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Role of *Anopheles subpictus* Grassi in Japanese encephalitis virus transmission in Tirunelveli, South India

Sir,

Japanese encephalitis (JE) distribution is significantly linked to irrigated rice production combined with pig rearing. The *Culex vishnui* subgroup of mosquitoes consisting of *Culex tritaeniorhynchus* Giles, *Cx. vishnui* Theobald and *Cx. pseudovishnui* Colless have been implicated as major vectors of JE virus (JEV)[1]. In India, however, JEV has been isolated from 17 species of mosquitoes; 10 species of *Culex*, three species each of *Anopheles* and *Mansonioides*, and one species of *Armigeres*[2].

In the genus *Anopheles*, the three species that carry JEV are *An. peditaeniatus* Leicester, *An. barbirostris* Van der Walp and *An. subpictus* Grassi. JEV has been isolated from *An. peditaeniatus* in Mandya, Karnataka[3]. It has been isolated from *An. subpictus* in Karnataka[4], Kerala[5] and Tamil Nadu[6]. JEV was isolated from *An. barbirostris* in Asansol, West Bengal[7]. *Anopheles subpictus* was first described by an Italian scientist, Grassi in 1899[8]. This is the most abundant anopheline species in most parts of India which can breed in a variety of habitats such as flowing or stagnant waters, clear or turbid waters, water with or without vegetation, unshaded or slightly shaded water bodies, wells, burrow pits, channels, ponds, tanks, ground pools, fallow and freshly flooded rice fields, cement cisterns, tree holes, lake margins and fresh or brackish waters, and the adult has a flight range of 1.5-6 km[9]. Here we report results of a longitudinal study carried out in Tirunelveli district, Tamil Nadu, India, on the role of *An. subpictus* in JEV transmission.

A longitudinal study of vector abundance and infection frequency was conducted during 2011-2013 in four villages of Tirunelveli district. The study villages, namely, Senthimangalam in Rajavallipuram Primary Health Centre (PHC), Ariyanayagipuram in Mukkudal PHC, Kuthalaperi in Manur PHC and one control village Magiladi in Thirukurungudi PHC of Tirunelveli Zone (based on no JE case incidence reported during the past 10 yr), were selected with the guidance of Tamil Nadu State Health Department, Zonal Entomological Team, located at Tirunelveli. The census data of the index villages were collected from the respective villages. Numerous Little Egret birds in the paddy fields and amplifying host pigs were observed.

Mosquito collection: Mosquitoes were sampled from the selected villages at bimonthly intervals during 2011 to 2013. Adult mosquitoes were collected resting on bushes and thatched roofs of cattle sheds during dusk hours and from human dwellings (indoor resting) and outdoor resting places during day time 0800-1000 h. Mosquito samples were transported to the field laboratory of Centre for Research in Medical Entomology (CRME), Madurai, India, lightly anaesthetized with ether, species identified[10] and sorted on ice into pools of <50 specimens/pool. Unfed mosquitoes were pooled on the same day of collection, whereas engorged female mosquitoes were held for 48 h for digestion of blood meals before pooling. Mosquito (only females) abundance was calculated as density (number collected per man-hour). Mosquito pools were stored at −80°C until processed for virus detection and isolation as described[11]. Two systems were used.

Antigen capture ELISA: Monoclonal antibody 6B4A-10 (reactive against all viruses in JE/WN/SLE/MVE complex) was used as capture antibody and monoclonal antibody peroxidase conjugate SLE MAB 6B6C-1 (reactive against all flaviviruses) as detector antibodies (supplied by Dr. T.F. Tsai, Centers for Disease Control and Prevention, Fort Collins Co., USA). A mosquito

pool was considered ELISA positive if its optical density value was ≥ mean + 4 standard deviation of the six normal pools.

Insect bioassay: *Toxorhynchites splendens* mosquito larvae were inoculated with ELISA positive pools intracerebrally and incubated for 7-10 days at 29°C and then tested by the indirect immunofluorescence assay (IFA) on head squeeze preparations (Toxo-IFA)[11]. Smears were tested with JEV-specific monoclonal antibody, MAB 112 (supplied by Dr. Kimura Kuroda, Tokyo Metropolitan Institute of Neurosciences, Japan) and detected by Fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin (Dakoppats, Denmark).

