Dengue is a deadly mosquito-borne infection warranting urgent attention for its containment particularly in the tropical and subtropical countries. In the absence of a vaccine or any specific drug for its treatment, an early diagnosis is considered indispensable to prevent any casualty. Detection of viruses in human sera particularly in endemic areas is cumbersome, difficult and also not desirable. Therefore, as an alternative approach, detection of the dengue virus antigen in mosquitoes has provided a reliable tool to (i) comprehend the types of viruses circulating in nature; and (ii) help in designing vector-specific control strategies. A mélange of diagnostic techniques are currently available with some advantages or disadvantages. Traditionally, cell cultures and suckling mice have been employed for virus isolations. While the virus isolation method in baby mice is time consuming, slow and expensive, the mosquito cell cultures offer a good degree of specificity. Mosquito inoculation techniques have been reported for detection and propagation of flaviviruses. Though this technique is sensitive for routine virological confirmation of dengue fever, it requires large number of infected mosquitoes, besides being time consuming. Insect bioassays (Toxo-IFA) are generally cumbersome requiring special facilities and are not suitable for large-scale epidemiological surveillance. ELISA has been shown to be a rapid and sensitive alternative to insect bioassays for monitoring arboviruses in wild populations. Reverse transcriptase polymerase chain reaction (RT-PCR) is a recent molecular diagnostic technology used for detecting virus infections in mosquitoes, which gives rapid results but is expensive and prone to contamination.

This review describes the development of various techniques involved in detection and isolation of dengue viruses in mosquitoes. Definite diagnosis of the impending dengue epidemic can be made using ELISA for virological surveillance system on dengue virus antigen in the mosquito vectors. Therefore, ELISA offers a potential tool and a convenient system for quickly screening large number of samples up to the serotype level which can be employed effectively and efficiently for large scale dengue surveillance programmes on wild caught mosquito vectors. ELISA positive samples can be screened further by Toxo-IFA system for virus isolation. On the other hand, techniques like mosquitoes cell culture, mosquito inoculation (Toxo-IFA) and RT-PCR techniques can be employed for dengue virus amplification.

**Key words** Dengue - diagnostics - vector mosquitoes - virus
Dengue, a mosquito-borne viral infection is regarded as a major public health problem globally. The two main clinical manifestations, namely dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), are responsible for exacting heavy morbidity and mortality every year and continues to be serious public health problem1,2. The severity of this disease can be judged from simple statistics that over 40 per cent (2.5 billion) of the world’s population in 100 tropical and subtropical countries, continue to live under the threat of contracting dengue infection3, while close to 50 million new infections and 24,000 deaths are reported annually worldwide. Besides, every year the disease forces nearly 500,000 people to hospitalization, of which 90 per cent are children. It is therefore, not surprising that disability adjusted life years (DALY) for dengue infection exacts a disease burden more or less equal to that of malaria (465,000)4. Mostly these deaths are due to lack of early diagnosis of dengue virus infection caused by four distinct serotypes, DEN-1, DEN-2, DEN-3 and DEN-4. DHF case fatality rate that can generally exceed 20 per cent in a non-endemic population, may be reduced to less than 1 per cent with the aid of modern supportive therapy based on early diagnosis of the type of viral infection5. Dengue viruses are, however, amongst the most difficult arboviruses to be isolated and propagated in vitro. In most parts of South Asia, including India, both *Aedes aegypti* and *Ae. albopictus* are considered as the two main vectors for dengue transmission. The geographical distribution of the disease is characteristically parallel to that of the principal vector species, *Ae. aegypti*6.

