Long-term study of Japanese encephalitis virus infection in *Anopheles subpictus* in Cuddalore district, Tamil Nadu, South India

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Summary

**OBJECTIVE** To investigate the role of *Anopheles subpictus* Grassi as a vector of Japanese encephalitis virus (JEV) transmission in Cuddalore, an area of Tamil Nadu endemic for the disease.

**METHOD** We collected 98 pools (4900 specimens) of wild adult male *An. subpictus* mosquitoes outdoors during dusk hours and screened them for JEV antigen by antigen-capture Enzyme Linked Immunosorbent Assay. Additionally, over a period of 1 year, we tested 166 pools (8300 specimens) of wild adult female *An. subpictus* mosquitoes collected indoors for JEV.

**RESULTS** Four pools of male *An. subpictus* tested positive. This indicates possible natural transovarial transmission of the virus through *An. subpictus*. Nineteen female pools were positive with a minimum infection rate of 2.3. From January through March the maximum infection rate was highest: 5.0 compared with 1.7 between April and September and 2.1 from October to December, although the difference was not statistically significant. From the 19 positive female pools, four isolates were confirmed as JEV by insect bioassay.

**CONCLUSION** The role of *An. subpictus* as a secondary vector in JEV transmission in Cuddalore, Tamil Nadu lends support to the hypothesis of periodic epidemics in the region.

**keywords** Japanese encephalitis, *Anopheles subpictus*, secondary vector, India

Introduction

Japanese encephalitis (JE), caused by a mosquito-borne virus was first recognized in India in 1955 and since then many major outbreaks from different parts of the country have been reported, predominantly in rural areas. Children are mainly affected, with morbidity rate estimated at 0.30–1.5 per 100 000 population. The case fatality rate ranges from 10% to 60%, and up to 50% of those who recover may be left with neurological deficits (Reuben & Gajanana 1997). JE is a seasonal disease: epidemics coincide with the monsoon and post-monsoon period (August–December), agricultural practices, high density of the mosquito vector (because of stagnant water) and presence of pigs and ardeid birds. The Japanese encephalitis virus (JEV) is a member of the genus Flavivirus and belongs to the JEV antigenic complex under family Flaviviridae. JEV is an enveloped virus about 40–50 nm in diameter, containing a molecule of infectious linear positive-sense single stranded RNA (Gajanana & Arunachalam 1998). JEV is maintained in nature by a complex cycle that involves pigs as amplifying hosts, ardeid birds as reservoirs and mosquitoes as vectors.

The *Culex vishnui* subgroup of mosquitoes consisting of *Cx. tritaeniorhynchus* Giles, *Cx. vishnui* Theobald and *Cx. pseudovishnui* Colless have been implicated as major vectors of JEV. In India, however, JEV has so far been isolated from 16 species of mosquitoes; 10 species of *Culex* and three species each of *Anopheles* and *Mansonioides* (Philip Samuel et al. 2000).

In the genus *Anopheles*, the three species that carry JEV are *Anopheles peditaeniatus* Leicester, *An. barbirostris* van der Walp and *An. subpictus*. While JEV has once been isolated from *An. peditaeniatus* in Mandya, Karnataka (Mourya et al. 1989), it has been isolated from *An. subpictus* in both Karnataka (George et al. 1987) and Kerala (Dhand & Kaul 1980). JEV was isolated from *An. barbirostris* only once, in Asansol, West Bengal (Chakravarty et al. 1975). During investigations of the first outbreak of JE in the Kuttanad area of Kerala state in 1996, our team along with Vector Control Research Centre, Pondicherry, detected JEV in one pool containing two *An. subpictus* mosquitoes (Dhand & Kaul 1980). *An. subpictus* breeds profusely in rain water accumulations and fallow rice fields, (Dhand & Kaul 1980), waste water...
disposal systems and irrigated sites (Mukhtar et al. 2003), and is also associated with floating and submerged aquatic vegetation in the vicinity of rice plants (Kant et al. 1996). Nighttime human biting collection studies by Tyagi and Yadav (2001) in Rajasthan, India, showed two feeding peaks for *An. subpictus*, one early in the night and the other just before dawn. *An. subpictus* is strongly zoophilic, feeding mostly on bovines (83%), rarely on pigs (0.6%) and humans (0.4%; CRME Annual Report 1997–98).

