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Using buffy coat for reverse transcriptase-polymerase chain reaction in the diagnosis of dengue virus infection: preliminary study

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Using reverse transcriptase-polymerase chain reaction (RT-PCR) to detect and type from viremic human serum samples for dengue virus infection is widely used today. However, a few false-negative results were reported due to very low titers of the virus particle in serum samples. As mononuclear cells, macrophages or monocytes are target cells for dengue virus infection, and the replication of virions can be observed in peripheral leukocytes frequently, the amount of virus particle in buffy coat should be higher than those in serum samples. Here, we describe a procedure in which RNA extraction from the buffy coat of a patient with a false-negative serum sample yielded specific viral RNA amplifiable by RT-PCR, thereby providing an alternative choice for the accurate diagnosis of dengue infection.

Key words: Buffy coat, dengue infection, reverse transcriptase-polymerase chain reaction (RT-PCR)

Dengue fever is caused by a mosquito-borne flavivirus, with four distinct serotypes (DEN 1-4). The common vector is *Aedes aegypti.* Infection with any serotype can result in a mild, self-limiting illness characterized by fever, headache, nausea, vomiting, abdominal pain, bone pain, myalgia, prostration, or rash [1,2]. However, a more severe form of the disease, dengue hemorrhagic fever-dengue shock syndrome (DHF-DSS), involving vascular and hemostatic abnormalities, is a serious problem with a high mortality rate in many tropical areas, especially in Southeast Asia [1,3-6]. Hence, a rapid and reliable diagnosis is urgently required.

Routine laboratory diagnosis often involves the detection of antibodies against dengue virus by hemagglutination inhibition (HI), complement fixation (CF), or plague reduction neutralization test (PRNT). These assays are neither rapid nor easy to manipulate, and their sensitivities and specificities are often low if the paired serum specimens (acute and convalescent) are collected inappropriately [7]. Immunoglobulin M (IgM)-capture enzyme-linked immunosorbent assay (ELISA) is more accurate [8,9], but it involves collecting samples for more than 7 days after onset of disease and is not type-specific [10,11], whereas viral culture for indirect fluorescence antibody assay (IFA) requires 7-14 days in preparation [11].

Recently, reverse transcriptase-polymerase chain reaction (RT-PCR) has been used widely and successfully in detecting and typing of viremic human serum samples [12-15]. The sensitivities for DEN-3 and DEN-4 were 100%. However, comparing with virus isolation, a few false-negative results for DEN-1 (sensitivity was 94-96%) and DEN-2 (sensitivity was 93%) were reported due to serum samples with virus titers lower than 10^2 of 50% infective doses per mL ($TCID_{50}$/mL) or those serum samples that contain an inhibitor of enzymatic amplification that copurified with template RNA if the specimens had long storage history [12,13].

Most evidence suggests that mononuclear phagocytic cells are primary target cells for dengue infection [16,17]. The virus has been found in peripheral monocytes and infected macrophages [17-19]. Replication of DEN-2 can be observed in peripheral blood leukocytes frequently within 10 days after onset of illness [20,21]. Hence, the amount of virus particles in buffy coat should be higher than those in serum samples in the acute stage.

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Using buffy coat for polymerase chain reaction (PCR) assay was successful for detection of cytomegalovirus and herpesvirus infection [22,23], but it has never been used for RT-PCR detection of dengue virus infection. Here, we describe a procedure in which RNA extraction from the buffy coat of a patient with false-negative RT-PCR serum sample yielded specific viral RNA amplifiable by RT-PCR.