Each year large numbers of dengue cases among children occur in many places coinciding with monsoon season and period of high vector prevalence. Sudden onset of dengue epidemics can be greatly mitigated using rapid and reliable diagnostic methods for the control and management of this disease. A definite diagnosis, which depends on isolation of the virus, can be made only in the laboratory by detecting viral antigen. Until recently detection of the virus relied solely on viral isolations, however, current procedures can detect dengue virus RNA and specific virus antigens in the different tissues. Surveillance of mosquitoes infected with dengue viruses can help monitor the infection rates within vector mosquito population harbouring specific serotype and provides an early warning sign to predict an impending epidemic7. Some workers have recently advocated use of virus infection rates in vector species as well as density of vectors in developing early warning tool8. This fact underscores the importance of vector mosquitoes in being employed to yield quick and reproducible results without any ethical implications. Various types of sensitive, specific and rapid diagnostic tools are available, each having its own advantages and disadvantages, and can be effectively used to find out the infected mosquitoes under a given set of laboratory conditions, albeit extensive cross-reactions among the flaviviruses obfuscating identification of the particular serotype. The best method for diagnosis is considered the one that is rapid, specific and inexpensive. Obviously there is no one ‘best’ method for all arboviruses, therefore, a melange of approaches fulfills the requirement. The present review brings together the available information on diagnostic methods employed for this purpose on vector mosquitoes and the relative significance of each technique to yield dependable results. This will help to get reliable estimate of the dengue infection in the mosquito vectors that can be included in the dengue surveillance programmes in future.

**Virus isolation**

Methods selected for virus isolation depend upon the laboratory facilities available. No single isolation system is adequate for all arboviruses. Identification of the infecting dengue virus serotype depends upon isolation of virus in a sensitive host system followed by serotype identification using reference monoclonal antibodies. A successful isolation of virus followed by serotyping usually takes more than two weeks which procrastinates the inevitable vector control under epidemic situations. Dengue viruses do not grow well in vitro normally. However, at present some more sensitive isolation systems comprising certain insect species such as non blood-sucking *Toxorhynchites* mosquitoes are available wherein the inoculation and isolation of dengue viruses are successfully accomplished.
(i) Suckling mice: All the four dengue viruses have been successfully isolated in BS-C-1 cells (African green monkey kidney cells) or 1-3 days old baby mice using a soup prepared from *Ae. aegypti*\(^9,10\). Baby mice are very insensitive before inculcating an evidence of infection\(^7\). In spite of this, suckling mice are important as it is generally not possible to detect the virus in other animal host body (e.g., mosquitoes, ticks) when in low quantity. Mice are inoculated intracranially with classified suspensions of clinical specimens or macerated arthropod pools or animal tissues. Since the suckling mice are readily available in all laboratories and have certain practical advantages over others, the supernatant of the mosquito soup after centrifugation is inoculated intracerebrally into suckling mice for virus isolation.

Dengue serotypes 1 and 4 were isolated from *Ae. aegypti* in 1961 from Vellore, in Tamil Nadu State, by inoculating infant mice\(^11\). In Singapore during 1960-1961, six strains of DEN-2 serotype were isolated, five from pools of *Ae. aegypti* and one from a pool of *Ae. albopictus*. The dengue virus infection rates based on minimum virus isolation rates per 1000 were 18.6 for *Ae. aegypti*, and 0.8 for *Ae. albopictus*. The majorities of the virus isolates were adapted to infant mice with difficulty and required many serial passages before illness appeared\(^12\). During 1962-1963 nearly 200 isolates of dengue viruses were recovered from human and arthropod materials in Thailand by passage in suckling mice and/or tissue culture\(^13\). All the five pools of *Ae. aegypti* mosquitoes from Rajasthan were positive in mice\(^14\). Using mouse inoculation test, many investigators were unsuccessful in isolating viruses from human cases during epidemics in India\(^15\)-\(^17\). However, using the same system, virus isolations were made from about 24 per cent\(^18\) and 30 per cent of patients\(^19\). Prasad Rao *et al*\(^20\) reported only 3 per cent isolation rate during 1978 outbreak\(^20\). One advantage of using baby mice, is that other arboviruses that cause dengue-like illness may be isolated with this system\(^2\). Virus isolation using suckling mice is time consuming, slow and expensive. Moreover, because of low sensitivity of this method many viruses cannot be isolated with baby mice. Those that are isolated frequently required numerous passages to adapt the virus growth in mice. This method is no longer recommended for isolation of dengue viruses\(^21\).