Mainly five species of Anopheles – *An. vagus*, *An. pallidus*, *An. peditaeniatus* and *An. barbirostris* are prevalent in the study area where *An. subpictus* was predominant almost round the year. In view of the above information, it is now considered opportune to examine whether *An. subpictus* is involved in JEV transmission in Cuddalore district, Tamil Nadu.

**Materials and methods**

**Study area**

We conducted a longitudinal study in five JE endemic villages of Cuddalore district: Kodikkalam, Pennadam, Soundarasalapuram, Eraiyyur and Neyvasal, between May 1997 and December 1999. These villages receive irrigation water through a canal from the Wellington Reservoir. In all villages animals live very close to human dwellings, and there are only makeshift cattle sheds, but no separate pig sties. At night pigs take shelter under small lean-tos next to human dwellings and often share human dwellings and cattle sheds. Tamil Nadu receives rain showers from June to August under the influence of the southwest monsoon, but heavier rainfall from September to December due to the northeast monsoon. There are no extremes of temperature and during the study period temperature ranged from 30 to 41 °C with relative humidity varying between 70% and 89%.

**Mosquito collections**

Mosquitoes were sampled from each village at biweekly intervals between May 1997 and December 1999. For virus detection, however, two collectors moved freely throughout the village and caught both blood-engorged and unfed adult mosquitoes resting on bushes and thatched roofs of cattle sheds and human dwellings. Mosquitoes were collected for 1 h after dusk by aspiration and flashlight, and subsequently transported to the field laboratory for various processing. As dusk collections yielded but a small number of *An. subpictus* during early part of the study in 1997 and 1998, more emphasis was given to collections of mosquitoes in the indoor resting sampling during 1999. Fully fed mosquitoes were held for 24–48 h for digestion of blood meals, anesthetized with ether, identified to species level using a key prepared by Reid (1968) and sorted on ice into pools of 50 specimens each, and transported in liquid nitrogen for further testing to the Centre for Research in Medical Entomology (CRME) head quarters at Madurai.

**Virus detection and isolation**

Mosquito pools were processed for virus infection by following two different and complementary systems. (i) Antigen-capture enzyme linked immunosorbent assay (ELISA): the procedure followed was that of Gajanana et al. (1995), using monoclonal antibody 6B4A-10 (reactive against all the viruses in the JE-WN-SLE-MVE complex) as capture antibody and monoclonal antibody peroxidase conjugate SLE MAB 6B6C-1 (reactive against all flaviviruses) as detector antibody. (ii) Insect bioassay: mosquito pools positive in ELISA were inoculated intracerebrally into *Toxorhynchites splendens* Wiedemann larvae, incubated at 29°C for 7 days, then tested by indirect immunofluorescence assay (IFA) on head squash preparations (Toxo-IFA). Smears were screened using a JEV-specific monoclonal antibody, MAB 112 (Kimura-Kuroda & Yasuri 1983), and detected by antimouse immunoglobulin conjugated with Fluorescein isothiocyanate (FITC) (Dakoppats, Denmark).

**Statistical analysis**

Virus infection rate in mosquitoes was expressed as minimum infection rate (MIR) per 1000 mosquitoes tested.

\[
\text{MIR} = \frac{\text{Number of positive pools}}{\text{Number of mosquitoes tested}} \times 1000
\]

Virus infection rates were calculated using SPSS 11.5 software package (SPSS Inc., Chicago, IL, USA) and chi-squared test by Epilinfo (CDC, Atlanta, GA, USA).

**Results and Discussion**

**Entomology**

Mainly five species of *Anopheles* (*An. subpictus*, *An. vagus*, *An. pallidus*, *An. peditaeniatus* and *An. barbirostris*) were prevalent in the study area of which *An. subpictus* was predominant almost round the year. Therefore, only this species was analysed. Its per man-hour density ranged between 2.83 and 199.05 in the study villages. The density of *An. subpictus* was higher in hot months than in cool months (CRME Annual Report 1997–98).
Virus infection

From March to December 1997, we collected 98 pools (4900 specimens) of wild adult male and 33 pools (1650 specimens) of adult female *An. subpictus* mosquitoes through outdoor sampling during dusk hours. They were screened for JEV antigen by ELISA and four male pools were found positive, indicating the existence of natural transovarial transmission of JEV in *An. subpictus*. No female pools were found positive (Table 1).

