Toxorhynchites-fluorescent antibody system for the detection of bluetongue virus from *Culicoides* midges (Diptera: Ceratopogonidae)[1]

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ABSTRACT

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A new system, the Toxorhynchites-fluorescent antibody (TFA) test in which the larvae of *Toxorhynchites splendens* mosquitoes were used for the detection of bluetongue virus (BTV) from *Culicoides* midges, was developed. Twenty-seven pools of *Culicoides* midges were collected from bluetongue-prone areas of Tamil Nadu by use of the light-trap and suction-trap methods. A suspension of each pool was injected intrathoracically into *T. splendens* IV instar larvae and inoculated onto Vero cell monolayers. An indirect fluorescent antibody technique and an immunoperoxidase test were used to detect BTV antigen in smears of crushed midges, crushed larval head smears after incubation for 7 d at 28° and cell monolayers showing cytopathic effects 48 h post inoculation. The suspensions were also injected intravenously into embryonated chicken eggs, and the characteristic BTV-induced lesion(s), *viz.* cherry-red appearance of embryos, were observed after 48 h. Virus was confirmed by a qualitative neutralization test conducted simultaneously in embryonated chicken eggs.

A total of seven out of 27 samples (26%) were positive for the presence of BTV antigen in all the diagnostic systems used. Since BTV propagates readily in experimentally infected *T. splendens* larvae and the BTV antigen can be detected by the fluorescent antibody technique with a sensitivity comparable to that for virus propagated in tissue culture and embryonated eggs, the TFA system can be adopted as a new method for the isolation of BTV from vectors. The advantages of the TFA system are discussed.

Keywords: Bluetongue virus, *Culicoides*, immunoperoxidase test, indirect fluorescent antibody technique, larvae, mosquito, *Toxorhynchites splendens*

INTRODUCTION

The role of *Culicoides* sp. as the biological vector of bluetongue (BT) has been well documented (Du Toit 1944; Sellers, Pedgley & Tucker 1978; Jennings & Mellor 1988; Boorman & Mellor 1992; Greiner, Mo, Homan, Gonzalez, Oviedo, Thomson & Gibbs 1993). In India, surveys have identified 29 *Culicoides* species (Natarajan, Grag & Mall 1982). Mohanty (1982) showed that the midges can transmit bluetongue virus (BTV) 7–10 d after ingestion of a BT-infected blood meal and remain infected till the end of their lives. The identification of BTV in vectors and the development and standardization of a new diagnostic test, *viz.* the *Toxorhynchites splendens* fluorescent antibody (TFA) system in which mosquito larvae are used for the detection of BTV antigen in vector samples, is presented in this paper.

Toxorhynchites mosquitoes have been shown to be useful for the isolation of field strains of Dengue and

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other arboviruses (Rosen 1981). They have advantages over other mosquitoes, *viz.*:

- large in size and hence easy to handle,
- neither male nor female adults feed on blood in nature and therefore do not become infected under natural conditions, and
- they do not possess biting mouth parts and hence cannot act as virus vectors in the event of an accidental escape from the laboratory.

Toxorhynchites mosquito eggs float singly on the surface of water and they hatch 2 d after deposition. At temperatures ranging from 20–30 °C, the larvae mature in 4–5 weeks after they had gone through four instar stages on days 2, 3, 4 and 21, respectively (Newkirk 1947). The adults live for at least 6 weeks.

Jennings, Boorman & Mellor (1984) demonstrated the use of adult mosquitoes of *Toxorhynchites brevipalpis* in a system for the propagation, isolation and identification of BTV antigen by indirect fluorescent antibody technique (IFAT), 5–7 d post inoculation (p.i.). Although there are no reports on the use of larvae for isolation and detection of BTV, Rajendran, Gajanaana, Samuel & Thenmozhi (1992) used *T. splendens* larvae in the TFA system to detect Japanese encephalitis virus infection in wild-caught mosquitoes in the South Arcot District of Tamil Nadu State.

In the present study, a TFA system was standardized for the detection of BTV and is compared with other systems. Its merits are discussed.

MATERIALS AND METHODS

Virus

A field isolate of BTV (BT/TN/91/2) adapted to Vero cells was supplied by the Vaccine Research Centre of the Tamil Nadu Veterinary and Animal Sciences University (TANUVAS). This virus was used for the production of positive controls in standardizing the tests.

Positive serum

Bluetongue virus type-1 antisera used for the identification of antigen in the test samples was produced in sheep and cattle free from antibodies against other agents. They were supplied by Veterinary Diagnostics Inc., USA.

