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Detection of dengue virus RNA in blood clots by multiplex nested reverse transcription-PCR

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ABSTRACT: Dengue is the most common vector-borne viral disease of humans globally. Detection of viral RNA from suspected patient specimens is rapid, specific and confirmative in laboratory diagnosis of dengue infections during the acute phase. In this study, a multiplex nested reverse transcription-PCR (RT-PCR) system was established for clinical specimens. While other nucleic acid amplification tests showed relatively low sensitivity, the multiplex nested RT-PCR assay detected 4 cases among blood clots from 8 serologically confirmed dengue patients. These results suggested that blood clots of dengue patients could be used in laboratory diagnosis, and that the multiplex nested RT-PCR assay, which simplified the detection procedure, could facilitate viral RNA detection of specimens in clinical laboratories.

KEY WORDS: dengue virus; multiplex nested RT-PCR; nucleic acid amplification test

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Dengue viruses (DENV), the causative agents for dengue, are members of the genus *Flavivirus* in the Family *Flaviviridae*. DENV, primarily transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes, consist of four antigenically and genetically distinct serotypes (DENV-1, -2, -3 and -4). Symptomatic infection of any serotype of DENV in human may range from dengue fever (DF) to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), although the majority of human infections are asymptomatic[1]. During the past 50 years, due to the global expanding of breeding sites for vectors, DENV have been spread to nearly seventy countries or districts, posing a threat to one-third of the world's population, hence, are listed as the most widespread and important arboviruses [1-2].

Among laboratory diagnosis approaches currently available for dengue infection such as virus isolation, detection of viral RNA, viral antigen assays and serological tests of virus-specific IgM/IgG antibodies, etc, molecular diagnostic that directly targets viral genomic fragments typically provides more rapid, sensitive and precise evidence for dengue infection than other methods, especially during the viremic phase[1,3]. Dozens of in-house established or commercially developed viral RNA detection systems have been reported in the past 2 decades, including one-step reverse transcription PCR (RT-PCR), nested RT-PCR, multiplex RT-PCR and diverse real time RT-PCR, etc. In particular, the nested RT-PCR system developed in 1992 by Robert Lanciotti et al has been evaluated and widely adapted in several international laboratories, although its tedious and labor-intensive operation procedure has hindered its application in clinical laboratories in that four separate PCR reactions are required for the second-round amplification and gel-analyses in parallel [4-8]. In order to establish a simple, sensitive diagnosis suitable for all DENV serotypes in clinical specimens, a modified multiplex nested RT-PCR assay was developed in this study based on these primers, its application in blood clots from suspected dengue patients was assessed and compared with other nucleic acid amplification assays.

Materials and Methods

Reference strains of DENV

Reference strains of 4 DENV serotypes, i.e., Hawaii (DENV-1), New Guinea C (DENV-2), H87 (DENV-3) and H241 (DENV-4), were maintained in our laboratory, propagated in C6/36 cells with RPMI 1640 medium supplemented with 2%

fetal bovine serum. Aliquots of cultured supernatants were stored at -70℃.

Clinical specimens

Whole blood samples from suspected dengue patients, confirmed by serological tests, were collected in our laboratory during 2011-2012. The timing of blood collection was shown in Table 1. Blood clots (BC01-BC08) were dispensed in microcentrifuge tubes after serum separation and stored in a -70℃ freezer.

Tab. 1 Diverse nucleic acid assays for dengue virus RNA in blood clot specimens

Sample ID	Days post-onset	OneStep RT-PCR	OneStep multiplex RT-PCR	OneStep SYBR Green real time RT-PCR	Multiplex nested RT-PCR	Serotypes
BC01	5d	−	−	−	+	Den2
BC02	3d	+	−	+	+	Den3 Den4
BC03	5d	−	−	−	−	/
BC04	9d	−	−	−	−	/
BC05	10d	−	−	−	−	/
BC06	10d	−	−	−	−	/
BC07	6d	−	−	−	+	Den1
BC08	6d	−	−	−	+	Den3

Instruments and reagents

A Biometra thermocycler (model T1, Germany) and an Applied Biosystems 7 500 real-time PCR system (USA) were used in nucleic acid amplification assays. The RNeasy Mini kit and a one-step RT-PCR kit (Qiagen, Germany) were used in RNA extraction and viral RNA detection. The one-step SYBR PrimeScript RT-PCR kit (version II), Ex Taq DNA polymerase and dNTPs were products from TaKaRa company (Dalian, China). Oligo primers as shown in literature were synthesized at Sangon Biotech (Shanghai) Co., Ltd[4].

RNA extraction and nuclei acid amplification

Blood clots were pre-treated as previously described [9]. Total RNA from clinical specimens or viral RNA from cultured supernatants were extracted by using a Qiagen RNeasy Mini kit, according to the manufacture's instructions. In brief, the extraction procedure started from 100 μg or 100 μL of samples, finally eluted in 50 μL RNase-free water. The purified total RNAs were stored in a -70℃ freezer until analysis.