In 1998, from January to August, outdoor sampling resulted in seven pools (350 specimens) of male *An. subpictus* collected, which tested negative for JEV. Higher numbers of mosquitoes were sampled in the indoor resting collections from September onwards. Seventy-four female *An. subpictus* pools (3700 specimens) were tested for JEV; 11 pools (MIR 3.0) were positive with four JEV isolates. JEV infection was present in two seasons in *An. subpictus*, from October to December (MIR 3.5) and from July to September (MIR 1.2). All four isolates were from October to December season (Table 2 and Figure 1).

In 1999, we collected 166 pools (8300 specimens) of wild adult female *An. subpictus* mosquitoes from indoors and tested them for JEV. Nineteen female pools were positive with MIR 2.3. JEV infection was present in all the seasons in *An. subpictus* with a higher MIR during January–March (5.0) than during other seasons (1.7, 1.7 and 2.1 for April–June, July–September and October–December respectively) but the difference in MIR between the four seasons was not statistically significant ($\chi^2 = 4.649$, $P = 0.199$; d.f. = 3). From the 19 positive female pools, four JEV isolates were confirmed as JEV by insect bioassay (*Toxorhynchites splendens* – immunofluorescent assay; Table 2). Season-wise minimum infection rate of *An. subpictus* for JEV is given in the Figure 1.

Discussion

In Vellore district, *An. subpictus* was the most dominant species after *Cx. vishnui* group and was collected throughout the year (Reuben 1971). JEV has been isolated from *An. subpictus* in Karnataka (George et al. 1987), Kerala (Dhanda et al. 1997). The reproductive rate of *An. subpictus* also varied from 1.38 in February to 57.2 in July (Panicker et al. 1981). In view of high prevalence of *An. subpictus* during the month of July (10.7%) when JE transmission was also high, *An. subpictus* has quite often been suspected to be involved in the epidemiology of JE transmission as predicted by Kanojia et al. (2003) in Gorakhpur district, Uttar Pradesh in North India.

Mukhtar et al. (2003) studied the role of wastewater irrigation in mosquito breeding in South Punjab, Pakistan and found that *An. subpictus* was the most predominant (11.8%) anopheline breeding during the months of July and September. Murty et al. (2002) also studied the prevalence of major vectors of JEV in an endemic district of Andhra Pradesh, India and their results confirmed those of Mukhtar et al. (2003), although in indoor resting collections the predominant species was the *Cx. vishnui* subgroup comprising 42.6% of the total collection, followed by *An. subpictus* (40.4%). In an entomological survey in Kurnool and Mehboobnagar districts of Andhra Pradesh state in January 2002, Sharma et al. (2003) suspected both *An. subpictus* and *An. hyrcanus* as secondary vectors for JE as they prevailed in high density (70/man-h).

| Table 1 Japanese encephalitis virus (JEV) minimum infection rate of *Anopheles subpictus* collected outdoor during dusk hours in Cuddalore district |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Month           | Male No. of pools tested* | Male No. pools positive | MIR/1000 | Female No. of pools tested* | Female No. pools positive | MIR/1000 | Total No. of pools tested* | No. pools positive | MIR/1000 |
| May 1997        | 10               | 2                | 4.0      | 3               | 0                | 0        | 13               | 2                | 3.1       |
| June            | 15               | 1                | 1.3      | 6               | 0                | 0        | 21               | 1                | 1.0       |
| July            | 27               | 0                | 0.0      | 5               | 0                | 0        | 32               | 0                | 0.0       |
| August          | 2                | 0                | 0.0      | –               | –                | –        | 2                | 0                | 0.0       |
| September       | 2                | 0                | 0.0      | –               | –                | –        | 2                | 0                | 0.0       |
| October         | 34               | 1                | 0.6      | 15              | 0                | 0        | 49               | 1                | 0.4       |
| November        | 7                | 0                | 0.0      | 4               | 0                | –        | 11               | 0                | 0.0       |
| December        | 1                | 0                | 0.0      | –               | –                | –        | 1                | 0                | 0.0       |
| Total           | 98               | 4                | 0.8      | 33              | 0                | 0        | 131              | 4                | 0.6       |