Vector density was calculated as the number of mosquitoes collected per man hour[11]. Virus infection rate in mosquitoes was expressed as minimum infection rate (MIR) per 1000 females tested[11].

MIR = Number of positive pools/Total number of mosquitoes tested × 1000.

The density of *Cx. tritaeniorhynchus* was compared with that of *An. subpictus* using independent *t* test with SPSS version 16.0 (Chicago, USA). The virus infection rates of *Cx. tritaeniorhynchus* and *An. subpictus* were compared by Fisher's exact test using Epi Info 3.5.3. (CDC software, Atlanta).

Seven species of *Anopheles – An. barbirostris*, *An. culicifacies*, *An. pallidus, An. peditaeniatus*, *An. subpictus*, *An. tessellatus* and *An. vagus* were prevalent in the study area whereas *An. subpictus* was predominant almost round the year. *Cx. tritaeniorhynchus* was found dominant in all the study villages, followed by *An. subpictus*. A total of 13,343 adult mosquitoes were collected, belonging to 24 species of mosquitoes of five genera: *Anopheles* (7 species), *Armigeres* (1 species), *Culex* (9 species), *Mansonia* (2 species) and *Aedes* (5 species) from the four villages. Greater numbers of JE vector *Cx. tritaeniorhynchus*, (9937), *An. subpictus* (1432), *Cx. gelidus* (992) and *Cx. vishnui* (337) were collected from the study villages (Table I). There was only one *Cx. pseudovishnui* collected from the study villages. Species compositions of mosquitoes are shown in Fig. 1. The density of *An. subpictus* ranged from 0 to 62 and the density of *Cx. tritaeniorhynchus* ranged between 0 and 313. The difference between the density of *Cx. tritaeniorhynchus* and *An. subpictus* was significant in Ariyanayagipuram and Senthimangalam (P<0.001, Table II). All the 527 pools were processed for JEV detection by antigen capture ELISA and 28 pools were found positive. JEV was detected from ten species of mosquitoes and 28 positive pools, namely, *Cx. tritaeniorhynchus* (10), *An. subpictus* (7), *Cx. infula* (2), *Mansonia annulifera* (2), *Ma. uniformis* (2), *Cx. bitaeniorhynchus* (1), *Cx. quinquefasciatus* (1), *An. pallidus* (1), *An. barbirostris* (1) and *Armigeres subalbatus* (1). JEV infection was high in Ariyanayagipuram (13), followed by Senthimangalam (8), Kuthalaperi (4) and Magiladi (3). Month-wise JEV infection in *Cx. tritaeniorhynchus* and *An. subpictus* in the study villages are given in Fig. 2. Among ten pools of *Cx. tritaeniorhynchus* and seven pools of *An. subpictus* positive in ELISA, seven and four pools were further confirmed as JEV by Toxo-IFA, respectively.

Night-time human biting collection studies in Rajasthan, India, showed two feeding peaks for *An. subpictus*, one early in the night and the other just before dawn[12]. *Anopheles subpictus* is strongly zoophagic feeding mostly on bovines (83%) and rarely on pigs (0.6%) and humans (0.4%)[13], and has quite often been suspected to be involved in the epidemiology of JE transmission as predicted in Gorakhpur district, Uttar Pradesh, in North India[14]. *Anopheles subpictus* was reported as a vector of JEV in Cuddalore, an area of Tamil Nadu, India, endemic for the disease[6]. In Vellore district, *An. subpictus* was the most dominant species after *Cx. vishnui* group and was collected throughout the year[13].