One-step RT-PCR and one-step SYBR Green RT-PCR assays were conducted in 25 μL and 20 μL volume, respectively, according to manufactures' recommended protocols. Briefly, for the one-step RT-PCR, the thermal cycler was programmed as follows: 50℃ 30 min for reverse transcription; 95℃ 15 min for PCR activation; 94℃ 30 sec, 55℃ 20 sec, 72℃ 40 sec for 35 cycles; with additional extension at 72℃ 10 min. For the one-step SYBR Green RT-PCR, the 7 500 real-time system was programmed at 42℃ 5 min for reverse transcription, followed by 95℃ 10 sec for PCR initiation. The PCR step was carried out in 45 cycles with 95℃ for 5 sec and 60℃ for 34 sec, followed by a standard dissociation protocol.

Results

Optimization of the multiplex RT-PCR system

There were six primers, including universal primers D1 and D2, four serotyping primers TS1-TS4, in the nested RT-PCR system established by Robert Lanciotti et al. In its second-round amplification, the combination of D1 with TS1-TS4 primers yields serotype-specific fragments of diverse lengths. In order to develop a more sensitive and simple multiplex assay with these primer sets, serial primer concentrations for D1 and TS1-TS4 combination were screened till an optimal system, with D1 at 0.4 μM and TS1-TS4 at 0.2 μM as final concentration, respectively, were determined. Therefore, with reference strain RNAs as templates, amplicons of 482, 119, 290 and 392 bp, respectively, were obtained for DENV-1 to DENV-4 under such conditions, indicating this multiplex RT-PCR assay was capable of precisely distinguishing DENV serotypes simultaneously in addition to de-

tecting viral RNA (Figure 1).

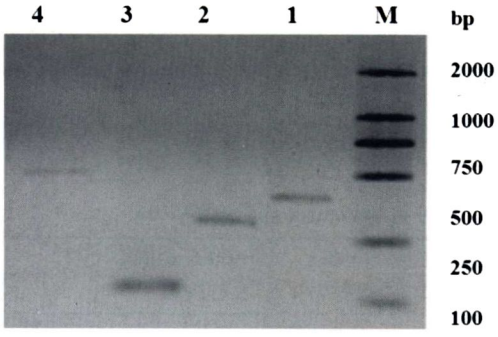

Fig. 1 Multiplex RT-PCR detection of dengue virus RNA from 4 serotypes

M: DL2000 DNA marker; Lane 1: DENV-4; Lane 2: DENV-3; Lane 3: DENV-2; Lane 4: DENV-1

Preliminary detection of blood clot specimens by one-step RT-PCR, one-step multiplex RT-PCR and one-step SYBR Green real time RT-PCR

Our first attempt to detect viral RNA in blood clots failed by the one-step multiplex RT-PCR, when no specific band was observed on agarose-gel analysis for the 8 specimens. Subsequently, we tested these specimens by another one-step RT-PCR using the universal primer pairs, D1/D2, and one-step SYBR Green real-time RT-PCR. Sample BC02 was positive in both assays, while the rest were negative (data not shown), suggesting limited sensitivity of the above nucleic acid amplification tests for clinical specimens from suspected dengue patients.

Multiplex nested RT-PCR detection of DENV RNA in blood clots

Thus, we alternatively detect these specimens by a multiplex nested RT-PCR, namely, universal primers D1/D2 were used in the one-step RT-PCR as the first-round reaction, while the second-round amplification was a multiplex PCR involving mixed primers D1 and TS1-TS4. It was revealed by agarose-gel analysis that specific bands appeared in 4 specimens (BC01, BC02, BC07 and BC08), shown in Figure 2. Based on relevant fragment lengths, it was obvious that BC01 was infected with DENV-2, BC07 was infected with DENV-1, BC08 was infected with DENV-3, while BC02 was co-infected with DENV-3 and DENV-4 in that both DENV-3 and DENV-4 specific bands were detected. This observation was additionally proved by conventional nested RT-PCR.

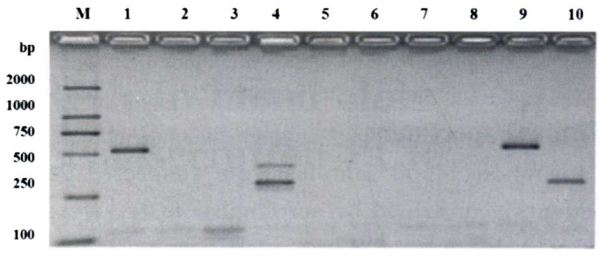

Fig. 2 Detection of dengue virus RNA in blood clots by multiplex nested RT-PCR

M: DL2000 DNA marker; Lane 1: DENV-1 positive control; Lane 2: Negative control; Lane 3-10: BC01-BC08.