* Pool size 50

MIR, Minimum infection rate.
Anopheles subpictus is known to transmit malaria and filariasis (Hoedojo et al. 1980; Amerasinghe & Amerasinghe 1999). In an isolated study of multiple host-feeding of *An. subpictus* in field populations and its specific role in transmitting malaria in Sri Lanka, Amerasinghe and Amerasinghe (1999) revealed that multiple blood feeding within the same gonotrophic cycle was attributed to a local ‘frequent feeding strategy’ in this primarily zoophagic and endophilic malaria vector. On the contrary, in Indonesia, *An. subpictus* is a potential vector of bancroftian filariasis in the Robek area, as 11.3% of this species that fed on microfilaraemia carriers harboured *Wuchereria bancrofti* larvae (Hoedojo et al. 1980).

The adult density of *An. subpictus* was higher in the hot months (199.05) than in the cool months whereas in the same area density of *Cx. vishnui* subgroup mosquitoes always remained high (1356.33) during JE transmission seasons, September to October (CRME Annual Report, 1997–98). *An. subpictus* was found to be strongly zoophilic, feeding mostly on bovines (83%). In contrast, blood meals of *Cx. vishnui* subgroup mosquitoes in the same area showed that 89% had fed on bovines, 3% on human and 3% on pigs (CRME Annual Report 1997–98). The JEV minimum infection rate in *An. subpictus* species of mosquito was 2.3, which is slightly higher than that of *Cx. vishnui* subgroup mosquitoes at 1.7 (CRME Annual Report 1997–98).

Among *Cx. vishnui* subgroup mosquitoes, we have proved *Cx. tritaeniorhynchus* to be the major vector for JEV transmission in Cuddalore district (CRME Annual Report 1997–98; Gajanana et al. 1997). With the isolation of JEV from *An. subpictus* in this study, it can be

### Table 2  Month-wise, season-wise Japanese encephalitis virus (JEV) minimum infection rate of female *Anopheles subpictus* collected indoors in Cuddalore district

<table>
<thead>
<tr>
<th>Year 1998*</th>
<th>No. of pools tested†</th>
<th>No. pools positive</th>
<th>MIR/1000</th>
<th>Year 1999</th>
<th>No. of pools tested†</th>
<th>No. pools positive</th>
<th>MIR/1000</th>
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<td>April–June</td>
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<td>August</td>
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<tr>
<td>September</td>
<td>17</td>
<td>1</td>
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<td>September</td>
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<tr>
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<td>1.2</td>
<td>July–September</td>
<td>59</td>
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<td>1.7</td>
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<tr>
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<td>33</td>
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<tr>
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<td>19</td>
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<td>November</td>
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<td>December</td>
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<tr>
<td>October–December</td>
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<td>3.5</td>
<td>October–December</td>
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<tr>
<td>January–December</td>
<td>74</td>
<td>11 (4)</td>
<td>3.0</td>
<td>January–December</td>
<td>166</td>
<td>19 (4)</td>
<td>2.3</td>
</tr>
</tbody>
</table>

MIR, minimum infection rate.

* From January 1998 to August 1998, only outdoor collections of *Anopheles subpictus* were carried out during which seven pools of males were collected and found negative for JEV. From September 1998 to December 1999, indoor resting collections were carried out.

† Pool size 50.

Figures in parantheses indicate number of isolates among the ELISA positives which were further confirmed as JEV by insect-Bioassay.

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**Figure 1** Japanese encephalitis virus (JEV) minimum infection rate of female *Anopheles subpictus* collected indoors in different seasons in Cuddalore district.

