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Notas Científicas

Molecular diagnosis of dengue virus infections in samples collected on filter paper

Diagnóstico molecular de infecciones por virus Dengue en muestras colectadas en papel de filtro

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SUMMARY

Dengue virus infections (DENV) are a severe public health problem due to the high rates of morbidity and mortality involved, and the fact that no clinical treatment or vaccines are available. In order to strengthen the laboratory diagnosis for surveillance systems in tropical countries with low resources, we report an optimized method using filter paper for blood spotting and subsequent molecular diagnosis of DENV serotypes. Control strains of all serotypes, as well as 35 whole blood patient samples dispensed on filter paper, were stored at room temperature for as long as 36 months. RT-PCR of 5'UTR-C fragment was amplified through adapted protocols to diagnose all dengue serotypes. Results showed amplification for all four viral serotypes, including control viral strains and 88.6 % of the samples. These results allowed determining the utility of filter paper for the preservation of samples regularly obtained from patients with clinical suspicion of dengue in settings where low resources do not permit an immediate analysis of the samples. Likewise, this study evidence the possibility of molecular diagnosis of DENV from multiple areas of the world where there are no laboratories with the capacity to confirm DENV cases.

Key words: DENV, acute infection, filter paper, molecular diagnosis, RT-PCR.

RESUMEN

Las infecciones por virus Dengue (DENV) representan un grave problema de salud pública debido a las altas tasas de morbilidad y mortalidad que causan, además no cuentan con tratamiento clínico específico, ni vacuna. Con el fin de reforzar el diagnóstico de laboratorio para los sistemas de vigilancia epidemiológica en países tropicales con recursos económicos limitados, se optimizó una metodología utilizando papel de filtro para la recolección de muestras y el subsiguiente diagnóstico molecular de los serotipos de DENV. Se emplearon cepas controles correspondientes a todos los serotipos virales, así como 35 muestras de sangre total dispensadas en papel de filtro que fueron mantenidas a temperatura ambiente por 36 meses. Las muestras fueron analizadas mediante RT-PCR para la amplificación de la región del genoma correspondiente a 5´UTR-C de los DENV. Los resultados mostraron la amplificación de los mencionados fragmentos en 88,6% de las muestras analizadas, así como de las cepas controles. Estos resultados evidenciaron la utilidad del papel de filtro para conservación de muestras obtenidas de pacientes con sospecha clínica de dengue ubicados en zonas donde no es posible realizar análisis de laboratorio de forma inmediata, así como su uso para el diagnóstico molecular. De este modo se reforzaría la vigilancia en áreas, donde no hay laboratorios con capacidad para confirmar casos de DENV.

Palabras clave: *DENV, infección aguda, papel de filtro, diagnóstico molecular, RT-PCR.*

Dengue is the most important arbovirus disease of humans, in terms of morbidity and mortality (Gubler, D. J., 1998. *Clin. Microbiol. Rev.* **11:** 480-496). It is caused by either Dengue virus (DENV) serotypes (DENV-1, DENV-2, DENV-3, DENV-4) and transmitted to humans primarily by the vector Aedes aegypti. Before 2009, it was reported that the disease progresses in asymptomatic or symptomatic

