Detection of Japanese encephalitis virus antigen in desiccated mosquitoes: an improved surveillance system

S. C. Tewari, V. Thenmozhi, R. Rajendran, N. C. Appavoo and A. Gajanana

Japanese encephalitis virus (JEV) is an important cause of epidemic encephalitis in South-East Asian countries including India. At present, surveillance for JEV in endemic areas is mainly based on clinical case reporting and abundance of vector mosquitoes. The monitoring of virus infection in vector mosquitoes should form an essential component of a surveillance system. However, conventional assay procedures (tissue culture, suckling-mice and mosquito-inoculation techniques) for detection of virus are cumbersome for the routine monitoring of infection status in mosquitoes. The antigen-capture enzyme immunoassay (EIA) (Peiris et al., 1992) offers a rapid and sensitive method for detection of JEV infection in wild-caught vector mosquitoes and hence is particularly useful for surveillance purposes.

The assay was validated in a large-scale field trial in southern India (Gajanana et al., 1997). Mosquito specimens were sent from the field to the Centre for Research in Medical Entomology (CRME), Madurai (India), in liquid nitrogen where they were stored at -80°C. Pools were first screened for flavivirus infection by EIA using broadly reactive antibody and the positive results were confirmed by insect bio-assay using virus-specific monoclonal antibody (MAB). But, the main limitation of this procedure for routine application in a public health programme is the need for a liquid nitrogen cylinder or other means for transportation and storage facilities under sub-zero conditions for field-collected specimens. Peiris et al. (1992) demonstrated that JEV antigens could be detected in dead mosquitoes by EIA. No further work on this important observation has been reported. Based on their finding we have developed a system for monitoring JEV infection in vector mosquitoes using EIA on desiccated specimens in endemic areas in Tamil Nadu (India) with the involvement of the field staff of the Public Health Department (PHD). The process of development of the system was in 3 stages. First, the effect of storage of dried, infected mosquitoes at room temperature on the stability of JEV antigen was studied in the laboratory. Second, natural infection rates in wild-caught mosquitoes transported in liquid nitrogen and stored at -80°C were compared with parallel desiccated specimens stored at room temperatures. Third, natural infection rates in wild-caught desiccated mosquitoes, sent by Zonal Entomological Teams (ZETs) of the PHD, were determined.

Laboratory study

A batch of 50, 3-day-old female Culex tritaeniorhynchus Giles from a laboratory colony were fed orally with JEV [P 20778; supplied by the National Institute of Virology (NIV), Pune, India] mixed with defibrinated goose blood as described previously (Philip Samuel et al., 1998). Full-fed mosquitoes (n = 42) were held at 29°C and 80% relative humidity, for 14 days, taking all precautions to prevent accidental escape of experimental insects from the holding cages. At the end of this period, one batch of 21 mosquitoes was crossed at -80°C (Batch A), and another batch of 21 mosquitoes was killed and then allowed to dry at room temperature (32-34°C) for different periods (Batch B). On days 7, 14, 21 and 28, sub-samples consisting of 5, 5, 5 and 6 mosquitoes, respectively, were removed from Batch B and transferred to -80°C until tested. All the 42 mosquitoes were individually homogenized and tested for JEV antigen by antigen-capture EIA (Gajanana et al., 1997) using the broadly reactive MAB 6B4A-10 as capture antibody and the broadly reactive MAB peroxidase conjugate, SLE MAB, 6B6C-1, as detector antibody (supplied by T. F. Tsai, Centers for Disease Control and Prevention, Fort Collins, USA). A mosquito was considered EIA positive if its OD value was ≥ mean + 4 SD of the negative control (normal uninfected laboratory colony mosquitoes). The cut-off OD for this experiment was 0.104 (mean and SD of the negative control were 0.068 ± 0.001, respectively). In Batch A, 19 (90.5%) and in Batch B, 20 (95.2%) mosquitoes tested were positive for JEV antigen. In Batch B, positivity rates of sub-samples tested after 7, 14, 21 and 28 days were 100, 100, 100 and 100%, respectively. The mean OD values of positive specimens of Batch A (0.37 ± SE 0.045) and Batch B (0.49 ± SE 0.064) were comparable (t = 1.53, d.f. 37, P = 0.13). Thus, storage at temperature between 32 and 34°C for at least 28 days did not appear to affect the stability of the JEV antigen.