Conjugates

The species-specific fluorescent isothiocyanate conjugate (Dako, USA) and horseradish peroxidase conjugate (Sigma, USA) were used for the IFAT and immunoperoxidase techniques (IPT).

Embryonated eggs

Embryonated chicken eggs were obtained from the Poultry Research Station, TANUVAS, Madras–18.

Toxorhynchites splendens larvae

Toxorhynchites splendens mosquito larvae were made available by the Mosquito Breeding Wing, Centre for Research in Medical Entomology, Indian Council of Medical Research, Madurai, Tamil Nadu State. Each larva was reared separately (to avoid cannibalism) in small plastic containers containing water. The larvae were fed individually with the freely available *Culex* mosquito larvae. Before inoculation, each larva was stunned by placing it on an ice cube which temporarily immobilized it. After inoculation, the larvae were returned to the individual containers where they regained their wriggling movements.

Sample collection and preparation

Insect traps

Light- and suction -traps recommended by the Centre for Research in Medical Entomology were used for the collection of the *Culicoides* midges. The light-trap used was a model of the New Jersey light-trap (Butts 1937) as modified by Boobar, Sardelis, Nelson & Brown (1987). It is used world-wide for the collection of certain insects and has been devised in such a way that when operated, the light source (a 2,5 V Krypton bulb) in the trap attracts insects in its vicinity towards the trap and the minifan (1,5 V battery operated) sucks and blows them into a special plastic meshed net in which they get trapped. The operation was carried out only at dusk (half an hour to one hour after sunset) by hanging the trap in sheep pens, sheep dwelling areas and water spots. The trapped insects were anaesthetized by placing a piece of cotton wool soaked in ether in the net. They were then, while immobilized, spread on a sheet of white paper and the *Culicoides* sp. were identified and separated from the pool.

The suction trap described by Du Toit (1944) was slightly modified. It consisted of a fibre-glass tube (with a mouth on one side and a tapering end on the other) to which a length of rubber tubing was attached. At the junction a mesh-net filter was placed in order to prevent insects from being sucked into the mouth. With this trap only target species are trapped —unwanted insects are avoided. A torch was used as a light source in the search for the *Culicoides* sp. midges while they were resting on objects in the animal premises or on animals themselves (ears, conjuctiva and base of tail). Once the insect had been identified, the mouth of the tube was placed directly over it and suction was produced by sucking on the rubber tube. The entrapped insect was transferred

to a conical flask from which it was prevented from escaping by covering the mouth with a cloth. The *Culicoides* sp. thus collected were anaesthetized with ether-soaked cotton wool.

Collection regions

The Tamil Nadu State was arbitrarily divided into seven agro-climatic zones (Fig. 1). From September 1994 to January 1995, midges were collected from government sheep farms, private farms and flocks in each zone (Fig. 1), as the recorded incidence of BT is highest during these months every year. A total of 27 different pools of insects were made.

Storage and preparation

The immobilized midges were collected in labelled screw-cap tubes and the preservative as described by Luedke & Jones (1972) was added. The preserved insect samples were transported in ice in a thermo cool box and stored at 4 °C in a refrigerator. Five midges from each of the 27 pools were crushed on glass slides, air dried and fixed in acetone for the indirect fluorescent antibody test (IFAT) and immunoperoxidase test (IPT). For inoculations, a pool consisting of 20 *Culicoides* midges was made from each of the 27 collections, triturated in 0,5 mℓ PBS containing 0,5 % BSA and antibiotics, and clarified by centrifugation (10 000 x *g*) for 30 min at 4 °C. The supernatant was used as the inoculum.

Virus identification

Indirect fluorescent antibody technique

One set of fixed smears was subjected to this technique as described by Jennings & Boorman (1980). After incubation in a humid chamber at 37 °C for 30 min with 10 µℓ of BTV bovine antiserum diluted 1:20 in PBS, smears were rinsed three times in PBS, treated with antibovine fluorescent isothiocyanate conjugate diluted 1:200 in PBS, incubated at 37 °C for 30 min, washed three times in PBS, mounted in buffered glycerol saline (pH 7,0) and examined under a fluorescent microscope (Leitz, Germany) for fluorescence.

Immunoperoxidase test

Another set of the acetone-fixed smears were subjected to IPT as described by Ellis, Luedke, Nunamaker & Haven (1991). Smears were flooded with methanol containing 3 % H_2O_2, incubated for 45 min to remove any endogenous peroxidase activity, washed thoroughly by means of PBS and treated with BTV bovine antiserum diluted 1:20 in PBS for 1 h at 37 °C. After they had been rinsed three times in PBS and dried in air, they were flooded with antibovine HRP conjugate diluted 1:500 in PBS, incubated at 37 °C for 1 h, treated with 0,05 % diamino benzidine in Tris-HCl buffer (pH 7,6) in the presence of 0,1 % H_2O_2 for 10 min at room temperature. After they had been washed with PBS, the smears were counter stained with haematoxylin, washed in tap water, air dried and examined under a light microscope for the presence of intracellular brown deposits.