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manner; as dengue fever (FD), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Later, it was established that the disease could be classified as severe and non severe dengue, which was classified into two groups according to the presence or absence of warning signs. The disease is endemic in more than 100 countries, with 50 million people infected each year and 500,000 severe cases reported (http://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf, accessed: December, 12, 2014). Since the decade of the 50s, the severe forms predominate in Asia. In America, specifically in the Caribbean and South America, cyclical epidemics FD, with sporadic cases of DHF (http://www.paho.org/Spanish/AD/DPC/CD/doc407.pdf, accessed: November, 10, 2014)) were presented. In 1981, Cuba had the first major epidemic in the region resulting in 158 deaths (Gubler & Clark, 1995. *Emerg. Infect. Dis.* **1:** 55-57; Guzmán *et al.*, 1999. *Rev. Cubana Med. Trop.* **51:** 5-13). Between 1989 and 1990, Venezuela experienced a second major epidemic and according to the official record it surpassed the 6000 DHF cases with 73 deaths (Pan American Health Organization, 1990. *Epidemiol. Bull.* **11:** 7-9). Since then, the country has high rates of the disease incidence, severe cases and deaths (http://www2.paho.org/hq/dmdocuments/2010/EGIDENGUE%20PARA%20LA%20SUBREGION%20ANDINA%2024_08.pdf, accessed: December, 10, 2014). From 2001-2007 the country experienced two DENV-3 epidemics (2001 and then 2005) (Schmidt *et al.*, 2011. *Infect. Genet. Evol.* **11:** 2011-2019) and co-circulation of DENV-1 was observed during these epidemics. During the epidemiological week 52 of 2009, the Ministry of Popular Power for Health (MPPS), reported a dengue cumulative incidence rate of 232/100,000 inhabitants. Among the federal agencies in alarm stage, was Lara state with 4984 and 1290 probable cases and hemorrhagic cases reported respectively. For the epidemiological week 52 of 2010, Lara state had overcome the alarm, but still reported cases and incidence rate for the country was 433.33/ 100,000 inhabitants, highlighting the emergency in relation to dengue (http://www.mpps.gob.ve/index.php?option=com_phocadownload&view=category&id=8:2009&Itemid=915 accessed December 14, 2014; http://www.mpps.gob.ve/index.php?option=com_phocadownload&view=category&id=9:2010&Itemid=915, accessed December 14, 2014). By 2014, during the eighth epidemiological week, Venezuela was the third country of the region with the highest number of reports, and the second with more severe cases, circulation of all the viral serotypes was confirmed (http://www.paho.org/hq/index.php?option=com_content&view=article&id=4494&Itemid=2481&lang=es, accessed December 14, 2014). Due to the epidemiological situation, affected countries have intensified surveillance, prevention and control active surveillance during interepidemic periods is based on early detection of dengue cases (Gubler, 2002. *Trends Microbiol.* **10:** 100-103.) and circulation of DENV in a community has been considered an indicator of future epidemics (Gubler., 1989. *Clin. Microbiol. Rev.* **11:** 480-496; http://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf, accessed: December, 12, 2014). However, detection of the viral serotypes is not always possible due to the absence of local laboratories with capacity for viral identification by virological and/or molecular methods. Furthermore, for the successful detection of DENV, it requires samples whose conditions of storage and transport are optimal, particularly if the location of patients is far from reference laboratories. For this, studies have been conducted which have shown that blood samples dispensed onto filter paper, could represent an interesting alternative for the diagnosis of DENV (Rigau-Perez *et al.*, 1998. Dengue and dengue haemorrhagic fever. *Lancet.* **352:** 971-977), conducting epidemiological screening studies involving many patients, the study of outbreaks in remote areas where conditions of storage and transport of samples are difficult, or for application in patients in whom the venipuncture process becomes difficult, such in children and elderly among others. In countries such as the French Guyana (Matheus *et al.*, 2007. *J. Clin. Microbiol.* **45:** 887-890), Brazil (Lima *et al.*, 2007. *Cad. Saude Publica.* **23:** 669-680), Paraguay (Matheus *et al.*, 2008. *Am. J. Trop. Med.* **79:** 685-687) and Vietnam (Anders *et al.*, 2012. *Am. J. Trop. Med. Hyg.* **87:** 165-170.) to name a few, analysis of samples absorbed on filter paper have allowed in some cases. The isolation of viral RNA, detection of NS1 protein (Matheus *et al.*, 2007. *J. Clin. Microbiol.* **45:** 887-890; Matheus *et al.*, 2012. *Am. J. Trop. Med. Hyg.* **86:** 159-165) or even genotyping by nucleotide sequencing of the envelope gene (Aubry *et al.*, 2012. *J Clin Virol.* **55:** 23-29) and the determination of IgM antibodies and / or IgG (Tran *et al.*, 2006. *BMC Infect. Dis.* **25:** 6-13; Lima *et al.*, 2007. *Cad. Saude Publica.* **23:** 669-680), which were detected without the rigors logistics and temperature required for

shipping, maintenance and transportation of fresh biological samples for viral diagnosis. The purpose of this study was to evaluate the use of the filter paper for collection, handling and transportation of blood samples and viral isolates from cell cultures for the molecular diagnosis of DENV through amplification of the 5'UTR-C fragment by Reverse Transcription coupled to the Polymerase Chain Reaction (RT-PCR) as a useful tool to strengthen epidemiological surveillance of dengue in Venezuela, as well as for the detection of outbreaks caused by any DENV serotypes.

