

DETECTION AND SEROTYPING OF DENGUE VIRUSES BY PCR : A SIMPLE, RAPID METHOD FOR THE ISOLATION OF VIRAL RNA FROM INFECTED MOSQUITO LARVAE

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Abstract. Dengue viruses pose a considerable global public health problem with an estimated 100 million cases of illness every year. This illustrates the need for rapid and reliable diagnostic methods for proper patient management and disease control. Currently, laboratory diagnosis depends on serology or virus isolation, with both methods having certain drawbacks. Alternatively, reverse transcription and polymerase chain reaction (RT-PCR) offers the potential for the rapid, highly sensitive and specific detection of dengue viruses. Since we occasionally encounter the problem of insufficient amounts of patient serum for the direct detection of dengue viruses, a method was developed for the extraction of viral RNA after biological amplification in mosquito larvae. Using this method, 15 of 19 clinical samples tested were correctly identified using RT-PCR.

INTRODUCTION

Dengue viruses are the aetiologic agents of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). They are members of the family Flaviviridae and can be classified into four serotypes (Den-1, -2, -3 and -4) on the basis of complement fixation (Kuberski and Rosen, 1977a) and plaque neutralization (Russell and Nisalak, 1967) tests.

Dengue viruses pose a considerable global public health problem with an estimated 100 million cases of illness every year (Halstead, 1988). Without the availability of a vaccine, eradication of the principal urban vector *Aedes aegypti* is important in controlling the spread of the disease. However, despite some success in the Americas in the 1950s and 1960s, *Aedes aegypti* has since been reintroduced into some regions that had been free of this mosquito. This fact underscores the importance of a fast and simple method for the laboratory diagnosis of dengue virus infections. Currently, laboratory diagnosis depends on the isolation of viruses or the detection of virus specific antibodies. Virus isolation from clinical samples can be achieved

using cultured mosquito cells (Tesh, 1979) or by inoculation of adult mosquitos (Kuberski and Rosen, 1977b) or mosquito larvae (Lam *et al*, 1986). These methods are sensitive but time consuming with incubation periods from 5 to 14 days. The serological diagnosis has improved considerably with the development of the dengue IgM ELISA (Lam *et al*, 1987), however, the detection of cross-reactive antibodies has been reported (Innis *et al*, 1989).

The polymerase chain reaction (PCR) offers the potential for rapid, highly sensitive and specific detection of viruses. This technique allows the *in vitro* enzymatic amplification of minute quantities of genetic material (Saiki *et al*, 1988) from different sources. Diagnosis of dengue virus infections by PCR has been reported using patients' serum (Deubel *et al*, 1990; Lanciotti *et al*, 1992) or after biological amplification of the virus in mosquito cell cultures (Morita *et al*, 1991). These approaches require up to 200 µl of clinical sample. However, we often encounter situations where after routine serological tests the amount of patient serum available is insufficient for infection of cell cultures or to attempt direct isolation of template. We therefore developed a method for the extraction of viral RNA from larvae which had been inoculated with patient's serum. This method is rapid and involves minimal sample handling, thereby also

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reducing the possibility of contamination. The salient feature of this method is the trapping of the template using silica particles (Boom *et al*, 1990).

MATERIALS AND METHODS

Inoculation of larvae

The fourth instar of *Toxorhynchites splendens* larvae was used for intracerebral inoculation (Lam *et al*, 1986). Prior to inoculation, the larvae were immobilized by immersion into ice water for 10-20 seconds. An aliquot of approximately 1 µl of patient's serum (diluted 1 : 2 with phosphate-buffered saline containing 5% fetal calf serum and 0.5% gelatin) was introduced into the dorsal epitome of the head capsule. Generally, eight larvae were inoculated per sample. The larvae were incubated at 30°C in containers of tap water and were fed with a constant supply of prey larvae (*eg Aedes aegypti*) to minimize cannibalism.