Toxorhynchites-fluorescent antibody technique

Standardization

A Vero cell-adapted BTV isolate (BT/TN/91/2), used undiluted as well as at dilutions of 10^{-1}, 10^{-2} and 10^{-3}, was inoculated into 40 laboratory-reared, early IV instar larvae of *T. splendens* (ten per dilution). Each larva (Fig. 2) was injected intrathoracically at the junction between its head and thorax while being viewed through a hand-held magnifying lens. Each larva received a dose of 10 µℓ (0,01 mℓ) of the inoculum. The inoculation was done by means of a capillary glass tube devised for this purpose at the Centre for Research in Medical Entomology, Madurai. After inoculation, the larvae were incubated at 28 °C as described by Rajendran *et al.* (1992). On each day from day 2–9 after inoculation, two larvae from each group were beheaded. The heads were squeezed individually and duplicate smears were made from the ensuing material. The smears were fixed in chilled acetone. Some of the larvae which had died naturally were processed in the same way on the day they died. The smears were subjected to IFAT and IPT. In addition, smears from uninoculated control larvae were made and treated in a similar manner.

Testing of samples

The inoculum prepared from each of the 27 pools of *Culicoides* midges was inoculated into eight laboratory-reared young IV instar larvae of *T. splendens.* They were placed in an incubator at 28 °C. On day 7 p.i., the heads of all the larvae were squeezed individually, and duplicate smears were made from the ensuing material. These were fixed in acetone and subjected to IFAT and IPT as described above. Coverslip cultures of Vero cells were infected with 1:10 dilutions of midge suspensions in accordance with established procedures. At 48 h p.i. a set of infected coverslips was fixed in cold acetone for IFAT and IPT. To act as a control, a set of uninfected coverslips was also fixed, stained and similarly examined.

Chicken embryos (11–13 d old) were inoculated intravenously with 10 µℓ (0,01 mℓ) of midge suspension. Three embryos were used per sample. Similarly, each sample of midge suspension was neutralized with a 1:5 dilution of BTV antiserum and inoculated into chicken embryos. The embryonated eggs, together with uninoculated controls, were incubated at 37 °C for 5 d after which they were killed and examined for BTV-induced lesions.

