COMPARATIVE EVALUATION OF BIOASSAY AND ELISA FOR DETECTION OF JAPANESE ENCEPHALITIS VIRUS IN FIELD COLLECTED MOSQUITOS

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Abstract. Comparative evaluation of enzyme-linked immunosorbent assay (ELISA) and bioassay (virus isolation in *Toxorhynchites splendens* larvae and identification by immunofluorescence test using virus specific monoclonal antibody) was carried out in order to define a suitable strategy for monitoring Japanese encephalitis virus infection in field mosquitos. A total of 8,850 adult female mosquitos in 177 pools (*Culex tritaeniorhynchus* 91, *Cx. vishnui* 59 and *Cx. fuscocephala* 27) collected from an endemic area of Tamil Nadu were examined by both the techniques. In ELISA, 9 pools which had optical densities (OD) equal to the mean of normal infected pools plus ≥ 4 standard deviations (SD) were considered positive and all of them were virus positive by the bioassay also. Sixty-five pools had OD = Mean + 2-3 SD and 103 pools had OD = Mean + < 2 SD of normal pools. From these groups, 12 (18.5%) and 8 (7.8%) pools respectively were found to be virus positive by the bioassay. In total 29 (16%) pools were positive by the bioassay as against 9 (5%) by ELISA.

This study demonstrated that the bioassay is sensitive for estimation of true positives and ELISA is a rapid screening system. A protocol has now been developed for surveillance in which field pools are first screened by ELISA and only those with OD = Mean + ≥ 2 SD are assayed in *Toxorhynchites*. By excluding a large majority of pools with low OD (Mean+ < 2 SD), which are likely to yield only a small percentage of true positives, the cost, time and labor involved are greatly reduced. The rationale behind this approach is discussed.

INTRODUCTION

Reliable estimation of natural mosquito infection with arboviruses forms a key element in any surveillance system and is essential to vector incrimination and for monitoring control measures. Traditionally, cell cultures and suckling mice have been employed for virus isolations. More recently mosquito inoculation techniques have been reported for detection and propagation of flaviviruses. (Kuberski and Rosen, 1977; Gubler *et al*, 1978; Gubler *et al*, 1979; Thet-win, 1982; Pang *et al*, 1983; Lam *et al*, 1986; Dhanda *et al*, 1989; Mourya *et al*, 1989). Bioassays are generally cumbersome, requiring special facilities (Scott and Olson, 1986) and therefore are not particularly suitable for large scale epidemiological surveillance. Enzyme-linked immunosorbent assay (ELISA) has been shown to be rapid and sensitive alternative to bioassays for monitoring arboviruses in wild mosquito populations (Scott *et al*, 1987; Tsai *et al*, 1987).

In an ongoing study of epidemiology of Japanese encephalitis (JE), a three-year longitudinal study of JE virus (JEV) infection in mosquitos was carried out by us in an endemic area for JE in Tamil Nadu. Initially, *Toxorhynchites splendens* larva was employed for detection of virus infection in field mosquitos (bioassay). While this study was in progress, Peiris *et al* (1992) reported JEV antigen detection using ELISA, but our initial attempts to use this technique for testing infected mosquito pools were unsuccessful. Subsequently, following certain technical improvements ELISA was finally adapted for field use. In order to define a suitable strategy for surveillance of JEV infection in natural mosquito populations, a comparative evaluation of bioassay and ELISA was carried out on field specimens. Standardization of the two techniques in the laboratory and their evaluation on field collected mosquitos are presented in this paper. No similar study has been reported previously.

MATERIALS AND METHODS

Larvae of *Tx. splendens* from a cycling laboratory colony were reared in individual plastic vials (ap-

proximately 30 ml capacity), and were fed with larvae of other species of mosquitos maintained in the laboratory. A cycling colony of *Cx. tritaeniorhynchus* was maintained in the laboratory. All inoculated and normal mosquitos were held at 28 ± 1°C and relative humidity of 80%.

Bioassay

In order to determine the time of appearance of JEV specific fluorescence and its nature, early fourth instar larvae of *Tx. splendens* were inoculated intracerebrally (Mourya, 1990) with 10^{-2} dilution of JEV (P 20778) (provided by the National Institute of Virology, Pune) in 0.75% bovine albumin phosphate buffered saline (BAPS) pH 7.4. Controls were inoculated with BAPS. From post infection (PI) day 2 to 7, headsqueeze smears of virus inoculated and control larvae were examined by indirect immunofluorescence (IIF).

