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Research Note

Temperature related storage evaluation of an RT-PCR test kit for the detection of dengue infection in mosquitoes

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Abstract. The rapid detection of dengue infection in mosquito vectors is important for early warning to forestall an outbreak. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) provides a rapid method for dengue detection in man and mosquitoes. An RT-PCR kit developed by the Medical Entomology Unit, Institute for Medical Research to detect dengue infection in mosquitoes, was tested for its shelf life at 3 storage temperatures: room temperature, refrigerator and freezer. Test kits were tested once every 3 days for kits stored at room temperature, and once every week for those stored at refrigerator and freezer temperatures. The results showed that the test kit could only be stored above its recommended storage temperature of -20°C for not more than 3 days. DNA 100bp markers in the kits appeared to be stable at the tested temperatures and were usable up to the 20th day when stored at 2 °C and below.

Dengue is a prominent disease in the tropics and subtropics. Dengue fever and dengue hemorrhagic fever is present in over 100 countries where 2.5 billion people are susceptible to it (Guzmán & Kourí, 2002). Approximately 50–100 million cases of DF and about 250,000–500,000 cases of DHF are officially notified annually (Guzmán & Kourí, 2002). The bulk of these cases occur in endemic regions such the Americas, Southeast Asia and the Western Pacific (Guzmán & Kourí, 2002).

The typical detection methods of dengue virus in the mosquito vectors include either the direct inoculation of the dengue virus into *Toxorhynchites* spp mosquitoes, or the inoculation of the virus into C6-36 cell culture. Virus identification is done by immunofluorescence assay with serotype-specific monoclonal antibodies (Kuberski & Rosen, 1977), or peroxidase anti-peroxidase staining (Okuno *et al.*, 1978). This is done 7-10 days post-inoculation for C6-36 cell cultures or 14 days post-inoculation for mosquitoes. Sensitivity to the virus is 36% with C6-36 cell lines or up to 80% by direct mosquito inoculation (Guzmán & Kourí,2002).

Hence the use of reverse transcriptase polymerase chain reaction (RT-PCR), for the detection of the dengue virus offers another potential method for sensitive, specific and rapid detection of small amounts of viral RNA, thus providing an attractive approach for the rapid identification of dengue infection (de Paula *et al.*, 2002). This is the case not only in terms of suspected human cases, but also on mosquito populations as well.

An RT-PCR test kit for dengue detection in mosquitoes has been developed by the Unit of Medical Entomology, Institute for Medical Research, Kuala Lumpur. The PCR products of this kit display diagnostic bands at the 480bp position if the test

amplicon is infected. To prepare the kit for future application, this experiment was conducted to determine the shelf life of the kit at various temperatures. These data are particularly important with regards to shipping and storage of the kit in the field. Typically the test kit is kept at -20°C, however, it is of particular interest to examine the shelf life of this test kit when stored in a household refrigerator, freezer and at room temperatures. This is because these 3 temperatures would be most readily available.

Three sets of test kits were prepared. Each kit was stored at room temperature, in a household refrigerator and in the freezer. The temperatures of the fridge and freezer used were monitored closely during the test period. Each kit consists of two parts, an extraction and a PCR component. Commercialized versions also includes an electrophoresis kit as well. The positive control was prepared by using culture fluid from C6-36 cell lines infected with dengue type-2. The fluid was tested using the in-house RT-PCR test method to determine the presence of the virus in the culture fluid. Fluid tested positive was used as the positive control for all of the test kits made. Uninfected culture fluid was used as the negative control.

The test kits were tested at regular intervals. To determine if they have retained their effectiveness, the kits were tested on the positive control ICF fluid in the kits themselves. The test results were then confirmed using in-house RT-PCR testing methods. The kit stored at room temperature was tested every 3 days over a one week period while the kits stored in the refrigerator and freezer were tested at weekly intervals. The temperature of the refrigerator and freezer for the duration of the test period are listed in table 1. The extraction, PCR and electrophoresis procedures are as follows.

All the following procedures were carried out individually on the kits tested and for the in-house method. A working solution was first prepared by adding 4.3 ml binding buffer and 86.7 µl poly A. The above volume is used for 20 samples. Fifty µl of dengue ICF fluid was then diluted with 300µl of ddH_2O. Two hundred µl of the diluted ICF was then pipetted into 200 µl working solution. Fifty µl of proteinase K was then added, and the mixture was then vortexed and incubated at 72°C for 10 minutes. One hundred µl of isopropanol was then added to the mixture and it was vortexed again. High pure filter tubes were then combined with collection tubes and the samples were pipetted into the upper reservoir. The samples were then centrifuged at 14,000rpm for 1 minute. The flow- through was then discarded and the filter tubes was then combined with new collection tubes. Five hundred µl of inhibitor removal buffer was then added to each upper reservoir. The samples were then centrifuged at 14,000rpm for another 1 minute. The flow- through was then

Table 1. Storage temperature in a household refrigerator and freezer during experiment duration