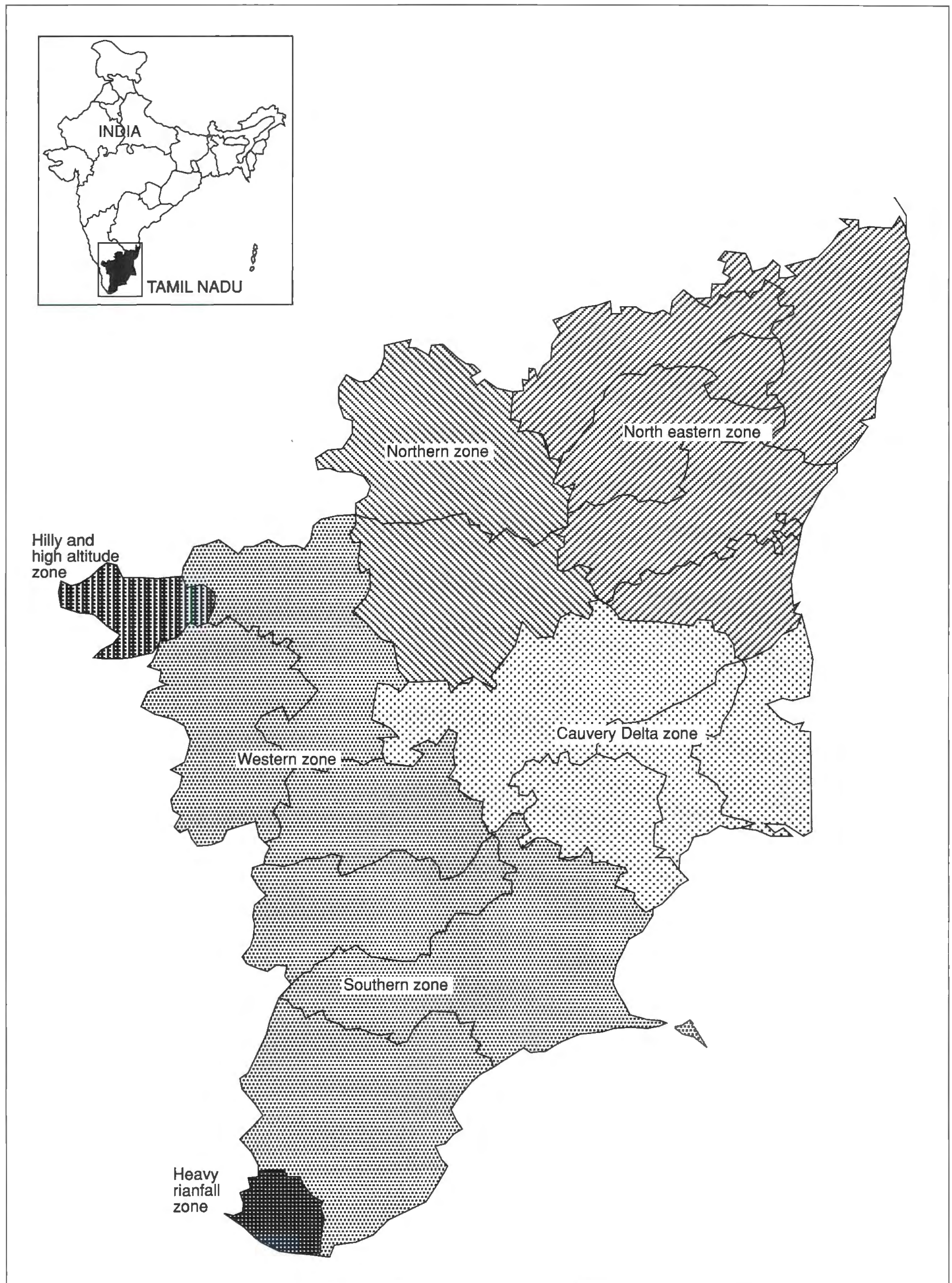

FIG. 1 Agro-climatic regions of Tamil Nadu, India

TABLE 1 The detection of bluetongue virus in experimentally infected *Toxorhynchites splendens* larvae with the use of IFAT

Inoculum	Results on day							
BT/TN/91/2	2	3	4	5	6	7	8	9
Undiluted	–	–	–	–	–	–	–	–
1:10	–	–	–	–	–	+	–	–
1:100	–	–	–	–	++	++++	+	–
1:1000	–	–	–	–	–	++	–	–
Negative control	–	–	–	–	–	–	–	–

– Negative
+ Low intensity of fluorescence
++ Moderate intensity of fluorescence
+++ High intensity of fluorescence
++++ Very high intensity of fluorescence

TABLE 2 Detection of bluetongue virus (BTV) in naturally infected *Culicoides* by five methods

Material tested	Result with				
	Direct		Vero cells (CPE)	Embryonated eggs	TFA
	IFAT	IPT			
Positive control					
BTV-inoculated ECE	–	–	–	(3/3)	–
BTV-infected Vero cells	(3/3)	(3/3)	(3/3)	–	–
BTV-infected larvae	–	–	–	–	(8/8)
Negative control					
Neutralized BTV in ECE	–	–	–	(0/3)	–
BTV-free Vero cells	(0/3)	(0/3)	–	–	–
Control larvae (not infected)	–	–	–	–	(0/6)
Culicoides					
Crushed *Culicoides*	(7/27)	(7/27)	–	–	–
Culicoides suspension injected into					
T. splendens larvae	–	(7/27)	–	–	(7/27
Embryonated eggs	–	–	–	(7/27)	–
Vero cell monolayers	(7/27)	(7/27)	(7/27)	–	–

( ) = Number positive/Number tested
IFAT = Indirect fluorescent antibody technique
ECE = Embryonated chicken eggs
TFA = *Toxorhynchites*-fluorescent antibody system
– = Not tested
IPT = Immunoperoxidase test
CPE = Cytopathic effect

RESULTS

Standardization of TFA

Toxorhynchites splendens larvae injected with Vero cell-adapted BTV became infected with BT virus as evidenced by specific intra-cytoplasmic apple-green fluorescence on day 7 p.i. Smears taken on other days gave inconclusive results as presented in Table 1.

Detection of BTV in naturally infected *Culicoides*

The results of the detection of BTV in naturally infected *Culicoides* by five methods, *viz.* IFAT, IPT, growth in Vero cells, infection of chicken embryos and TFA are presented in Table 2 and are briefly discussed below.