(ii) Mosquito cell cultures: The choice depends on the availability of a host-cell cultures or mice that serve an indicator of virus infection, i.e., cytopathic effects in cell cultures, sign of illness or death in mice. This is the most common method for virus isolation. Grace\(^22\) reported the establishment of the first mosquito cell line in the world from *Ae. aegypti* mosquitoes. Singh\(^23\) established two cell lines at the National Institute of Virology (NIV) Pune, from the larval tissues of *Ae. aegypti* and *Ae. albopictus*. The first *Culex* cell line in the country was established from the embryonic tissue of *Cx. bitaeniorhynchus* mosquitoes\(^24\). Cell lines from other two species of Culicine mosquitoes viz., *Cx. infusa*, *Cx. ambigouir*, which are members of *Cx. bitaeniorhynchus* complex were also established subsequently\(^25\). These cell lines also supported the multiplication of other arboviruses of public health importance in India viz., chikungunya, dengue and sindbis viruses\(^26\). A new cell line from the forest mosquito *Ae. krombeinii* was established in the early 1990s, which proved highly sensitive to several arboviruses\(^27\). It was extremely sensitive to Japanese encephalitis (JE) and dengue viruses and very low titre of these viruses could be detected using indirect immunofluorescent technique (IFA) while *Cx. bitaeniorhynchus* and C6/36 cell lines could not detect these viruses when infected with same dilution\(^28\). Recently, a new cell line from the embryonic tissue of *Cx. tritaeniorhynchus* mosquitoes was established. IFA technique is now routinely being used in the detection of virus antigen in infected cell lines and mosquitoes\(^29,30\). Mosquito cell cultures particularly C6/36 (*Ae. albopictus*), AP-61 (*Ae. pseudoscutellaris*), *Ae. krombeini*, TR-248 (*Toxorhynchites amboinensis*), and other established mammalian cell culture lines (LLC-MK2 cells), are commonly methods for virus isolation\(^27,31-33\). These are not only the most widely used but also provide a relatively simple and economical method for dengue virus isolation and assay\(^34\). The development of direct plaque assay using LLC-MK2 cells provided faster and more sensitive method, but still had the disadvantage of requiring adoption of the virus to the cell culture\(^35\). Several continuous mosquito cell lines have been shown to be highly susceptible to dengue virus infection. The C6/36 clone of *Ae. albopictus* cells was chosen for virus isolation because it demonstrated high sensitivity to dengue
virus infection and ease of handling\textsuperscript{31,36}. These cell lines are highly stable and have optimal growth at lower temperatures than mammalian cells. The virus titre can be determined in all groups of mosquitoes using BHK-21 cells\textsuperscript{38}. In Peninsular Malaysia, virus isolation was carried out using cell culture (C6/36 clone) of \textit{Ae. albopictus} and detection of dengue virus by peroxidase staining. All positive isolations were further confirmed by the reverse transcriptase-polymerase chain reaction (RT-PCR)\textsuperscript{39}.

For dengue virus isolation mosquito cell cultures have proved to be more sensitive than mice or mammalian cell culture systems. Some prefer to use cytopathic effect to detect infection especially with AP-61 cells. This has the disadvantage of not producing cytopathic effects (rounding, refraction of light, detachment from the substrate) and requires secondary step for recognizing presence of virus in the culture. Intra-thoracic inoculation of \textit{Toxorhynchites} mosquitoes (which do not take blood meals) or \textit{Aedes} mosquitoes has also been used. In routine diagnosis, the C6/36 cell lines have become most widely used. Following a period of 7-10 days post-inoculation in cell lines or 14 days post-inoculation in mosquitoes, virus identification is done by immunofluorescence assay with serotype-specific monoclonal antibodies\textsuperscript{35,40,41}. Viral isolation rates were obtained up to 36 per cent with C6/36 cell lines or up to 80 per cent by direct mosquito inoculation\textsuperscript{41,42}. A rapid centrifugation assay for dengue virus improved the isolation rate, even in tissue samples\textsuperscript{43}. IFA has become an important tool in the detection of virus antigen with its simplicity and specificity\textsuperscript{44}. Several virus isolations were made in mosquito cell lines from field collected mosquitoes at NIV, Pune\textsuperscript{45-48}. The \textit{Cx. bitaeniorhynchus} cell line for JE virus and \textit{Ae. albopictus} cell line or C6/36 cells\textsuperscript{31} for DEN viruses are mainly being used in the isolation of these viruses from mosquitoes collected during epidemic periods. Ravi \textit{et al.}\textsuperscript{49} reported isolation rates of 3.7 per cent using \textit{Ae. albopictus}\textsuperscript{49} and 7.5 per cent using \textit{Ae. pseudocutellaris} cell cultures\textsuperscript{50}.