Case Description

A 50-year-old Taiwanese man was admitted to the hospital because of intermittent fever and chills for 4 days. The patient was well apart from being an asymptomatic chronic hepatitis B virus carrier. One month earlier, he had attended a meeting in the Philippines. After returning to Taiwan, he developed profound tenderness over both thighs, subsequent fever of up to 40 °C and chills with profuse sweating for 4 days prior to admission. Physical examination on admission revealed a moderately ill looking with a body temperature of 37.8 °C, pulse rate: 94/min, respiration rate: 16/min, and blood pressure: 122/78 mmHg. His neck was supple. Lungs were clear. The heart was normal. The abdomen was flat and soft without palpable liver or spleen. Neurologic examination was negative. No petechiae was found on the skin. Initial laboratory examination revealed a hematocrit of 44.2%, white blood cell count of 4,400/mm^3 with 75% neutrophils, and platelet count of 75,000/mm^3. Radiograph of the chest was normal. Abdominal plain film and sonogram were negative. Initial serum biochemistry revealed the following: aspartate aminotransferase (AST): 138 U/L (normal range, 0-35 U/L), alanine aminotransferase (ALT): 69 U/L (normal range, 0-42 U/L), and lactate dehydrogenase (LDH): 558 U/L (normal range, 95-213 U/L). Total bilirubin was 1.1 mg/dL (normal range, 0.2-1.6 mg/dL). Three days later, AST and ALT were increased to 1084 U/L and 289 U/L, respectively. Prothrombin time was 13.4 sec (control 12.5 sec). Activated partial prothrombin time was 33.8 sec (control 31.5 sec). Blood culture was negative. Blood smears were negative for malaria. IgM capture ELISA for cytomegalovirus (CMV) was negative. Epstein-Barr viral capsid antigen (EB VCA) test (IgM) was less than 1/10. Widal test was less than 1:40. Weil-Felix test was less than 1:40. Heterophil antibody presumptive test was negative. Anti-hepatitis A virus (HAV) IgM and anti-hepatitis C virus (HCV) IgM were negative as well. HI titer for dengue virus and Japanese encephalitis (JE) virus were less than10; IgM capture ELISA for dengue virus was negative. Using the patient's serum, the RT-PCR assay for dengue virus was negative. Nevertheless, a viral syndrome was highly suspected, and supportive treatment with acetaminophen and intravenous fluid was prescribed. The patient's fever subsided on the fifth hospital day, and he was discharged on the seventh day in a stable condition. Unfortunately, we could not obtain a convalescent serum after patient discharged from the hospital. However, 1 year later, a serum sample collected from this patient had HI titer of 80 for dengue virus.

Materials and Methods

Serum collection and tissue culture

The acute stage serum collected during the second day of patient's hospitalization was diluted to 1:10 with phosphate buffer solution (PBS) and passed through bacteria filter (0.45 μm). The negative control serum was collected from a normal healthy male staff in our laboratory. Tissue culture cells were inoculated on 0.5 mL/6 well plates (Falcon, Franklin, Lakes, NJ, USA). These cells were prepared from mosquito cell line (C6/36), rhesus monkey kidney cell line (LLC-MK2), and a human promonocyte cell line (HL-CZ) which was established in our virology laboratory and proven to be effective for dengue virus isolation [24,25]. The C6/36 cell line was obtained from the Vector-Borne Infectious Diseases Division, Centers for Disease Control and Prevention (Fort Collins, CO, USA) and cultured at 28 °C in Leibowitz L15 medium (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FCS). The HL-CZ cell line was cultured in Falcon plastic flasks with RPMI 1640 medium (Boehringer, Mannheim, Germany) containing 10% heat-inactivated FCS supplemented with 100 U/mL of penicillin and 100 mg/mL of streptomycin. Both HL-CZ cell line and LLC-MK2 cell line were cultured at 37 °C in a 5% CO_2 incubator and observed for cytopathic effect (CPE) every 2 days. The virus culture was prepared four times in blind passage every 5 days in LLC-MK2 cells, C6/38 cells, and HL-CZ cells for the detection of dengue virus by RT-PCR, and the suspended cell smear was made for the indirect immunofluorescence test. The DEN-2 monoclonal antibody produced by hybridoma cell (ATCC, HB46, 3H5) was used for identification, in which anti-mouse IgG FITC conjugate (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was used as a secondary antibody.