Extraction of viral RNA

The contents of at least four larvae heads per sample were extruded by squeezing them against the wall of an Eppendorf tube. The tissue was suspended in 100 µl of lysis buffer (8 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 6.4, 36 mM EDTA and 0.2% Triton-X100) and 30 µl of a suspension of silica particles (Boom *et al*, 1990). The mixture was vortexed and left at room temperature for 10 minutes to allow binding of the nucleic acids. The particles were sedimented by centrifugation at 13,000 rpm for 30 seconds and the pellet washed once with 100 µl of washing buffer (10 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 6.4), twice with 500 µl of 70% ethanol and once with 500 µl acetone. After the final washing step, the pellet was dried at 56°C for 10 minutes. The nucleic acid was eluted for 10 minutes at 56°C with 50 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The particles were sedimented by centrifugation at 13,000 rpm for 2 minutes and the supernatant recovered. An aliquot of 10 µl was used for reverse transcription.

Reverse transcription and PCR

The RNA was first heat denatured at 95°C for 5 minutes. Complementary DNA was synthesized in a final reaction volume of 20 µl PCR buffer

(50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.0 mM MgCl₂) containing 0.5 mM each of the four deoxyribonucleoside triphosphates, 100 ng of random primers, 15 units of RNAsin (Promega) and 5 units of AMVRT (Stratagene). The mixture was incubated at 42°C for 60 minutes after which the reaction volume was adjusted to 50 µl with PCR buffer.

Amplification was carried out in separate tubes with 50 pmoles each of Den-2 or Den-3 serotype specific primers, respectively (Deubel *et al*, 1990). The mixture was heat denatured for 10 minutes, quick chilled on ice and 2 units of Taq polymerase (Stratagene) added. The reaction was initially maintained at 72°C for 90 seconds, followed by 35 cycles of denaturation at 94°C, annealing at 55°C and extension at 72°C for 60 seconds each. After the last cycle, the samples were kept at 72°C for 10 minutes. A tube containing all the reaction components except the template (blank) was always included.

Aliquots (5 µl) of the PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

RESULTS

To determine the earliest time point when dengue viruses could be detected, larvae were infected with a virus positive patient's serum (dengue-3, strain 03472) and the RNA extracted from a pool of four larvae from day 1 to day 5 post-infection. Fig 1 shows that the RT-PCR product of 257 bp could be detected by agarose gel electrophoresis as early as day 2 post-infection.

To determine the sensitivity of the extraction method known numbers of dengue virus (New Guinea C) plaque forming units were added to uninfected larvae heads prior to RNA extraction. Fig 2 shows that as few as 4 PFU could be detected by agarose gel electrophoresis. A total of 19 clinical samples [17 sera and two cerebrospinal fluid (CSF) samples] from which viruses had been isolated in C6/36 cell culture earlier were inoculated into larvae. On day 3 post-infection RNA was extracted for RT-PCR from a pool of four larvae per sample. PCR products were analyzed by agarose gel electrophoresis and bands of the expected sizes (266 bp for Den-2 and 257 bp for Den-3) were detected after staining with ethidium bromide.

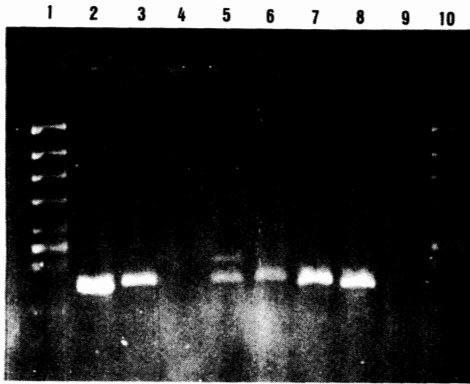


Fig 1—Analysis of RT-PCR products after template isolation from infected mosquito larvae. Composite agarose (1%) and NuSieve® agarose (3%) gel. Lanes 1 and 10: ϕ X174 *Hinc* II digested DNA; lane 2: dengue-3 (strain 03472) isolated from cell culture; lane 3: dengue-3 (strain 03472) isolated from serum; lanes 4, 5, 6, 7 and 8: template (dengue-3, strain 03472) extracted from mosquito larvae on Day 1 to Day 5 post-infection; lane 9: uninfected larvae. The size of the PCR product is 257 bp.