A new cell line from \textit{Cx. tritaeniorhynchus} supported the multiplication of JE and West Nile viruses but not of any of the dengue serotypes\textsuperscript{51}. Due to the presence of certain cytoplasmic inclusions in the cells\textsuperscript{52}, this cell line was not used for further research especially for isolation of virus from field-collected mosquitoes\textsuperscript{53}. However, this system was less sensitive than mosquito inoculation\textsuperscript{44}. The sensitivity of the mosquito cell lines may also vary with the strain of the virus. In samples from an epidemic in Mozambique, more than twice as many DEN-3 viruses were isolated by mosquito inoculation than by the use of mosquito cells\textsuperscript{55}. None of the standard laboratory animals nor mammalian cell cultures are sufficiently susceptible to dengue virus infection to use for routine dengue virus isolation\textsuperscript{56}. Although the use of this method continues in some laboratories, it is not recommended\textsuperscript{41,54}.

(iii) Mosquito inoculation: Mosquito inoculation techniques are reported for detection and viral amplification\textsuperscript{45,46,57-62}. This technique provided for the first time a sensitive method for isolation and assay of dengue viruses. \textit{Tx. splendens}, a non haematophagous mosquito, was evaluated as a bioassay host for the detection and propagation of dengue viruses. All dengue virus serotypes and strains attained titres in \textit{Tx. splendens} comparable to those observed for 2 strains of \textit{Ae. aegypti}. Peak virus titres occurred in \textit{Tx. splendens} approximately 6 days post-inoculation; however, specific fluorescence for all viruses was not observed in 100 per cent of mosquito heads until 12 days post-inoculation. A 100 per cent correlation was noted between specific fluorescence in \textit{Tx. splendens} heads and the recovery of virus from corresponding thorax, abdomens. The volume of inoculum tolerated by \textit{Tx. spendens} was approximately 5 times greater than that injected into \textit{Ae. aegypti}. The overall survival rate for \textit{Tx. splendens} following intra-thoracic inoculation with dengue viruses was higher compared to \textit{Ae. aegypti}. These findings imply that \textit{Tx. splendens} would be more efficient than \textit{Ae. aegypti} as a laboratory assay host for detecting dengue viruses in blood of infected patients and for use in experimental investigations\textsuperscript{63}. The mosquito inoculation technique was used for all titrations\textsuperscript{64}. After incubation for 10 days at 32\degree C individual mosquitoes were examined for the presence or absence of viral antigen in the salivary glands and brain tissue by the direct immunofluorescence antibody technique (IFA).
Mourya demonstrated fluorescence 24 h after intracerebral inoculation of *Tx. splendens* larvae. This is a simple technique to determine dengue virus infection in the mosquito. Variations of the mosquito inoculation technique include inoculation of adult and larval *Toxorhynchites* mosquitoes. Isolation of viruses by intracerebral inoculation of the fourth instars of *Tx. splendens* larvae is routinely followed for dengue diagnosis. The viruses are detected by indirect immunofluorescence (IIFA) using a type-specific dengue monoclonal antibody. Mosquito inoculation techniques have been shown to be sensitive for isolation of flaviviruses. Inoculation of *Tx. splendens* larvae is relatively simple and safe and has been employed for isolation of dengue virus and JE virus from field-caught mosquitoes.