RT-PCR

The patient's buffy coat in the acute stage was prepared from 10 mL of peripheral blood drawn into a heparinized-collection tube, then centrifuged at 1,800

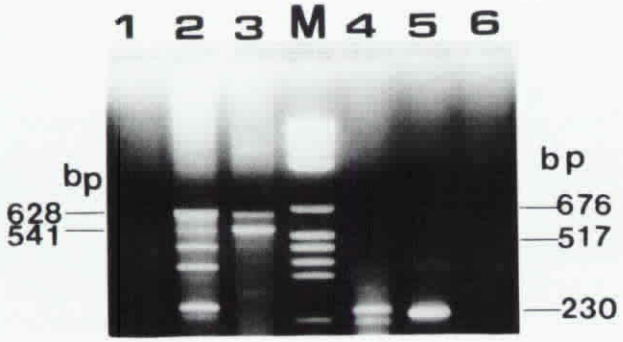

Fig. 1. Photograph of an agarose gel stained with ethidium bromide, containing RT-PCR products for detection of DEN-2 genome from patient's buffy coat by using universal primer pairs YF-1, YF-3 (lane 1,2, and 3), and specific primer pairs D2-S, D2-C (lane 4, 5, and 6). Lane 1: negative control (noninfected buffy coat); Lane 2: patient's buffy coat (universal D-2); Lane 3: positive control (universal D-2); M: marker (PGEM DNA); Lane 4: patient's buffy coat (specific D-2); Lane 5: positive control (specific D-2); Lane 6: negative control (non-infected buffy coat). Using universal primers, two fragments of DNA, 628 bp (in size) and 541 bp were amplified. A 230 bp fragment was seen when specific primers (D2-S and D2-C) were used in RT-PCR.

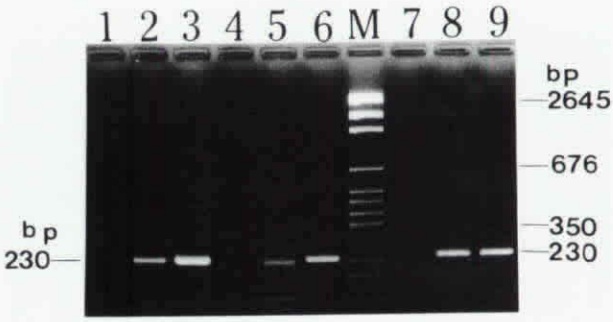

Fig. 2. Identification and specificity of DEN-2 with specific primer pair by RT-PCR. DEN-2 infected cells were amplified with specific primer pairs and amplified product was detected in ethidium bromide-staining agarose gel. Lane 1: negative control (noninfected serum in C6/36 cells); Lane 2: patient's serum infected C6/36 cells; Lane 3: positive control (DEN-2 infected C6/36 cells); Lane 4: negative control (noninfected serum in HL-CZ cells); Lane 5: Patient's serum infected HL-CZ cells; Lane 6: positive control (DEN-2 infected HL-CZ cells); M: marker (PGEM DNA); Lane 7: negative control (noninfected serum in LLC-MK2 cells); Lane 8: patient's serum infected LLC-MK2 cells; Lane 9: positive control (DEN-2 infected LLC-MK2 cells).