Blood meal analyses of *An. subpictus* were collected from different places of India such as Assam, Poona (Pune), Jaypore hills, South-East India and Delhi with anthropophilic index of 2.3, 0.4, 0.0, 3.1, 0.0 and 2.4 per cent, respectively[15]. In the present study, the anthropophilic index was calculated to be 25 per cent. The duration of gonotrophic cycle was 98, 102 and 88 h in rainy, winter and summer seasons, respectively, and the average being 96 h. Proportion parous, daily survival rate and daily mortality rate were 0.51, 84 and 16 per cent, respectively. Among the female population, 14.5 per cent passed three or more gonotrophic cycles in natural conditions. Both *An. subpictus* and *An. hyrcanus* were suspected as secondary vectors for JE as they prevailed in high density[16]. During JE season, substantial densities of *An. subpictus* and *An. peditaeniatus* suggest the supportive role of these species[17]. In the present study seven of the 28 positive pools (25%) were from *An.*

Table I. Japanese encephalitis virus infection in mosquitoes in Tirunelveli district (2011-2013)

Species	Village														
	Senthimangalam			Ariyanayagipuram			Kuthalaperi			Magiladi			Total		
	Number of mosquitoes	Number of pools	Number of pools positive	Number of mosquitoes	Number of pools	Number of pools positive	Number of mosquitoes	Number of pools	Number of pools positive	Number of mosquitoes	Number of pools	Number of pools positive	Number of mosquitoes	Number of pools	Number of pools positive
Culex bitaeniorhynchus	23	2	0	2	2	1	15	3	0	4	2	0	44	9	1
Cx. fuscanus	1	1	0	0	0	0	1	1	0	2	1	0	4	3	0
Cx. fuscocephala	3	2	0	0	0	0	15	3	0	27	3	0	45	8	0
Cx. gelidus	690	26	0	183	12	0	3	3	0	116	6	0	992	47	0
Cx. infula	19	5	1	82	7	1	16	3	0	2	1	0	119	16	2
Cx. pseudovishnui	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
Cx. tritaeniorhynchus	3772	89	3	3167	76	3	1368	34	2	1630	46	2	9937	245	10
Cx. vishnui	79	9	0	38	10	0	117	5	0	103	8	0	337	32	0
Cx. quinquefasciatus	38	6	0	18	4	1	0	0	0	3	3	0	59	13	1
Mansonia annulifera	32	8	1	15	9	1	0	0	0	0	0	0	47	17	2
Ma. uniformis	51	9	2	11	4	0	0	0	0	0	0	0	62	13	2
Anopheles barbirostris	16	5	0	6	3	1	0	0	0	0	0	0	22	8	1
An. culicifacies	3	2	0	0	0	0	0	0	0	0	0	0	3	2	0
An. nigirimus	0	0	0	6	1	0	0	0	0	0	0	0	6	1	0
An. pallidus	7	4	0	12	4	0	2	1	0	7	3	1	28	12	1
An. peditaeniatus	28	4	0	30	5	0	34	1	0	0	0	0	92	10	0
An. subpictus	177	13	0	620	23	5	312	12	2	323	19	0	1432	67	7
An. tessellatus	2	1	0	1	1	0	0	0	0	0	0	0	3	2	0
Armigeres subalbatus	65	10	1	2	1	0	6	3	0	37	7	0	110	21	1
Grand total	5007	197	8	4193	162	13	1889	69	4	2254	99	3	13343	527	28

Table II. Statistical analysis on mosquito density (number of mosquitoes collected per man hour)

Area	*Culex tritaeniorhynchus*	*Anopheles subpictus*	*P*
Ariyanayagipuram	8.38	3.38	<0.001
Kuthalaperi	7.46	4.16	0.054
Magiladi	5.72	2.75	0.035
Senthimangalam	7.14	1.55	<0.001
All villages	7.25	3.01	<0.001