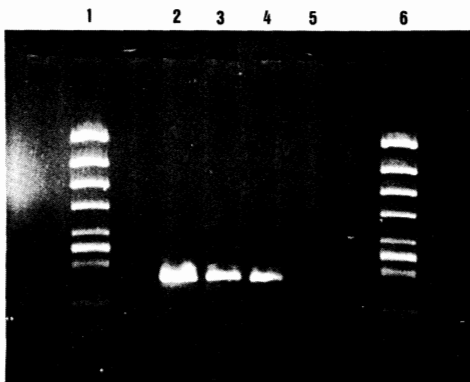


Fig 2—Sensitivity of the RT-PCR in the detection of dengue-2 (New Guinea C) viruses in the presence of larval head tissue. Composite agarose (1%) and NuSieve® agarose (3%) gel. Lanes 1 and 6: *Hinc* II digested ϕ X174 DNA; lanes 2, 3 and 4: 400, 40, 4 PFU added; lane 5: blank. The size of the PCR product is 266 bp.

Table 1 shows that 15 of the 19 samples, including the two CSF specimens, were correctly identified.

Table 1

Comparison of the detection and identification of dengue viruses by larvae inoculation-RT-PCR and virus isolation.

	Virus serotype	
	Den-2	Den-3
Virus isolation ^a	5	14
larvae-RT-PCR	3	12 ^b

^a viruses were isolated in C6/36 cells and the serotype determined by indirect immunofluorescence using monoclonal antibodies (Henchal *et al*, 1982)

^b includes two CSF samples

DISCUSSION

Isolation of dengue viruses by intracerebral inoculation of the fourth instar of *Toxorhynchites splendens* larvae (Lam *et al*, 1986) has been routinely carried out in our dengue diagnostic laboratory. The viruses are detected on day 5 post-inoculation by indirect immunofluorescence using a flavivirus-specific broad reacting monoclonal antibody and subsequently identified using a type-specific dengue monoclonal antibody. We reasoned that larvae inoculation together with RT-PCR should overcome the problem encountered when insufficient serum is received for direct amplification, since only 1 to 2 μ l of a diluted (1 : 2) sample is required.

Initially, proteinase-K and phenol/choroform treatment were used for the extraction of template from infected larvae heads (Maguire, 1986; Tardieux and Poupel, 1990), however, amplification could not be achieved. Finally, a simple, rapid and reproducible protocol was developed which takes advantage of the nucleic acid binding properties of silica beads (Boom *et al*, 1990). This method significantly reduces the number of steps required in sample handling and therefore reduces the possibilities of contamination which is a concern in PCR.

The method was sensitive enough to detect 4 PFU of dengue-2 (New Guinea C) on ethidium bromide stained agarose gels. Dengue-3 viruses could be detected from a pool of four infected lar-

vae as early as 2 days post-inoculation. However, viral RNA extraction on day 3 post-inoculation proved to be most reproducible.

When applied to the analysis of 19 virus isolation positive samples, 15 of them were correctly identified by RT-PCR. Both of the CSF samples that had previously been negative by direct RT-PCR were positive after biological amplification of the virus in larvae. This would indicate that the initial virus titer in the CSF was too low for direct detection by RT-PCR. The failure to detect dengue viruses in four of the samples could be attributed to the loss of virus infectivity after prolonged storage or repeated freeze-thaw cycles of the specimen.

The method presented here allows the rapid identification of dengue viruses even from specimens that are not suitable for direct RT-PCR or inoculation of cell cultures due to insufficient amounts or to bacterial or fungal contamination of the specimen. The results can be obtained several days earlier than serological identification with monoclonal antibodies which is routinely done on day 5 post-inoculation.

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