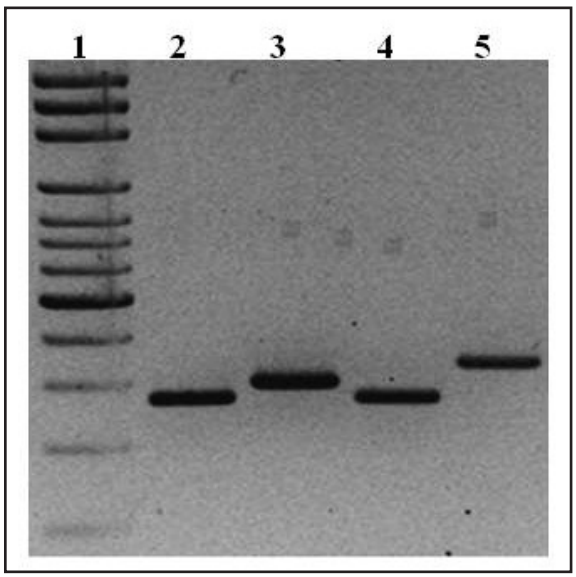
Four strains of DENV were used; 16007 (DENV-1), 16681 (DENV-2), H87 (DENV-3) and 8887 (DENV-4). These were cryopreserved at -70 ° C, after being amplified in the cell line C6 / 36-HT and were used to optimize the elution conditions of the viral particles absorbed on filter papers, prior to analyzing the stored samples of whole blood. For this, they were dispensed into cellulose filter paper No. 1 (Whatman Ltd.©), 200 µL of cell culture supernatant from each of the viral strains diagnosed by RT-PCR technique. Strains absorbed on this paper were allowed to dry at room temperature for two hours to finally place them within a microvial and conserved at room temperature until processing. This type of paper was used because of availability in the lab at the time it was possible to start the analysis of samples material. One hundred whole blood samples were collected from febrile patients with suspicion of infection with dengue virus, who presented symptoms compatible with all clinical forms of the disease, proceeding from various health care centers (public and private) in the cities of Barquisimeto and Cabudare, Lara state. Most patients who entered the study came from the service of immediate medical attention at the Pediatrics University Hospital "Dr. Agustín Zubillaga" (HUPAZ) of Barquisimeto. Each of the samples was accompanied by a personal informed consent or from a valid parent assessment, depending on the patient's age. Samples were collected into tubes containing anticoagulant (EDTA) by venipuncture procedure and transferred to the lab at UCLA within the hour. Once in the laboratory and upon mixing of the sample, we proceeded to dispense three drops of whole blood on filter paper Schleicher & Schuell (S & S) 903®. These were allowed to dry at room temperature and placed in groups of ten with paper separators between samples in plastic waterproof ziplock bags. In order to prevent disturbance of the sample due to humidity, the bags carried inside a moisture indicator and desiccants. Finally, the samples were stored at room temperature in the dark for a period of three years until the processing was possible. All whole blood samples that had been collected in tubes and dispensed onto filter paper were previously evaluated through RT-PCR, in their corresponding plasmas using Lanciotti's protocol (Lanciotti *et al.*, 1992 . *J. Clin. Microbiol.* **30:** 545-551), from which 70% were positive to DENV. Of these, due to the availability of resources, 35 positive samples were randomly selected for elution from the filter paper and subsequent molecular diagnosis of DENV (Lanciotti *et al.*, 1992. *J. Clin. Microbiol.* **30:** 545-551; Camacho *et al.*, 2012. Salus online. http://salus-online.fcs.uc.edu.ve/amplificaciongenomavirusdengue.pdf).

In order to optimize the elution conditions of the samples absorbed on filter paper, the control strains of the different serotypes were initially analyzed and subsequently the whole blood samples. For this, 200 µL of the lysis solution (0.5 M Tris-HCl pH 8, Triton X-100 0.5% 2 M guanidine isothiocyanate) were added to the three circles of paper inside of the microvials (150 uL) of each of the samples absorbed on filter paper, which were placed under constant stirring at 200 rpm for 2 h at 37ºC. After this time, it was processed by centrifuging the microvials at 14,000 rpm during 15 minutes at room temperature. From the supernatants obtained, viral RNA extractions were performed using the RNA extraction kit QIAGEN® QIAamp (QIAGEN Inc., CA, USA) following the manufacturer's instructions. The extraction products were diluted in 50 uL of nuclease-free water and stored at -70ºC. We proceeded to analyze the RNA corresponding to the cell cultures of viral strains, according to the methodology described (Camacho *et al.*, 2012. Salus online. http://salus-online.fcs.uc.edu.ve/amplificaciongenomavirusdengue.pdf). in order to obtain fragments corresponding to the starting region of the genome of DENV and part of the gene encoding the capsid (5'UTR-C). Briefly, the methodology consisted of adding 5 µL of RNA extracted in 45 µL of reaction mixture to RT-PCR [oligonucleotides (0.5 µM for DENV-1 and DENV-3, 1 µM for DENV-2 and 0.75 µM for DENV-4), MgCl2 (0.5 mM), deoxynucleotides (0.02 mM), Taq polymerase (1.25 U) and reverse transcriptase (2.5 U)] which was subject to the conditions of time and appropriate temperature in a thermocycler PTC-100™ (MJ Research, Inc.). After obtaining the