Both the inoculation techniques have similar sensitivity to intrathoracic inoculation of adult mosquitoes, but are more difficult and labour-intensive. The main advantage is that viruses can be isolated in a few days. Inoculation of samples directly into mosquitoes specially adult or larval inoculation in *Tx. amboinensis* and *Tx. splendens* is the best isolation system in terms of sensitivity. NIV, Pune and Centre for Research in Medical Entomology (CRME), Madurai have developed necessary expertise in this discipline. Detection of virus antigen is a promising tool for surveillance. Distinct advantage is that it can be performed under field conditions. With the availability of a fluorescence microscope objective attachment to a standard laboratory microscope for field use, it should now be possible to perform the virus antigen detection test even in peripheral laboratories. Insect bioassays are cumbersome and special facilities are required. It has the disadvantage of being labour-intensive and requires rearing and maintenance of mosquitoes, the expertise for which is not available in most virology laboratories. The mosquito inoculation technique has the disadvantages of being labour-intensive and requiring an insectary to produce large numbers of mosquitoes for inoculation. Also, unless strict safety precautions are maintained, the chance of laboratory infections increase, although this risk can be eliminated by using male *Aedes* mosquitoes or non-biting *Toxorhynchites* species for inoculation.

However, the possibility of laboratory acquired infections through the bite of mosquitoes should be borne in mind and, therefore, rigid safety precautions must be followed in utilizing this technique. The use of non-biting mosquitoes can eliminate the risk of laboratory infection through infective bite, but the slow breeding and protracted life history of this mosquito make it rather difficult to get them in sufficiently large numbers for experimental purposes. Unfortunately, this method is not available in most endemic countries.

**Direct visualization (detection) of viral antigens in field-collected mosquito vectors of dengue**

(i) Detection of antigen by enzyme linked immunosorbant assay (ELISA): ELISA is the most widely used in routine practice. Dengue viruses are difficult to isolate and propagate, as they do not grow well in laboratory animals or cultures of mammalian cells. Although there are very sensitive methods to isolate dengue viruses in mosquito cell lines and mosquitoes, they are time consuming, labour-intensive and expensive. Isolation and recovery of arboviruses from field-collected mosquitoes can be most efficiently accomplished by pooling adult specimens prior to testing in various *in vivo* and *in vitro* assay systems. Such pooling of larvae has also become a routine practice for evaluating the role of transovarial transmission in the ecology of arboviruses. It is sometimes important to detect virus in individual mosquitoes, although this is more labour-intensive than detection in pooled specimens. Use of individual specimens greatly reduces the chance of mistakes caused by introducing a misidentified specimen into a large pool. More importantly, evaluation of individual mosquitoes can generate a precise estimate of vector infection rate. This parameter, combined with survival rate and human biting rate, can be used to make a meaningful estimation of local transmission risk.

Direct detection of virus can provide a rapid diagnosis. In the epidemic situations, the early detection will help to devise appropriate public health measures. Therefore the more rapid, sensitive and specific diagnostic methods would be better in situations for which time is a critical element. In
order to develop dengue-specific ELISA, dengue serotype-specific monoclonal antibody (MABs) that captures only dengue DEN1-DEN4 was used. By this method, studies undertaken in the rural dengue surveillance in Vellore district and dengue epidemic in Chennai, south India, were investigated. In Vellore, pools of mosquitoes of adult *Ae. aegypti* and *Ae. albopictus* were tested by ELISA and found positive for flavivirus antigen. By employing this ELISA and/or Toxo-IFA system, the natural vertical transmission of dengue viruses in *Ae. aegypti* in Vellore district was also investigated and DEN-2 virus was identified in Vellore. ELISA can be used as an inexpensive way to screen large numbers of mosquito specimens with relatively little effort. Detection of virus infection in wild-caught *Aedes* species should form an essential component of a surveillance system for dengue. ELISA technique is shown to be a sensitive alternative to insect bioassay for monitoring arboviruses in wild mosquito population. This technique will be useful in dengue virus surveillance for monitoring the dengue virus activity in endemic areas and also to develop an early warning to plan control strategies. Thus ELISA-based methods using specific MABs can also lead to definite diagnosis. The antigen capture ELISA for virus detection is the most useful procedure currently available and it is widely recommended for virological surveillance. During routine surveillance in our study, initially some 600 pools were tested by Toxo-IFA and those found positives were confirmed by ELISA. This procedure took nearly 1 yr. After adapting the revised strategy, in about 6 months, over 4,000 pools were first screened by ELISA and more than 70% cent of about 300 pools were examined by Toxo-IFA also.