μg for 20 min and aspirated with a pipette. The negative control buffy coat was collected from a healthy male volunteer. The flavi-universal primer pair (primer code YF-1: 5'-GGTCTCCTCTAACCTCCAG-3' and YF-3: 5'-GAGTGGATGACCACGGGAAGACATGC-3') and the DEN-2 specific primer pair (primer code D2-S: 5'-GTTCCTCTGCAAACACTCCA-3' and D2-C: 5'-GTGTTATTTTGATTTCCTTG-3') were used [26,27]. Two hundred microliter of buffy coat from 10 mL of blood was extracted as RNA template, then 10 μL of RNA was heated at 80 °C for 5 min and added to 1.5 mircoliter of complementary primer (100 pmol) in a 500 μL Eppendorf tube. The mixture was cooled down to 42 °C and incubated for 60 min at the same temperature followed by the addition of 90 μL of RT-PCR mixture (0.2 mM) deoxynucleoside triphosphate, 10 mM Tris (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per mL, 10U of reverse transcriptase (Promega Corporation, Madison, WI, USA), and RNasin (1% Nonident P-40, 10U RNase inhibitor) (Promega). The mixture was incubated at 53 °C for 10 min for RT followed by the addition of 95 μL of the following mixture: 100 pmol of sense primer, 0.2 mM deoxynucleoside triphosphate, 10 mM Tris (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per mL, and 2U of Taq DNA polymerase (Finnzyme Inc., Estoo, Finland). The reaction mixture was covered by three drops of mineral oil. The PCR process comprised an initial step at 53 °C for 10 min for flavi-universal primers, then heating to 94 °C, 1 min; 53 °C, 1 min, 72 °C, 1 min for a total of 34 cycles, whereas for the DEN-2 specific primers, an initial step at 94 °C for 3 min, then, the following thermal cycles was started: 94 °C, 1 min; 53 °C, 10 min; 72 °C, 3 min for a total of 34 cycles, followed by a final extension at 72 °C for 3 min. After PCR amplification, 15 μL of product was electrophoresised in a 2% agarose gel and visualized with UV light after ethidium bromide staining.

Results

The results of flavivirus genomic RNA detection with flavi-universal primers and specific primers are shown in Fig. 1. The RNA was extracted from buffy coat, followed by a RT-PCR, the detection of dengue RNA can be completed successfully within 6 h. Since the primer code YF-1 was expected to anneal with two conserved sequences near 3'-end of the viral genome, except YF genomic RNA, two amplified DNA bands with 628 and 541 basepairs (bp) in sizes were observed as shown in lane 2 (patient's buffy coat) and lane 3 (DEN-2 control) on the agarose gel in the presence of ethidium bromide. As for lane 1 (noninfected buffy coat), no visible band could be detected. Afterwards, DEN-2 specific primer pairs (D2-S and D2-C) were used, a specific cDNA band 230 bp in size was amplified

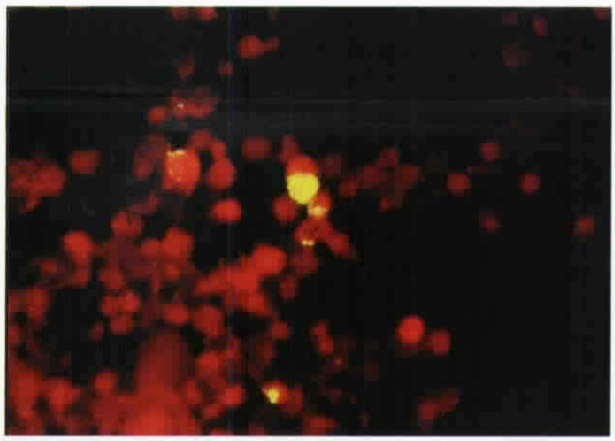

Fig. 3. Immunofluorescence stain of DEN-2 virus in the fifth passage of HL-CZ cell (x 400).

by RT-PCR in lane 4 (patient's buffy coat) and lane 5 (positive control with specific D-2). This band was also seen in the fourth passage of the isolates with C6/36, HL-CZ, and LLC-MK2 cell culture (Fig. 2).

Dengue virus was isolated from C6/36, HL-CZ, and LLC-MK2 cell culture successfully with the patient's acute stage serum. The cultured virus was identified as DEN-2 by the monoclonal antibody in immunofluorescence test. Specific immunofluorescence was seen in the fifth passage of the virus in HL-CZ cells (Fig. 3) with DEN-2 positive response on day five.

This result indicated that dengue virus could be detected by using buffy coat from infected patient. The virus was also cultivated with C6/36, HL-CZ, and LLC-MK2 cell cultures and reidentified by RT-PCR and specific immunofluorescence antibodies.