Field evaluation

Between September 1995 and February 1997, female adults of Culex species were collected during dusk hours in 3 villages—Kodikkalam, Pennadam and Soundarasapuram—in Cuddalore district (highly endemic for JE), sorted by species and made into pools containing 5-50 mosquitoes per pool, and transported to Madurai. From each species, half the number of pools was transported in liquid nitrogen and stored at -80°C, and the other half was dried, transported and stored at ambient temperature. A total of 977 pools (3 449 specimens) were collected and 80°C were tested by EIA: 33 were found positive for flavivirus antigen, 29 of which were confirmed as JEV by inoculation to Toxorhynchites splendens larvae followed by immunofluorescence using the JE-specific MAB, MAB 112 (supplied by Dr Kimura Kuroda, Tokyo Metropolitan Institute of Neurosciences, Japan) (Mourya et al., 1989). Similarly, out of 850 pools (3 642 specimens) of desiccated specimens tested by EIA, 30 were positive of which 2 with high OD values (1.055, and 1.185) were tested by inhibition ELISA (Tsai et al., 1987) using JE and/or West Nile virus (WN) specific MAB (supplied by NIV, Pune, India). One was confirmed as JE and the other as WN. The remaining positive pools were not tested as their OD values were not sufficiently high to perform inhibition ELISA. The minimum infection rate (MIR: infection rate per 1000 females tested) of mosquitoes stored at -80°C (0.84) was comparable with that of the desiccated specimens (0-80) (x² 0.06, d.f. 1, P = 0.80). The MIRs of the dominant vector, Cx. tritaeniorhynchus, by the 2 storage methods were also comparable (x² 0.26, d.f. 1, P = 0.60) (Table).

Surveillance in Tamil Nadu

Nine districts in Tamil Nadu each having a ZET were chosen for the monitoring of JEV infection in vector mosquitoes by EIA. Of these, Trichy, Perambalur and Cuddalore are endemic for JE. In Madurai, Vellore and Coimbatore there were outbreaks of JE in the past but no cases are being reported at present; in Salem, Virudhunagar,
Table. Comparison of Japanese encephalitis virus infection rates (revealed by enzyme immunoassay) in mosquitoes after desiccation or after storage at -80°C

<table>
<thead>
<tr>
<th>Species</th>
<th>Stored at -80°C</th>
<th>Desiccated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested*</td>
<td>Positive*</td>
</tr>
<tr>
<td>Cx. tritaeniorhynchus</td>
<td>626 (31 210)</td>
<td>30</td>
</tr>
<tr>
<td>Cx. gelidus</td>
<td>128 (47 23)</td>
<td>3</td>
</tr>
<tr>
<td>Cx. vishnui</td>
<td>79 (23 41)</td>
<td>0</td>
</tr>
<tr>
<td>Others*</td>
<td>46 (875)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>879 (39 149)</td>
<td>33</td>
</tr>
</tbody>
</table>

*Values are number of mosquito pools (number of specimens in parentheses).

Thanjavur and Dindigul, only sporadic cases of encephalitis clinically diagnosed as JE are reported. Staff of the ZETs were given training in collection, identification, desiccation and transportation of JE vector mosquitoes. Samples of wild-caught females were pooled by species (7–50 specimens per pool) and sent in small polythene packets with complete collection data to CRME by post. At CRME, they were re-identified and stored at room temperature until tested. From March 1996 to February 1999, a total of 318 pools (15 489 specimens) of dried mosquitoes belonging to Cx. vishnui subgroup (established vectors of JE in southern India) sent by the monitoring stations were examined by EIA. The time interval between collection and examination by EIA was about 30 days. The number of pools received from each station was, however, small, ranging from 4 (Dindigul) to 97 (Virudhunagar). Four EIA-positive pools (1 each from Cuddalore and Perambalur districts, and 2 from Virudhunagar) were detected in the months of October to January (1 in each month) which is the transmission season for JE in southern India. The surveillance work is continuing.

Comments

Our study confirms the observation of Peiris et al. (1992), that JEV antigen can be detected in dried mosquitoes by EIA, and indicates its potential as a surveillance system for monitoring natural JEV infection in wild mosquitoes in known endemic areas. JEV infection in mosquitoes in nature follows a Poisson distribution—a rare and random event (Sokal & Rohlf, 1981). For example, in the present study the MIR of vector species in Cuddalore was 0.84 in specimens stored at -80°C, and 0.80 in desiccated specimens. Similarly in a previous study in the same area (Gajanana et al., 1997), the MIR was 0.32. Therefore thousands of specimens need to be examined in order to detect 1 infected mosquito. Hence, the success of the system depends upon the number of mosquito samples sent by monitoring teams to the central laboratory for testing.

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References