In order to study the value of the bioassay to detect virus infection in pools of mosquitos, a batch of 2-day-old female *Cx. tritaeniorhynchus* from the laboratory was inoculated intrathoracically (Rosen and Gubler, 1974) with 10^{-2} dilution of JEV (P 20778). After 10 days incubatiion, pools of 50 mosquitos each were prepared by addition of 1 infected adult to 49 uninfected colony adults, triturated in cold porcelain pestle and mortar in 0.6 ml of BAPS and the suspension centrifuged at 6,000*g* at 4°C for 30 minutes. The supernatant of each pool was inoculated intracerebrally to a group of 30 *Tx. splendens* larvae. From PI days 3-7, headsqueeze smears of 5 inoculated larvae per pool were examined by IIF.

IIF

Headsqueeze smears were fixed in chilled acetone for 5 minutes. A drop of either (1) rabbit anti JE immune serum (RIS) (raised in the laboratory following the protocol of the National Institute of Virology, Pune) or (2) JEV specific mouse monoclonal antibody, MAB 112, (kindly provided by Dr Kimura-Kuroda, Japan), diluted 1 : 100 in phosphate buffered saline (PBS) pH 7.2, was added to cover the smear, incubated in a humid chamber at 37°C for 30 minutes followed by 3 washes in PBS. Then 1 : 200 dilution of fluorescein isothiocyanate (FITC) conjugated antirabbit immuno-globulin (DAKO Catalogue No.F205) or 1 : 100 dilution of FITC conjugated anti-mouse gammaglobulins (Immunodiagnostics) as the case may be was added, incubated at 37°C for 30 minutes, followed by 3 washes in PBS. The smears were mounted in 90% glycerol in PBS pH 7.2 and examined under Leitz fluorescence microscope (Aristoplan). A smear of a JEV infected *Toxorhynchites* larva and a smear of an uninoculated larva were included as positive and negative controls respectively in each day's work. A test smear if positive when stained with RIS indicated flavivirus infection. JEV specificity was ascertained by positive fluorescence in MAB 112 stained smear.

Antigen capture ELISA
Standardization

Two-day-old females of *Cx. tritaeniorhynchus* were inoculated intrathoracically with 10^{-2} dilution of JEV (P 20778) and were incubated for 10 days. Pools of 50 mosquitos each (1 infected plus 49 uninfected adults) were prepared and used for ELISA. In the first experiment, 5 pools were individually ground in cold porcelain pestle and mortar in 0.6 ml BAPS, centrifuged and the supernatant tested by ELISA.

In the second experiment, 9 pools were triturated individually in cold glass tissue homogenizer (Ten Broeck) in 0.6 ml BAPS, frozen and thawed twice, centrifuged and the supernatant examined by ELISA.

In the third experiment, 22 pools were used. Ten pools were individually ground in cold glass homogenizer and 12 pools were individually triturated in separate plastic microcentrifuge tube with glass pestle fitted to high speed motor. The homogenates, after freezing and thawing twice, were centrifuged and the super-natants examined by ELISA.

ELISA

The procedure followed was essentially that of Peiris *et al* (1992). The inner 60 wells of a flat bottomed microtiter plate (Titertek) were coated with monoclonal antibody, 6B4A-10, (reactive against all the viruses in the JE/WN/SLE/MVE Complex) diluted 1 : 10,000 in carbonate bicarbonate buffer pH 9.0. The coated plate was held at 4°C overnight and next day after emptying the wells, 1% gelatin in PBS 7.4 was added to block non-specific binding and the plate left at room temperature for 1 hour. This was followed by the addition of 25µl of mosquito sus-