(ii) Detection of antigens by immunofluorescence: Identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay was developed. The method of choice for dengue virus identification is IFA with serotype-specific monoclonal antibodies produced in tissue culture or mouse ascitic fluids and an anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate. This test can be easily performed with infected cell cultures, mosquito brain or tissue squashes, mouse brain squashes, or even on formalin-fixed tissues embedded in paraffin and sectioned for histopathological testing. It is a simple and reliable method which allows the detection of multiple viruses in patients with concurrent infections with more than one serotype.

In those laboratories that do not have immunofluorescence capability, dengue viruses can also be identified using monoclonal antibodies in an antigen capture ELISA. These methods usually take two or more weeks delaying after the laboratory diagnosis for initiating the control operation.

(iii) Immune electron microscopy: Its advantages include rapidity and lack of requirement for specific reagents. High viral density in liquids is required. Only small tissue areas can be examined by this sectioning, however, techniques have been described for enhancing chances for virus detection. The most difficult task in diagnosing viruses by electron microscopy is to determine whether unusual structure are indeed viruses rather than spherical structures and membrane debris in negative stains and normal cellular organelles in thin sections. Electron microscopy can be an important adjunct to other methods for virus identification, but possibly cannot be relied on its own. Recently structural identity of Chandipura virus was confirmed by electron microscopy from a large encephalitis outbreak in children in many districts of Andhra Pradesh.

Modern diagnostic technologies

Polymerase chain reaction (PCR) has been applied to dengue diagnosis with sera, tissue from fatal cases, mosquito pool, infected cell cultures, and mosquito larvae, mosquito surveillance and genetic strain characterization. Several PCR protocols for dengue detection have been described that vary in the RNA extraction methods, genomic location of primers, specificity, sensitivity and the methods to detect PCR products and to determine the serotype. Reverse transcriptase (RT)-PCR has provided one of the most important steps in the molecular diagnosis of dengue virus. A rapid assay has been developed followed by a second PCR with specific primers allowing serotype identification (DNA products) of different sizes according to the dengue serotype obtained.
(i) Polymerase chain reaction (PCR): In recent years RT-PCR has been developed for a number of RNA viruses, including dengue viruses\textsuperscript{109}. Unlike most other techniques which require screening of pools of mosquitoes to detect viruses, RT-PCR carries out the job with solitary specimen. The technique allows for the multifold biological amplification of viral nucleic acid and has been used to rapidly diagnose viral diseases\textsuperscript{34,110-112}. The primary advantage of this molecular tool lies in the speed at which specimens can be screened for the presence of dengue viruses and also by its highly sensitive and specific detection. It is able to monitor the infection rate in mosquitoes, both adults and larvae, with a high degree of precision\textsuperscript{1,113}. RT-PCR can also detect small quantities of virus\textsuperscript{3}. This method has also been employed for detecting and typing dengue virus RNA in the field caught \textit{Aedes} mosquitoes, besides determining the infection rate in local \textit{Aedes} mosquitoes\textsuperscript{114}. Chung \textit{et al}\textsuperscript{114} have developed a single RT-PCR followed by a semi-nested PCR using an upstream consensus primer and four type-specific primers within the non-structural protein gene (NS3) of dengue viruses to type dengue viruses in field populations of female \textit{Aedes} mosquitoes. Alternatively, RT-PCR offers the potential for the rapid, highly sensitive and specific detection of dengue viruses up to the serotype level.

In the dengue-sensitive areas in Singapore, a rapid and sensitive, semi-nested, RT-PCR assay using non-structural protein 3 gene primers for the type-specific detection of dengue viruses in artificially infected and in field-collected adult \textit{Aedes} mosquitoes were employed\textsuperscript{115}. In laboratory experiments, the assay was sensitive enough to detect one virus infected mosquito head in pools of up to 59 uninfected heads. Use of RT-PCR in epidemiological investigations has been intensified recently as geographic locations of the virus-infected \textit{Aedes} mosquitoes, detected as early as six weeks before the start of the dengue outbreaks, were traced to have a correlation with the residence or workplaces of patients. Virologic surveillance using RT-PCR for detecting dengue virus-infected \textit{Aedes} mosquitoes in the field may serve as an additional early warning monitoring system for predicting dengue outbreaks\textsuperscript{115}.