Discussion

Dengue virus infection is becoming an increasingly important etiology of fever in returning travelers from endemic areas especially in Southeast Asia [6]. As there are frequent air travels between Taiwan and other Southeast Asian countries, a sensitive and specific tool for rapid differential diagnosis is urgently required for the physician to insure prompt treatment if dengue hemorrhagic fever is suspected.

Although HI test was previously recommended as the gold standard of dengue serologic assay by World Health Organization (WHO) [28], there exist some disadvantages in its application. The required paired serum samples prevent the test from providing clinicians with rapid diagnosis. Furthermore, the collection of properly paired sera is often difficult, because patients without complications are discharged early from the hospital before the convalescent serum samples are obtained. In this case, we found that the HI titer was less than 10 in the acute stage. Unfortunately, we could not obtain a convalescent serum after patient discharged from our hospital. However, 1 year later, a serum sample obtained from the patient had HI titer of 80. In our previous study, more than 60% of the sera with low titers (1:10) had been detected when collected within the first 10 days of the onset of fever. Even in 19 isolates of dengue virus proven from HL-CZ cells culture, eight (42%) were from acute sera without detectable HI antibodies [25]. Furthermore, the HI is often complicated by the presence of cross-reactivity with other flavivirus and not type-specific [2,11].

Virus isolation in tissue culture, followed by immunofluorescent staining with DEN type-specific monoclonal antibodies, provides the definitive diagnosis, but the isolation rate has a wide range from 10.5% to 80% [7,29,30]. In this study, dengue virus had been isolated successfully from C6/36 cells, HL-CZ cells, and LLC-MK2 cells, and the cultured virus was identified as DEN-2 by the type-specific monoclonal antibody. However, the procedure was laborious and took many weeks to perform.

The development of a PCR-based assay, the RT-PCR method in particular, has improved the accuracy and speed in the diagnosis of dengue infection and epidemiological surveillance. It provides a sensitivity range of 80% to 100% for detecting dengue viruses in serum samples [12,13,15,31,32]. A few false-negative results were reported when compared with virus isolation [12,13]. Using blot hybridization of the DNA product from RT-PCR amplification of quantitated dengue virus RNAs, Lanciotti *et al* attained a sensitivity level of between 10^3 and 10^5 viral genome equivalents, and suggested that serum RT-PCR assay may yield false-negative results if $TCID_{50}$/mL is less than 10^2 [12]. It may explain the negative result for RT-PCR for our patient's serum.

Dengue virus is predominantly a lymphotropic agent. From animal study, it appears that the virus replicates in dermal histiocytes at the mosquito bite site initially, then spreads to macrophages in draining lymph nodes, spleen (macrophages), liver (Kupffer cells), bone marrow (mononuclear cells) and blood (monocytes) [33]. Using electron microscopy, Hase *et al* demonstrated the entry modes of JE and DEN-2 viruses into C6/36 cells [19]. The dengue virions are attached to the plasma of host cells by their envelope spikes, then, their envelopes overlap the host plasma membrane and dissolve the attachment sites. Finally, these virions penetrate into the cytoplasm through membrane disruptions created at the adsorption sites. As for the

entry of DEN-2 virus into human peripheral blood monocytes, the virions penetrate into the cytoplasm through plasma or macropinocytic vacuolar membrane in the same manner [19]. Comparing with bacterial and other viral infections, there was a high percentage (20-50%) of atypical blast transformed lymphocytes in the buffy coat from DHF patients [34]. Ultrastructural studies of DEN-2 infected lymphoblastoid Raji cells showed that the replication of the virus was confined to the cisternae of the rough endoplasmic reticula [35]. Hence, this replication pattern may produce more virus particles in buffy coat than those in serum samples.

In this study, serum RT-PCR was negative, but RT-PCR assay of the buffy coat yielded a positive result. Therefore, using buffy coat instead of serum sample for RT-PCR assay would provide an alternative choice in accurate diagnosis of dengue virus infection. Because of sample size limitation, a large controlled study for further assessment of this approach is required.

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