pension and 25µl of (0.05%) Tween-20 in PBS (Wash buffer) and the plate was incubated at 4°C overnight. Next day it was washed gently with wash buffer and the detector monoclonal antibody peroxidase conjugate [SLE MAB 6B6C-1, (Division of Vector-borne Infectious Diseases, CDC, USA.) reactive against all flaviviruses] (Tsai *et al*, 1987) diluted 1 : 2,000, in buffered saline (containing 2% bovine serum albumin, 0.1% Tween-20 and 3,000 units of heparin per 100 ml of PBS pH 7.4) was added, incubated at 37°C for 90 minutes and after 6 further washes with wash buffer, the substrate (O - phenyllenediamine 2 mg, and urea peroxidase 1 mg in 5 ml phosphate citrate buffer, pH 6.0) was added and left at room temperature in dark for 1 hour. The reaction was stopped by addition of 2M H_2SO_4, and optical density (OD) read at 490 nM in a BIOTEK ELISA reader (EL 307C). Each plate contained known positive (JEV infected larval or suckling mouse brain homogenate) and negative (homogenate of uninfected adult mosquito pool) controls and a substrate blank. In order to allow for variation in the assay system, OD of the substrate blank was subtracted from others and the results interpreted. A pool was declared positive for virus antigen if its OD was equal to the mean plus ≥4 standard deviations (SD) of OD of uninfected pools (n = 22) from the laboratory mos-quito colony.

Comparison of bioassay and ELISA on field specimens

Culicine mosquitos were captured in dusk collections in and around cattle sheds in villages of South Arcot District. They were held for 48 - 72 hours in the field station at Vriddhachalam, identified, sorted into pools of 50 and transported on liquid nitrogen to Madurai to be stored at -70°C till further studies.

Pools were homogenized in BAPS in plastic centrifuge tubes and divided into two aliquots. One aliquot, after two cycles of freezing and thawing, was centrifuged and the supernatant examined by ELISA. The second aliquot was centrifuged and the supernatant inoculated to 10 *Tx. splendens* larvae per pool. After 7 days incubation headsqueeze smears from 6 larvae were prepared in duplicate and the remaining 4 larvae were pooled and stored at -70°C for further passages if needed. One set of smears was first screened by IIF using RIS and the positives were tested for JEV specificity using MAB 112 on corresponding duplicate smears.

RESULTS

Bioassay

All the control *Tx. splendens* larvae inoculated with BAPS were negative in IIF. Those inoculated with 10^{-2} dilution of JEV (P 20778) were positive for fluorescence from PI day 2. Three types of fluorescence were generally seen when stained with RIS; large neurocytes with bright perinuclear fluorescence, discrete fluorescing masses of varying sizes and diffuse 'smear' like fluorescence. A similar picture was observed when smears were stained with MAB 112 except that the fluorescing structures were less intense and less abundant.

Results of IIF on headsqueeze smears of *Tx. splendens* larvae inoculated with experimental *Cx. tritaeniorhynchus* pools containing infected adults are shown in Table 1. Three sets of inoculated larvae were positive on days 3 and 4 and two more sets were positive on days 5 and 6 PI respectively. On day 7 PI all the 5 sets were positive and more than 50% of ino-culated larvae showed fluorescence. These results indicated that the bioassay system was sensitive enough to detect one JEV infected adult in a pool size of 50 mosquitos.

ELISA

In the first experiment, all the 5 infected pools of *Cx. tritaeniorhynchus* ground in pestle and mortar, were negative by ELISA. In the second experiment 7 out of 9 pools homogenized in glass tissue grinder, were positive by ELISA. In the third experiment 5 out of 10 pools (50%) homogenized in glass tissue grinder and 7 out of 12 pools (58%) homogenized in plastic micro-centrifuge tubes, gave positive reactions and the mean OD of positive pools were 0.084 ± 0.007 SD, and 0.085 ± 0.007 SD respectively. These results indicated that there was loss of virus in homogenates prepared in pestle and mortar, and homogenates prepared in glass or plastic homogenizer were equally suitable for performing ELISA.

Comparison of bioassay with ELISA on field specimens

A total of 16,250 adult female mosquitos in 325 pools (*Cx. tritaeniorhynchus*, 184; *Cx. vishnui*, 106;

Table 1

IIF test on headsqueeze smears of *Tx. splendens* larvae with JEV infected *Cx. tritaeniorhynchus* pools.