results and verified the amplification of viral RNA, we proceeded to the analysis of RNA obtained from whole blood samples absorbed on filter paper using same technique in both cases. The reaction products were analyzed on 2% agarose gels and were compared with the molecular marker (100 bp DNA Step Ladder, Axygen). The size expected for the products was 275 bp (DENV-1), 300 bp (DENV-2), 274 bp (DENV-3) and 339 bp (DENV-4). Statistical analysis was descriptive, the relative and absolute frequency of positive samples divided by the total strains and analyzed samples was calculated. Additionally, the frequency of positive results for each serotype was obtained.

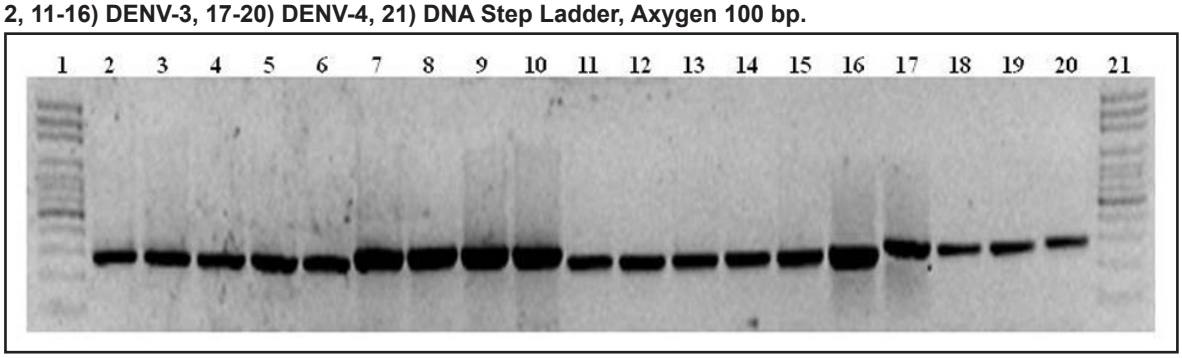
From the samples analyzed, 31/35 (88.6%) showed the same result as previously obtained when using the viral strains. The clustering of the samples by serotype allowed determination of 100% matching of results for DENV-1, DENV-2 and DENV-4, while in the case of DENV-3, 75% positivity was observed for the samples tested. The results from the analyzed samples have shown an absolute positivity of the DENV strains selected, demonstrating in principle, evidence of the possibility of obtaining RNA of DENV from the elution procedure used (Fig. 1). In this figure, the presence of a sharp band was observed in correspondence with expected molecular sizes according to the methodology (Camacho *et al.*, 2012. Salus online. http://salus-online.fcs.uc.edu.ve/amplificaciongenomavirusdengue.pdf). With the established working conditions, analysis of the 35 whole blood samples adsorbed on filter paper which were previously diagnosed for DENV was performed. In Fig. 2, the sharp bands are observed corresponding to some of the samples that were positive, in all cases, in correspondence with expected molecular size for each serotype.

Fig. 1. Agarose gel electrophoresis of the products of amplification of DENV 5'UTR-C fragment from RNA extracted from viral isolates.1) Step DNA Ladder, Axygen, 100bp. 2) DENV-1. 3) DENV-2. 4) DENV-3. 5) DENV-4.



The choice of technique for amplifying 5'UTR-C for the molecular diagnosis of DENV, was selected due to the fact that it is done in one step, resulting in cost and time savings. Likewise, specificity (Camacho *et al.*, 2012. Salus online. http://salus-online.fcs.uc.edu.ve/amplificaciongenomavirusdengue.pdf), analytical sensitivity (Reyes *et al.*, 2013. Segundo Congreso Venezolano de Ciencia, Tecnología e Innovación. Caracas, Venezuela) and agreement of this RT-PCR with others employed commonly in diagnosis have been demonstrated, which makes it a robust

Fig. 2. Agarose gel electrophoresis of the products of amplification of DENV 5'UTR-C fragment from RNA extracted from whole blood samples. 1) DNA Step Ladder, Axygen 100 bp. 2-6) DENV-1, 7-10) DENV-2, 11-16) DENV-3, 17-20) DENV-4, 21) DNA Step Ladder, Axygen 100 bp.



and reliable methodology (Camacho *et al.*, 2012. Salus online. http://salus-online.fcs.uc.edu.ve/amplificaciongenomavirusdengue.pdf).

