



The use of serum spotted onto filter paper for diagnosing and monitoring Chikungunya virus infection



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ABSTRACT

Background: The recent emergence of Chikungunya Virus (CHIKV) in the Americas constitutes a major public health problem on this continent, where the mosquito vector is widespread. The rapid diagnosis of suspected cases is essential for the monitoring and control of this ongoing outbreak. However, this requires reliable tools that are difficult to establish in areas without specialized laboratories.

Objectives: The aim was to evaluate the performances of serum samples spotted onto filter paper for molecular and serological diagnosis of Chikungunya infection.

Study design: Analyses were performed from frozen sera and serum spotted onto filter paper provided from 121 Chikungunya suspected cases collected at a biological laboratory on Saint-Martin Island.

Results: This approach performed well in comparisons with standard methods, with a sensitivity of 100% and a specificity of 93.6% for the combined technical approaches (RT-PCR and serological results). Comparisons of serum samples spotted onto filter paper and frozen samples showed a concordance rate of 94.8% in molecular tests and 98.2% in serological tests.

Conclusions: This simple sampling technique could overcome the problems of the lack of efficient CHIKV diagnosis tools in remote regions, providing good results regardless of the molecular or serological approach used. This simple filter paper-based method can be used to diagnose both chikungunya and dengue infections, as previously demonstrated following transport at ambient temperature to specialized laboratories. Given the set-up costs and high performance of this method, it could be recommended for the monitoring and control of Chikungunya virus expansion in the Americas and in other affected regions.

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1. Background

Chikungunya is a mosquito-borne disease caused by an Alphavirus from the *Togaviridae* family. The virus is transmitted by the bite of *Aedes* mosquitoes, including *Aedes aegypti* and *Aedes albopictus* [1]. The disease is typically an acute illness characterized by sudden-onset fever, rash, and incapacitating arthralgia, which may persist for weeks, months or years after the acute phase of infection [2]. No licensed vaccine or specific treatment for Chikun-

gunya infection is currently available. The only effective mean of prevention is to protect individuals against mosquito bites.

Chikungunya has spread across Africa, South Asia, and the Indian Subcontinent. In 2005, Chikungunya Virus (CHIKV) emerged in the Indian Ocean region and caused major epidemics, on Reunion Island in particular, where it affected about 300,000 inhabitants [3]. In December 2013, the circulation of CHIKV was reported for the first time in French part of Saint-Martin Island in the Caribbean region [4,5]. Investigations of the first confirmed cases revealed that the virus concerned was of the Asian genotype [6]. The emergence of CHIKV on Saint-Martin was rapidly followed by outbreaks on other neighboring islands, such as Saint-Barthelemy, Martinique, Guadeloupe, and the British Virgin Islands, and a large outbreak is now considered to be underway in the Americas [7,8]. As of June 2015, the World Health Organization reported that there had been more than 1 million suspected cases and around 30,000 confirmed CHIKV

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cases in the Americas [8]. This rapid expansion constitutes a major problem for public health services in the Americas, because the mosquito vector is present in a wide geographic zone extending from North America to Brazil [9]. In the absence of an effective vaccine or specific treatment, the rapid diagnosis of suspected CHIKV cases is important, for monitoring and controlling this expansion, particularly in regions not yet affected, and to distinguish between CHIKV and dengue virus infections, dengue virus being another endemic arbovirus transmitted by the same vector in the Americas. The following strategy is used for CHIKV diagnosis: for serum collected during the first seven days after the onset of symptoms, real-time reverse transcription-polymerase chain reaction (RT-PCR) is used to detect the viral genome; for serum collected on or after day 5, serologic techniques are used to detect IgM and IgG responses to the virus [10]. Molecular diagnosis is sensitive and specific, but requires specialized technical support and equipment that are difficult to obtain in remote areas. Moreover, commercially available serologic diagnostic tests for CHIKV have been shown to have a low sensitivity and specificity [11]. All these difficulties hamper the diagnosis, monitoring and control of CHIKV infections in the Americas and other tropical regions in which this virus is circulating.