Since a change in serotype is particularly important to bring about a surge in DF/DHF cases, RT-PCR is further advantageous in detecting the specific serotype circulating in natural populations of mosquitoes\textsuperscript{116}. The PCR can be designed to be broadly cross-reacting or extremely specific. The extraction techniques used to isolate RNA destroy the infectivity of the virus, therefore, the risk of exposure to bio-hazardous materials is eliminated once the RNA is extracted from the sample. Despite its advantages, the PCR is not routinely employed in arbovirus diagnostic laboratories because it is expensive. Improvements in automated handling of PCR as well as detection of product are currently being developed but are not available to most laboratories performing arbovirus diagnosis. PCR is notoriously prone to contamination. Every batch of assays of mosquitoes should accompany a no-DNA (negative control) sample, and stringent precautions must be taken to avoid carryover contamination\textsuperscript{117}. PCR is used for quick detection and it requires a small quantity of the sample.

Although RT-PCR has similar sensitivity to virus isolation systems that use C6/36 cell cultures, poor handling, storage and time of sample collection and quantum of virus present in the samples greatly affect the results. The presence of antibody usually do not influence the outcome of PCR as they do in virus isolation. A number of methods involving primers from different locations in the genome and different approaches to detect the RT-PCR products have been developed over the past several years\textsuperscript{118,35,41}. Since RT-PCR is highly sensitive to amplicon contamination, without proper controls false-positive results may occur.

(ii) Hybridization probes for detection of viral nucleic acid: Recent advances in the molecular technology such as RT-PCR and hybridization, nucleic acid probes are made possible for the detection of viral genomic materials in mosquitoes. Hybridization techniques are used for the detection of many important arboviruses. Hybridization probe method detects viral nucleic acids with cloned hybridization probes\textsuperscript{119}. Probes with variable specificity ranging from dengue complex to serotype-specific can be constructed depending on the
genome sequences used. A PCR-amplified serotype-specific complimentary DNA (cDNA) was cloned and used as a non-radioactive nucleic acid hybridization probe in subsequent assays. Hybridization techniques are rapid and specific and relatively simple especially good for diagnosis of viruses that are difficult to cultivate. Using degenerate primers, RT-PCR forms a sensitive and specific method for the detection of dengue viruses in clinical specimens. The use of radiolabelled serotype probes allows for an additional level of specificity (96-100% specific), unavailable in assays using RT/PCR alone (89-100%). Despite several advantages, hybridization techniques have certain drawbacks like lack of sensitivity. Moreover, a labelled probe specific for each virus to be detected is required.

Preliminary data suggest that this method is less sensitive than RT-PCR, but like PCR the outcome of the test is not influenced by the presence of inhibitory substances. Difficulties encountered during working with RNA and the technical expertise required to obtain reproducible results make this method more suitable as a research tool than as a routine diagnostic test. Moreover, in this technique as a pre-requisite, viraemia levels should also correlate with mosquito infection rates.

Discussion and conclusion

Reliable estimation of natural mosquito infection with arboviruses forms a key element in any surveillance system and is essential for vector incrimination and monitoring control measures. A laboratory diagnosis of dengue infection can be accomplished by detecting either the virus or anti-dengue antibodies. Methods selected for virus isolation depend much upon the laboratory facilities available. In the case of virus isolation the baby mice virus isolation method is very time-consuming, slow and expensive. Moreover, because of the low sensitivity of the method, many wild-type viruses cannot be isolated. Those that are isolated frequently require numerous passages to adapt the viruses to grow in mice. This method is no longer recommended for isolation of dengue viruses.

Mosquito cell cultures are a recent addition to dengue virus isolation methodologies. Three cell lines of comparable sensitivity are most frequently used. The C6/36 clone of Aedes albopictus cells is less sensitive than the mosquito inoculation method. Use of these cell lines has provided a rapid, sensitive and economical method for dengue virus isolation. The sensitivity of mosquito cell lines may vary with the strain of the virus. Even though cell cultures are less sensitive than mosquito inoculation, large number of samples can be processed in a relatively short time. Mammalian cell cultures have many of the same disadvantages as baby mice for isolation of dengue viruses, such as being expensive, slow, and intensive.