PI day	*Cx. tritaeniorhynchus* pool No.					Total
	S1	S2	S3	S4	S5	
3	0/5*	2/5	1/5	0/5	4/5	7/25
4	0/5	5/5	3/5	0/5	2/5	10/25
5	0/5	0/5	1/5	1/5	4/5	7/25
6	1/5	4/5	1/5	2/5	4/5	12/25
7	3/5	3/5	2/5	4/5	5/5	17/25
						53/125

* Number of larvae positive in IIF/Number examined.

and *Cx. fuscocephala*, 35) collected in the months of September and October 1992, in villages of South Arcot District, were tested by ELISA; 253 pools had OD equal to mean + < 2 SD of OD of normal pools (Group 1), 65 had OD equal to mean + 2 - 3 SD (Group 2) and 9 pools had OD equal to mean + ≥ 4 SD (Group 3). All the 74 pools in Groups 2 and 3, and 103 pools from Group 1 were examined by bioassay also. In total, 177 pools were examined of which 27 were positive by bioassay and 9 by ELISA (Table 2). It was further observed that 8 out of 103 pools (7.8%) in Group 1, 12 out of 65 (18.5%) in Group 2 and 7 out of 9 (77.8%) in Group 3 were positive by bioassay. The two bioassay negative pools in Group 3 also became positive after two passages in *Toxorhynchites*, indicating that these pools contained viable virus.

In total the bioassay detected 29 (16%) positive pools as against 9 (5%) detected by ELISA. All the 29 positive pools have undergone at least two passages in *Toxorhynchites* and all of them have been confirmed as JEV by MAB 112 - IIF.

DISCUSSION

Previous studies have shown that adult mosquito inoculation techniques are very sensitive for isolation of flaviviruses from clinical specimens (Kuber-

Table 2

Comparison of ELISA and IIF on field collected mosquito pools.

Group	OD of test pool equal to	ELISA +/-	No. of pools tested	IIF +	IIF -
1	Mean + < 2SD*	-	103	8 (7.8%)	95
2	Mean + 2-3SD	-	65	12 (18.5%)	53
3	Mean + ≥ 4SD	+	9	7 (77.8%)	2
	Total		177	27 (15.3%)	150

* Refers to the mean and standard deviation (SD) of optical density (OD) of normal uninfected mosquito pools (n = 22) which were 0.057 and 0.006 respectively.

ski and Rosen, 1977; Gubler *et al*, 1978; 1979; Thetwin, 1982) and from naturally infected mosquitos (Dhanda *et al*, 1989; Mourya *et al*, 1989). Pang *et al* (1983) and Lam *et al* (1986) demonstrated the advantages of using larval stages of *Tx. splendens* for isolation of dengue viruses. The work presented here also shows that intracerebral inoculation of *Tx. splendens* larvae coupled with IIF is a very sensitive technique for isolation of JEV from mosquitos infected in nature.

Mourya (1990) demonstrated fluorescence 24 hours after intracerebral inoculation of *Tx. splendens* larva with a prototype JEV (P 20778). We found that when high concentrations of the same strain of JEV was used, fluorescence could be visualized as early as day 2 PI (earliest examined). But, when pools of experimentally infected adult *Cx. tritaeniorhynchus* were used for inoculating *Toxorhynchites* larvae, 3 sets of inoculated larvae were positive on PI days 3 and 4 and all the sets on days 6 and 7. On day 7 more than 50% inoculated larvae showed fluorescence. These results suggested that pools differed in their virus content, a situation analogous to that observed in the field, and indicated that minimum holding time for *Tx. splendens* larvae inoculated with field pools to be 7 days. Therefore, subsequently larvae inoculated with field pools were examined on day 7 PI routinely.

Initial attempts to use ELISA on adult mosquito pools were unsuccessful, due to technical problems. Firstly, porcelain is known to adsorb microorganisms, and therefore it was suspected that the virus concentration of pools ground in porcelain pestle and mortar decreased below the threshold level detectable by the ELISA. Therefore, pestle and mortar was replaced by glass or plastic homogenizer. The OD values of pools ground in glass or plastic homogenizers were found to be similar. For convenience plastic homogenizers are preferable to glass for large scale testing of field samples. Secondly, it was suspected that vigorous washing steps might have resulted in the loss of bound molecules from the solid phase (Venkatesan and Wakelin, 1993). Therefore in the modified protocol, gentle hand washing steps were incorporated. Thirdly, in the modified procedure mosquito suspensions were subjected to two cycles of freezing and thawing prior to centrifugation (Peiris *et al*, 1992). Freezing and thawing disrupt cells and release the virus to the medium thereby rising the virus titer. It is possible that more than one technical improvement might have contributed finally to a successful ELISA protocol.