In smears of crushed *Culicoides,* seven of the 27 different pools of samples tested were positive for the

FIG. 2 *Toxorhynchites splendens* larva. Size comparison with *Culex* sp. larva (left)

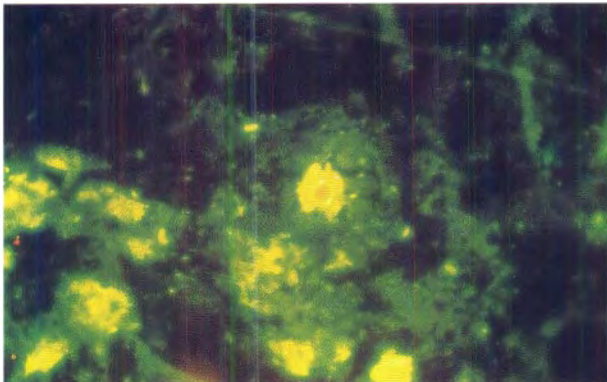

FIG. 3 IFAT on a squeezed smear of a BTV-infected *Toxorhynchites splendens* larva. Intra-cytoplasmic apple-green fluorescence is seen

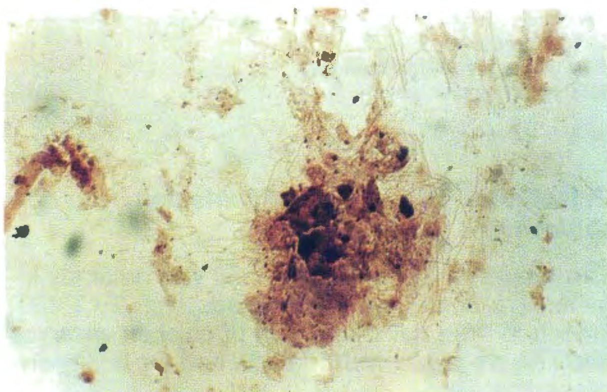

FIG. 4 ITP on a squeezed smear of a BTV-infected *Toxorhynchites splendens* larva. Intra-cytoplasmic brown substrate deposits are seen

presence of BTV antigen by IFAT, showing intra-cytoplasmic apple-green fluorescence, and IPT, showing intra-cytoplasmic brown substrate deposits.

In infected Vero cells, the same seven pools of BTV-positive samples induced cytopathogenic effects in Vero cell monolayers at 48 h p.i. as was evidenced by syncytial formation, vacuolation of the cytoplasm and the presence of intra-cytoplasmic inclusions. The presence of BTV antigen was confirmed by IFAT and IPT.

In inoculated embryonated chicken eggs, all seven positive samples induced a cherry-red appearance of the chicken embryos. Since there was no death of embryos until 5 d after infection, they were killed and examined. Neither the embryos inoculated with samples neutralized by specific antiserum nor the uninoculated controls, showed any lesions.

The same seven samples that were positive for BTV antigen as detailed above, were also positive in the TFA system on day 7 by IFAT (Fig. 3) and IPT (Fig. 4).

DISCUSSION

In the present study, IFAT was performed on smears of whole crushed *Culicoides* midges, and specific fluorescence, confirming the presence of BTV antigen, was observed. Jennings & Boorman (1980) detected fluorescence in head, thorax and abdomen, and Ballinger, Jones & Beaty (1987) detected BTV antigen in midgut cells and secondary target tissues. The technique described here for the detection of BTV directly in crushed smears of the midges is simple, rapid and inexpensive.

This is the first record of IPT detection of BTV antigen in crushed midge smears, although the technique has been employed for the demonstration of BTV antigen by Wechsler, Austin & Wilson (1990), Ellis *et al.* (1991) and Prasad, Minakshi, Kakker & Srivastava (1994), who used other tissues and cell-culture systems. The immunoperoxidase technique is simpler to perform and is less time consuming and more cost effective in antigen detection than IFAT, but may be less sensitive.

In the present study, the propagation of BTV in the larvae of *T. spendens* and its subsequent identification by IFAT and IPT were demonstrated for the first time. These results indicate that this sytem could also be employed for primary isolation and identification of BTV from vectors. Though the sensitivity of the system was found to be comparable to the other methods used in this study, the testing of large numbers of individual samples is required to ascertain the order of sensitivity of various test systems.

Propagation and detection of BTV in Vero cells is expensive, laborious and time consuming. Inoculation

of chicken embryos is a sensitive method for detecting BTV in vector samples, but the intravenous route of inoculation is again time consuming. *Toxorhynchites splendens* larvae were found to support the propagation of BTV originating from vector samples. Should this system be as sensitive as the embryonated chicken-egg system, it could be one of the choices for primary isolation of BTV from vectors if the necessary laboratory facilities are available. Further tests, however, are needed to compare the sensitivity of the different systems.

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