Mosquito inoculation is a method sensitive enough for routine successful virologic confirmation of dengue. Moreover, there are many endemic dengue virus strains that can be recovered only by this method. Four mosquito species have been used for virus isolation, viz., Aedes aegypti, Aedes albopictus, Tx. amboinensis, and Tx. splendens. Male and female mosquitoes are equally susceptible. A recent variation on this method involves intracerebral inoculation of larval and intrathoracic inoculation of adult Toxorhynchitis mosquitoes. However, intracerebral inoculation not provided any advantage over intrathoracic inoculation method since being rapid. The mosquito inoculation technique has the disadvantages of being labour-intensive and requires an insectary to produce large numbers of mosquitoes for inoculation. The possibility of laboratory acquired infections through the bite is possible and therefore safety precautions must be followed in utilizing this technique. Unless rigid safety precautions are maintained, the chance of laboratory infections increases, although this risk can be eliminated by using male Aedes mosquitoes or non-biting Toxorhynchites species for inoculation. Conventional methods, i.e., culture of virus in suckling mice and the use of microinoculation of Toxorhynchites adults or larvae and subsequent detection with fluorescent antibody staining, for detecting viruses in the vector are laborious and time-consuming. Virus detection in the mosquito, regardless of the species, is generally performed by the IIFA test on mosquito tissues, usually brain or salivary glands. Another recent advance is the development of specific monoclonal antibodies for identification of dengue viruses. For large-scale
surveillance on wild-caught mosquito vectors of dengue to estimate true positives. ELISA technique can be used as a convenient and rapid system for screening large number of samples\(^{125}\). For the diagnosis up to serotype level, if the quantity of the sample is sufficient, serotype-specific antibodies can be used for ELISA.

In comparison to foregoing description of various techniques employed in detection/isolation of dengue viruses, the RT-PCR method is rapid, highly sensitive, simple, reliable and reproducible for the specific detection of dengue viruses. It can be effectively used to detect viral RNA in mosquitoes\(^1\). The improvements in RT-PCR technology are being continuously improvised which make it even more useful in its application for dengue virus detection. When the sample quantity is very less or insufficient all the positives can be propagated by mosquito inoculation technique/tissue culture/RT-PCR for further virus isolation studies.

Dengue virus do not grow well in any of the laboratory animals or mammalian cell cultures normally used in virology laboratories. Dengue viruses were first isolated by inoculation of baby mice. This system is very insensitive and several blind passages are usually required to allow adaptation of the virus. The development of mosquito inoculation technique provided, for the first time a sensitive and relatively rapid method for isolation and assay of dengue viruses. The IFA test provided a simple technique to determine dengue virus infection in the mosquitoes, but it requires expertise to maintain a mosquito colony, which is not available in many laboratories. This can be done using mosquito cell lines that are less labour-intensive. Even though mosquito cell lines are highly susceptible to dengue virus infection, the technique is less sensitive than the mosquito inoculation technique for routine isolation of dengue viruses. *Toxorhynchites* inoculation technique followed by IFA is very sensitive for the detection of infection in wild-caught vector mosquitoes and gives an estimate of true positives. But it is time causing and cumbersome for testing large number of samples. ELISA test on the other hand is a convenient and rapid system and can be used for large scale screening of vectors. Further ELISA positives should be again screened by Toxo-IFA system. By this new strategy, a large number of pools can be screened within a short time. The cost and labour involved are greatly reduced. Thus utilization of these two techniques will give a better estimate of mosquito infection rates and help to forecast the associated risk of dengue epidemic. Other methods can be employed for propagation of these viruses and can be used for virus isolation later. According to the laboratory facility available, certain technical improvements can be made on the diagnostic procedures which can also be standardized and selected for the routine surveillance use. This system allows the dengue viruses being transmitted in an area to be monitored with a minimal amount of effort and provides the early warning capability necessary to predict epidemic dengue.

**References**


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