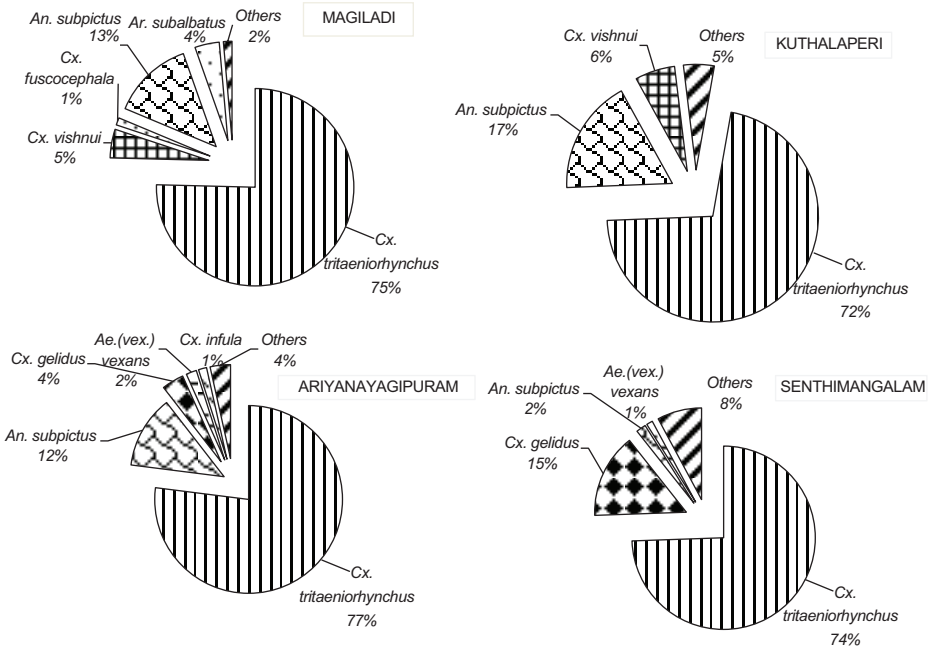

Fig. 1. Mosquito species compositions (January 2011 - November 2013).

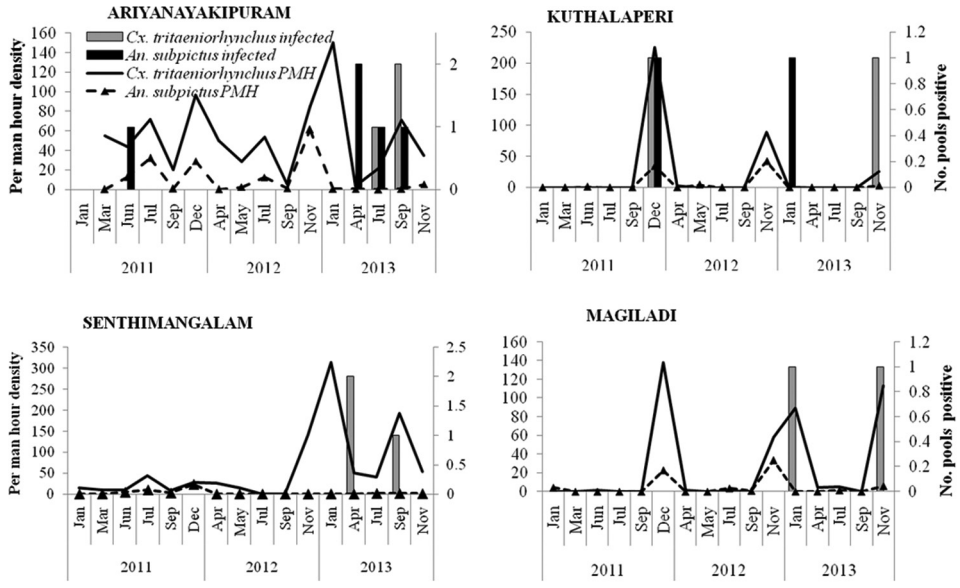

Fig. 2. Mosquito density and Japanese encephalities virus infection (village-wise).

subpictus and also next to the JE primary vector *Cx. tritaeniorhynchus* (10/28, 36%).

Anopheles subpictus has a great adaptability to survive with many other mosquito species in almost all types of breeding habitats. Its man-hour density was higher than other anophelines in most part of its distribution. Although the cattle blood is the first choice, its moderate anthropophilic index and high survival rate in all seasons are indicative for its role as disease transmitters.

With the isolation of JEV from *An. subpictus* in this study, it was demonstrated that this species acquired the infection in nature and might transmit this infection and act as a secondary or bridge vector in JEV transmission in Tirunelveli as they prevailed in high density.

***Conflicts of Interest*:** None.

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