Discussion

Virus components are usually presented in dengue patients' sera, plasmas, circulating blood cells and autopsy tissues such as liver, brain of fatal cases within 4-5 days post-onset. Virus isolation, viral nucleic acid detection or viral antigen assays are confirmative in diagnosis at the early stage, however, post-acute-phase evidence for DENV infection has to mainly rely on serological tests of virus-specific antibodies in the wake of viremic period [1,3]. We detected viral RNAs in 4 specimens among eight blood clots from serological positive patients, the timing of sample collection indicated that all positive specimens were collected within 4-6 days post-onset, in contrast, the negative specimens were collected between 5-10 days post-onset. It is notable that Sample BC02, which was positive in three nucleic acid amplification assays, was collected on day 3 post-onset. This observation indicates viral RNA detection for samples collected at earlier days of the acute phase is valuable in early diagnosis of dengue infection.

A variety of molecular approaches, including RT-PCR derived assays such as conventional RT-PCR, nested RT-PCR and real-time RT-PCR, other nucleic acid amplification methods such as reverse transcription loop-mediated amplification (RT-LAMP) and nuclei acid sequence based amplification (NASBA), and high throughput devices such as microarrays and next-generation deep sequencing platforms, are accessible for DENV diagnosis thus far. RT-PCR derived tests have been first options for clinical virological laboratories in that it's fast, specific and low-cost. The nested RT-PCR assay established by Robert Lanciotti et al in 1992 has been introduced and modified in lots

of international laboratories [5-8]. It has been reported that diverse multiplex RT-PCR assay systems were established in several laboratories, however, few of them was sensitive enough to be applicable to clinical specimens. Indeed, it is revealed in several attempts of our study that such systems are suitable for identification of virus isolates, rather than clinical specimens of low virus load.

Apart from conventional serum or plasma specimens from suspected dengue patients, utilization of other clinical specimens or sources for viral genome detection may develop novel categories of specimens that is more accessible, sensitive and with longer positive durations, in particular, spares more sera or plasmas in subsequent virus isolation procedures, if necessary. Genomic fragments of DENV have been documented detectable in paraffin-embedded tissues, saliva and dry blood spotted on filter papers [10-13]. Recently, it was reported that Japanese researchers detected DENV RNA from patients' urine specimens, even viral genomes have already been proven to disappear in serum specimens [14]. After our successfully detection of Japanese encephalitis virus RNA from blood clots of clinically suspected patients, using an in-house established SYBR Green real-time RT-PCR assay, we collected these blood clots from suspected dengue patients over one year. Comparison of detection efficiencies of several nucleic acid amplification assays for total RNAs extracted from blood clots, the multiplex nested RT-PCR showed the highest sensitivity. This observation suggests that the byproducts of serum separation, blood clots, which comprise plenty of solid blood components such as red blood cells, white blood cells, platelets and fibrins, etc, may be another source for DENV RNA detection in dengue diagnosis at clinical laboratories. Although both blood clots and serum specimens from the same patient are not systematically examined and compared in parallel in this study, it is not clear which specimen is more sensitive or has a more prolonged positive duration, hence, suitable for DENV RNA detection. It is advantageous for blood clots in that several host housekeeping genes such as GAPDH may be employed as internal controls in nucleic acids assays, while no internal control is available in serum specimens. Therefore, application of nucleic acid tests to such clinical specimens other than conventional serum and plasma samples, with standardized sample processing protocols, will facilitate DENV RNA detection in more clinical laboratories, enhance their diagnostic capacity, and eventually contribute to local public health.

In summary, the multiplex nested RT-PCR assay is ideal for early diagnosis of dengue due to its high sensitivity, easy-to-perform, low-cost and rapidity. Blood clots are valuable clinical materials, especially in small laboratories, for clinical diagnosis of several pathogens that appears in blood during certain period of their life cycles.

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多重套式 RT-PCR 检测患者凝血块中登革病毒 RNA*

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摘 要:目的 建立简便、灵敏、适合于全部血清型登革病毒核酸检测的多重套式 RT-PCR 体系,检测临床样品中登革病毒 RNA,作为实验室辅助诊断的依据。**方法** 利用登革病毒标准株核酸,建立多重 RT-PCR 检测方法。提取患者凝血块总 RNA,分别用一步法 RT-PCR 及多重套式 RT-PCR 检测。**结果** 通过对检测体系进行优化,多重 RT-PCR 能够同时检测 4 种血清型登革病毒核酸。采用多重套式 RT-PCR 方法,从 8 例登革热患者凝血块中有 4 例检测到病毒 RNA,而其它核酸检测方法仅检出 1 例阳性。**结论** 多重套式 RT-PCR 的方法能够从临床凝血块样品中检测到登革病毒核酸,并同时进行血清学分型,简化了登革病毒核酸检测步骤,有利于对临床样品开展病毒核酸检测。

关键词:登革病毒;多重套式 RT-PCR;核酸检测

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法可用于隐孢子虫的流行病学调查研究及临床诊断。

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