As described, the surveillance systems have been implemented for early DENV outbreak detection, monitoring the introduction of new viral serotypes, and the distribution of viral circulation in endemic areas (Gubler & Costra-Velez., 1991. *Pan. Am. Health Organ.* 25: 237-247; Rigau-Perez and Gubler, 1997. Dengue and dengue hemorrhagic fever. Oxford: CABI International). The reason why, in some endemic populations diagnosis of infections with DENV is not timely done, obeys to the lack of specialized laboratories with ability to isolate DENV or detect its genome, in the place of implementation of the study. In other cases, there are limitations in obtaining resources for maintenance and transport of samples in adequate cooling conditions to reach the specialized laboratories (Matheus *et al.*, 2008. *Am. J. Trop. Med.* **79:** 685-687). In order to solve the situation that represents the absence of specialized laboratories, some authors have conducted studies to determine antibodies (IgM and / or IgG) against DENV (Tran *et al.*, 2006.. *BMC Infect. Dis*. **25:** 6-13; Lima *et al.*, 2007. *Cad. Saude Publica.* **23:** 669-680), as well as, for the detection of infectious indicators such as the antigen NS1 and / or viral genomes using samples absorbed on filter paper by using various methods. In their works, and in this report, analyzed blood samples were previously collected on filter paper. The eluate obtained in all cases, worked as a source of anti DENV antibodies, viral RNA, and viable viral particles DENV (Matheus *et al.*, 2008. *Am. J. Trop. Med.* **79:** 685-687; Matheus *et al.*, 2012. *Am. J. Trop. Med. Hyg.* **86:** 159-165; Aubry *et al.*, 2012. *J Clin Virol.* **55:** 23-29). The results from this report open the possibility of studying outbreaks caused by DENV and evaluation of large populations from samples collected on filter paper without the constraints of maintenance and transport conventionally employed. Likewise, in this study, the specific molecular detection of DENV acute phase samples and the stability of viral RNA in whole blood samples absorbed on filter paper were demonstrated. Such fact is corroborated by positivity obtained (88.6%) in the samples evaluated, despite the storage time and temperature of these, which reiterates the usefulness of filter paper to the conservation of viral RNA under the conditions laid down in this study, where the quality of the elution procedure was observed, as well as the robustness of the RT-PCR employed. Importantly, the only samples which did not correspond 100% with the previous results were those for DENV-3, which were striking to us, considering the value of analytical sensitivity reported for the technique in use, the ability to detect even a viral particle of DENV-3 has been demonstrated (Reyes *et al.*, 2013. Segundo Congreso Venezolano de Ciencia, Tecnología e Innovación. Caracas, Venezuela). The result obtained for DENV-3 may suggest that the lack of absolute coincidence, could be associated with the loss of quality in some of the samples of whole blood absorbed on filter paper, perhaps due to a long storage and the temperature at which were maintained (≈26 ° C), or to deterioration of some samples by failure of the humidity control system employed, only on the bags which held those particular samples.

As described by other authors (Matheus *et al.*, 2008. *Am. J. Trop. Med.* 79: 685-687; Matheus *et al.*, 2012. *Am. J. Trop. Med. Hyg.* 86: 159-165), this work evidenced the value of using venous blood samples collected on filter paper for molecular diagnosis of DENV infections, due to the ease of collection, maintenance and transportation compared to conventional practices. Finally, although this study analyzed few samples, it represents the first report carried out in the country about the use of filter paper for the collection and preservation of blood samples, under the nontraditional conditions described, for DENV RNA amplification.

Our results confirmed previous observations indicating that viral particles remain in the filter paper, which in this case, confirmed the detection of virus at the molecular level, but that may well be employed for the detection of other indicators of viral infection, such as NS1 antigen (Matheus *et al.*, 2007. *J. Clin. Microbiol.* **45:** 887-890; Matheus *et al.*, 2012. *Am. J. Trop. Med. Hyg.* **86:** 159-165) in febrile patients samples and IgM and / or IgG (Lima *et al.*, 2007. *Cad. Saude Publica.* **23:** 669-680). It could also be used to detect virus in epidemiological studies of outbreaks; studies with retrospective and/or prospective designs, allowing time to obtain economical resources while it could be stored and decisively in routine analyzes performed by epidemiological surveillance systems in the country, considering the benefits that this methodology represents for the control and prevention of DENV infections in the community, regardless of their location.

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Ethical approval

The Ethical committee of the BIOMED of Carabobo University gave approval for this study. The Bioethical Committee from HUPAZ, which gave approval for the study, as well.

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