Days of storage	Temperature (°C)	
	Fridge	Freezer
1	1	-10
2	1	-10
3	1	-10
4	1	-10
5	1	-10
6	1	-10
7	1	-10
8	2	-10
9	2	-10
10	1	-20
11	1	-20
12	1	-20
13	1	-20
14	1	-20
15	1	-20
16	1	-20
17	1	-20
18	2	-10
19	2	-10
20	2	-10

discarded and the filter tubes were combined with new collection tubes. Four hundred and fifty µl wash buffer was then added to each upper reservoir and the samples were centrifuged at 14, 000rpm. The flow through was discarded and the filter tubes were combined with new collection tubes. Another 450 µl wash buffer was added and the samples were centrifuged at 14,000rpm. The filter tubes were then combined with 1.5 ml eppendorf tubes. Fifty µl of elution buffer was then added to the middle spot of the filter tubes and the samples were centrifuged at 14,000rpm for 2 minutes. The samples collected in the eppendorf tubes were then stored at -80 °C if necessary.

For PCR amplification, 13 µl of the kit master mix was transferred into PCR tubes. AMV RT, Taq polymerase, UPC primer and UPS primer were added in that sequence to the master mix at 0.5 µl each. Ten µl of extracted samples were than added to the PCR mixture. The mixtures were then vortexed and spun down for 10 seconds. All samples were transferred to a thermocycler and the following program was carried out: 51 °C for 45 minutes for amplification, 92 °C for 3 minutes as initial denaturation. The following 3 step cycle was then repeated for 41 times: 92 °C for 30 seconds as the denaturation step, 53 °C for 30 seconds as annealing step, 72 °C for 1 minute as the extension step. As a final extension, the samples were heated to 72 °C for 5 minutes. To visualize the PCR products, all samples were run on a 3% agarose gel with 15 µl of ethidium bromide. The electrophoresis was carried out for 45 minutes at 100 volts. One hundred base pair markers were used from each test kit along with one from the in-house test kit.

Test results are shown in table 2. The 100bp markers remain stable regardless of the storage temperatures. Of worthy note is the marker stored at room temperature, which was still usable and showed clear bands up to two weeks. The kit stored at room temperature displayed positive results three days from the date of kit creation (25/3/2005). On the 6th day of testing (28/3/2005), the kit stored at room temperature no longer yielded any positive results, although in-house testing showed that the positive control stored with the kit still contained viral RNA. Beyond that point, all further testing with this kit yielded negative results. The kits stored in the refrigerator and freezer showed negative results for the positive control ever since their first testing one week from kit creation and storage (28/3/2005) and this might be due to the denaturation of the enzyme components of the kit (particularly proteinase K), which are normally stored at -20°C. This possibility is enforced considering that the proteinase K stored in the refrigerator showed precipitation since 28/3/2005. However it cannot be concluded that the kits have lost their effectiveness since the in-house testing method shows similar negative results when used to test the positive control ICF of both kits.

Table 2. Test results according to days of storage

Days of storage	Room Temperature						Fridge						Freezer					
	Positive control		Negative control		Marker		Positive control		Negative control		Marker		Positive control		Negative control		Marker	
	TK	IH	TK	IH	TK	IH	TK	IH	TK	IH	TK	IH	TK	IH	TK	IH	TK	IH
3	+	+	–	–	+	+	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t
6	–	+	–	–	+	+	–	–	–	–	+	+	–	–	–	–	+	+
13	–	n/t	–	n/t	+	+	–	n/t	–	n/t	+	+	–	n/t	–	n/t	+	+
20	n/t	n/t	n/t	n/t	n/t	n/t	–	n/t	–	n/t	+	+	–	n/t	–	n/t	+	+

+ = positive; – = negative; n/t = not tested; TK = test kit; IH = in-house.

The loss of RNA in the positive control ICF fluid might be due to complications associated with RNA storage in a cell rich medium. Endogenous RNases, which are found in cell rich samples such as blood and buffy coat, may have a role in the degradation of the viral RNA and thus resulting in a lower detection result by RT-PCR (De Paula *et al*., 2002). This problem would be significant in this experiment considering the positive control ICF was stored for a week, albeit at low temperatures, before use. Storage time is the crucial factor here since the positive control ICF stored at room temperature still contained enough RNA to be detected by the in-house method one week since storage (28/3/2005).

Of the other components of the kit, those that involve enzymes are of particular concern. Any rise from the recommended storage temperature of -20ºC may result in enzyme denaturation and loss of effectiveness. Even the *Taq* polymerase used, which is theoretically stable up to 70 ºC, has sufficient activities at lower temperatures that non-specific products are frequently made by extensions of low stringency primer binding events that occur during the time that samples are being prepared or that thermal cycling is being initiated. These products are then propagated during subsequent rounds of thermal cycling. This reduces the sensitivity of the PCR procedure in diagnostic applications (Daiss *et al*., 1995).

In conclusion, the test kit can be stored above its recommended storage temperature of -20ºC, but only temporarily and for a duration of not more than 3 days. This is because long term storage of kit enzymes at higher storage temperatures lead to their loss of effectiveness. Furthermore, the positive ICF control is susceptible to RNA loss through hydrolysis, hence a more stable form of virus RNA has to be found as the positive control. The 100bp markers, however, are very stable and should pose no storage problems as long as the storage temperature is below 2 ºC.

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