpictus*, *Cx. whitmorei* and *Ma. annulifera*) and an out group (*Phlebotomus papatasi*) were retrieved from the GenBank database and used for phylogenetic analysis. There were no stop codons or frame shifts, indicating that the sequences were not pseudogenes (NUMTs). Molecular analysis verified morphological identity, and phylogenetic relationships were discovered. The illustration of the evolutionary relationships within the selected taxa was represented by Maximum Likelihood (ML) tree (Fig. 3). The tree exhibited distinct conspecific clades differentiated by branch values shown in the tree. The tree constituted three distinctive congeneric clusters. *Anopheles* group (*An. barbirostris* and *An. peditaeniatus*) formed a monophyletic clade in the phylogenetic tree. Among monophyletic *Culex* genus, *Cx. infula* and *Cx. bitaeniorhyncus* formed a single clade with high bootstrap (98) value as they show minute difference morphologically in their abdominal banding pattern. Similarly, closely related species *Cx. vishnui* and *Cx. pseudovishnui* differing only in speckling pattern of hind femora formed a single clade with a bootstrap value 97. All the *Mansonia* species clustered together with 81% confidence.

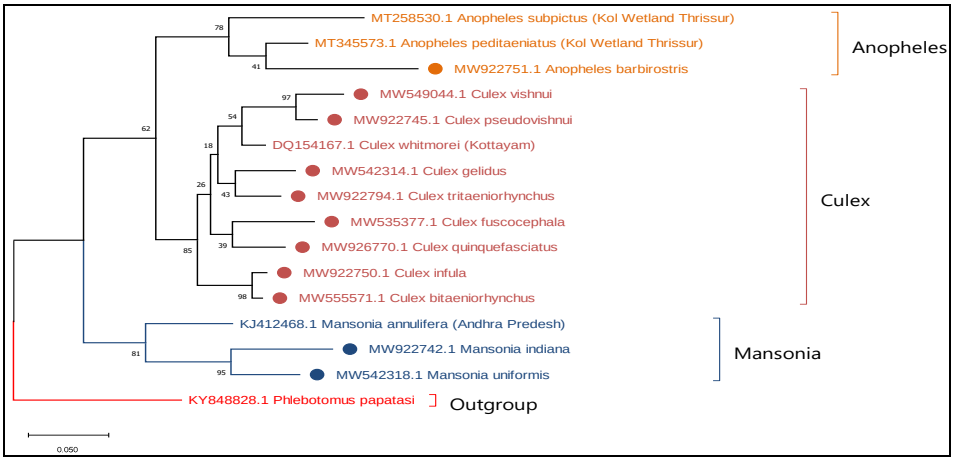

Fig: 3 Phylogeny tree representing the relationship between the Japanese Encephalitis vectors from India based on partial COI gene sequences with *Phlebotomus papatasi* as outgroup (Maximum-Likelihood method).

Discussion

Japanese encephalitis is a global public health issue and its early outbreaks goes undetected due to lack of proper identification and control of potential JE vectors. It results spreading of this vector borne disease throughout the world threatening to become an epidemic. The transmission of the Japanese encephalitis virus (JEV) is attributed to environmental and ecological factors. Agricultural activities linked to irrigation, anthropogenic flooding, drainage, and harvest cycles may have a major impact on *Cx. vishnui* subgroup (*Cx. tritaeniorhynchus*, *Cx. vishnui* and *Cx. pseudovishnui*) numbers in locations with high rice production, irrespective of climatic considerations (Pearce *et al*., 2018). As observed, Mananthavady taluk has large areas that are under rice cultivation or fields with banana plantations that are always water logged. Various plantations (coffee, tea, rubber, cashew etc), intermittent rain and suitable climatic conditions also add to the increase in multiplication of mosquitoes. This would be the contributory factor for the occurrence of 12 out of 16 species of Japanese encephalitis vectors in the area. So, the area should be classified as high JEV endemic.

Conclusion

There is no specific treatment for JE; only prevention can control the disease. Control may be possible only after developing a strong surveillance system through vector control programs together with a high-quality immunization program. In the present study we have made an attempt to find out the JE mosquito vectors of Mananthavady Taluk, Wayanad, a place which is known for its abundance in various kinds of diseases. Molecular barcodes provided in the study would serve as an effective tool for identification and thereby helps in the control of JE vectors.

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Disclosure Statement

The authors declare that they have no competing interests.

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