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*Anopheles subpictus* is known to transmit malaria and filariasis (Hoedojo et al. 1980; Amerasinghe & Amerasinghe 1999). In an isolated study of multiple host-feeding of *An. subpictus* in field populations and its specific role in transmitting malaria in Sri Lanka, Amerasinghe and Amerasinghe (1999) revealed that multiple blood feeding within the same gonotrophic cycle was attributed to a local...
demonstrated that the species is feasible to acquire the infection in nature, and may transmit the infection. Thus it can be safely surmized that An. subpictus may also play a role as a secondary or bridge vector in the months when An. subpictus density is very high, maintain the virus circulation in nature and also maintain the JEV by transovarial transmission. Further work is required on this particular species of mosquito to validate the role in JEV transmission.

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**Etude de long terme de l’infection par le virus de l’encéphalite japonaise chez *Anopheles subpictus* dans le district de Cuddalore, Tamil Nadu**

**Objectif** Investiger le rôle de *Anopheles subpictus* Grassi comme vecteur dans la transmission du virus de l’encéphalite japonaise à Cuddalore, une région de Tamil Nadu, endémique pour la maladie.

**Méthodes** Nous avons collecté 98 pools (49 000 spécimens) de mâles adultes et sauvages du moustique *An. subpictus* à l’extérieur, au coucher du soleil, et les avons testé pour rechercher l’antigène du virus de l’encéphalite japonaise au moyen d’un test ELISA de capture d’antigène. Nous avons également testé sur une période d’une année, 166 pools (83000 spécimens) de femelles adultes sauvages du moustique *An. subpictus* collectés à l’intérieur pour JEV.

**Résultats** 4 pools de *An. subpictus* mâles étaient positifs au test. Cela indique une transmission trans-ovarienne possible du virus chez *An. subpictus*.

19 pools de femelles étaient positifs avec un taux d’infection minimum de 2.3. De janvier à mars le taux maximum d’infection était le plus élevé: 5.0 comparé à 1.7 entre avril et septembre et 2.1 entre octobre et décembre, quoique les différences n’étaient pas statistiquement significatives. Des 19 pools femelles positifs, 4 souches ont été confirmées pour le virus de l’encéphalite japonaise par un test biologique.

**Conclusion** Le rôle de *An. subpictus* comme vecteur secondaire dans la transmission du virus de l’encéphalite japonaise à Cuddalore, Tamil Nadu procure un soutien à l’hypothèse d’une épidémie périodique dans la région.

**Mots clés** encéphalite japonaise, *Anopheles subpictus*, vecteur secondaire, Inde

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**Estudio a largo plazo de la infeccio´ n del Virus de la Encefalitis Japonesa en *Anopheles subpictus* en el distrito de Cuddalore, Tamil Nadu**

**Obietivo** Investigar el papel del *Anopheles subpictus* Grassi como vector transmisor del virus de la encefalitis Japonesa en Cuddalore, un área en Tamil Nadu, endémica para esta enfermedad.

**Método** Se recolectaron en el exterior, durante horas de penumbra, 98 grupos (49000 especímenes) de mosquitos machos adultos salvajes de *An. subpictus*, y se probaron para el antibiogéno del virus de la encefalitis japonesa mediante un ensayo inmunoadsorbente ligado a enzimas de captura de antígenos. Adicionalmente, durante un periodo de 1 año, se probaron 166 grupos (83000 especímenes) de mosquitos hembras adultas salvajes de *An. subpictus* recolectadas intradomiciliariamente.

**Resultados** Cuatro grupos de machos de *An. subpictus* fueron positivos. Esto indica una posible transmisión natural transovariana del virus a través de *An. subpictus*. Diecinueve lotes de hembras fueron positivos, con una tasa de infección mínima del 2.3. Entre Enero y Marzo la tasa de infección máxima fue 5.0; la mayor, comparada con 1.7 entre Abril y Septiembre, y 2.1 entre Octubre y Diciembre, aunque la diferencia no fue estadísticamente significativa. De los 19 lotes de hembras positivos, 4 aislados fueron confirmados como virus de la encefalitis japonesa mediante bioensayo con insectos.

**Conclusión** El papel del *An. subpictus* como un vector secundario en la transmisión del virus de la encefalitis Japonesa en Cuddalore, Tamil Nadu, apoya la hipótesis de epidemias periódicas en la región.

**Palabras clave** encefalitis japonesa, *Anopheles subpictus*, vector secundario, India