Hildreth and Beaty (1984) and Tsai *et al* (1987) have reported that enzyme immunoassay to be less sensitive than cell culture system during early phase of EEE and SLE virus infections. The present comparative study also showed that bioassay detected more positive pools (29) than ELISA (9). The 29 isolates in bioassay are considered as true positives as they could be serially passaged and all of them confirmed as JEV. The two pools in Group 3 which were positive by ELISA but initially negative by bioassay may be considered as false negatives, because upon serial passages in *Toxorhynchites* they also became positive by IIF. Two explanations are possible for this discrepancy. Firstly, the original pools contained an overwhelming proportion of non-infectious virus particles and only a small viable fraction. Upon passages, the virus titer increased giving positive IIF test. Alternatively, the critical portions of the brain tissue of inoculated larvae were not represented in headsqueeze preparations - a possibility suggested by Kuberski and Rosen (1977). The second explanation appeared to be valid because, subsequently in routine surveillance, 44 ELISA positive pools were identified and all were found to be positive by bioassay also (Unpublished data, CRME).

Out of a total 325 field pools tested by ELISA, 253 gave OD values of less than mean +2SD (Group 1). From this group only 103 pools were tested by bioassay and 8 (7.8%) were positive. However, this represented 28% of the total 29 bioassay positive pools. This could be an underestimate because had we examined the remaining 150 pools in Group 1, an estimated 12 bioassay positives (7.8% of 150) might have been detected. Number of positives from Group 1 would then be 20 out of a total 41 (49%). Whether this percentage holds good for all seasons needs further study.

Based on this study a protocol has now been developed in which field pools are first screened by ELISA and all those with OD = Mean + $\geq$ 2 SD (Groups 2 and 3) are tested by bioassay to detect true positives and the rest (Group 1) are discarded. By excluding Group 1 pools from bioassay a proportion of true positives is likely to be missed. But, the reasoning for following this procedure is as follows: the labor, time and the cost involved for testing of large number of pools in Group 1 which is likely to yield only about 8% positives in bioassay,

are far greater than those required for testing of Groups 2 and 3 which are likely to detect 20 and 78% positives from their respective groups. Further, low OD values of Group 1 implies low virus content in individual positive pools, which most likely is due to low virus titers in individual infected mosquitos in which infection has not disseminated. This group may therefore be epidemiologically less dangerous than Groups 2 and 3 with higher OD values and hence by implication have high virus contents in positive pools. A positive correlation between the quantity of virus in mosquitos and their ability to transmit has been observed in case of Rocio viruses (Mitchell *et al*, 1981). Also, Hardy *et al* (1983) have observed that the mean viral titers in *Cx. tarsalis* females that were competent transmitters of WEE virus were 100-fold higher than in females that were incompetent transmitters. They have quoted similar observations by earlier workers for La Crosse virus in *Aedes* sp, Rift Valley Fever virus in *Cx. pipiens* and Venezuelan encephalitis virus in *Culex (Melanoconion) taeniopus*. Finally, by following the revised strategy, large number of pools can be tested expeditiously. As an illustration, during routine surveillance in the study area, initially some 600 pools were first tested by bioassay and those positives were confirmed by ELISA. This procedure took nearly 1 year. After adapting the revised strategy, in about 6 months, over 4,000 pools were first screened by ELISA and more than 70% of about 300 pools belonging to Groups 2 and 3 were examined by bioassay also. Details of these studies will be published separately.

In conclusion, present study demonstrates that the *Toxorynchites* inoculation technique followed by IIF is very sensitive for detection of JEV infection in wild-caught mosquitos and gives an estimation of true positives. But it is very time consuming, and cumbersome for testing a large number of samples. The ELISA, on the other hand, is a convenient and rapid system, but less sensitive than bioassay. Therefore, utilization of the two techniques in a manner that is feasible for large scale surveillance gives a better estimation of mosquito infection rates. Further empiric observations are, however, needed to determine the threshold mosquito infection rate associated with risk of epidemic transmission.

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