We have already shown that dried blood spotted onto filter paper is a useful alternative to frozen samples for the diagnosis of dengue infection in patients living in low-income countries [12,13]. Other studies have also demonstrated that this approach is suitable for the molecular and serological diagnosis of bacterial and parasitic diseases [14,15].

2. Objectives

To facilitate testing and to limit the costs of transporting samples for CHIKV testing, in the cold, to specialized laboratories, we evaluated the use of serum spotted onto filter paper for the molecular and serological diagnosis of CHIKV.

3. Study design

3.1. Specimen collection

Due to the lack of local specialized laboratories able to diagnose CHIKV infection in Saint-Martin, a biological laboratory located in the French part of the island organized, in collaboration with the Regional Health Agency, the transport of all samples from patients with suspected CHIKV to the French National Reference Center (FNRC) for Arboviruses in Marseille, France (Marseille-Lab) for biological investigations [16]. Molecular and serological diagnosis techniques were used; depending on the time elapsed between the onset of symptoms and sample collection for each patient [10]. In parallel to the shipment of frozen serum to Marseille-Lab, serum samples spotted onto filter paper (Whatman 3 M; Scheilcher & Schuell, Germany) were also obtained for 121 patients with suspected CHIKV infection. Briefly, venous blood samples were collected from these 121 patients. They were rapidly centrifuged and the resulting serum sample was divided in two. One portion of the sample was stored at -20°C until shipment to Marseille-Lab and the other was spotted onto filter paper (three spots of $20\ \mu\text{L}$). These serum samples spotted onto filter paper were placed in individual, labeled and hermetic plastic bag, respecting the shipping guidelines for dried blood spots [17]. These samples were sent by post at ambient temperature ($20\text{--}25^{\circ}\text{C}$ and $85\text{--}90\%$ relative humidity) to the French National Reference Center for Arboviruses in Cayenne (Cayenne-Lab). After, an average delay of reception of 10 days, these samples were stored at -80°C during 8 days before the beginning of molecular and serological analyses. For each patient: one of

the spots was used for molecular diagnosis, another for “in house” serological analysis, and the final spot was retained as a potential control. In accordance with the information provided by the patients (date of onset of disease and date of sample collection), the two laboratories analyzed 61 samples by real-time RT-PCR, 25 by immune-capture method and 35 by both techniques.

3.2. Molecular and serological analyses performed by Marseille-Lab

For molecular diagnosis, RNA was extracted from $140\ \mu\text{L}$ of serum with the QIAamp[®] Viral RNA kit, as recommended by the manufacturer’s protocol (Qiagen, Hilden Germany), and the CHIKV genome was detected by real-time RT-PCR targeting the E1 gene, as previously described [18]. For IgM detection, we used an “in-house” IgM antibody-capture enzyme-linked immunoabsorbent assay (MAC-ELISA), with precipitated and inactivated virus produced in cell culture as the antigen, as previously described [19].

3.3. Molecular and serological analyses performed by Cayenne-Lab

For each patient tested, two spots of serum spotted onto filter paper (SFP) were cut out and placed in two independent $1.8\ \text{mL}$ sterile tubes for molecular and serological analyses.

For the molecular investigation, viral RNA was extracted from the serum spot on filter paper for each patient and from $10\ \mu\text{L}$ of serum from a CHIKV-positive control. RNA was extracted with the QIAamp[®] Viral RNA kit (Qiagen, Hilden Germany). Each sterile tube containing a cut serum spot from a patient ($20\ \mu\text{L}$) and those containing control samples were incubated in $560\ \mu\text{L}$ of AVL buffer for 15 min for lysis. We added $560\ \mu\text{L}$ of ethanol (96–100%) and mixed by pulse-vortexing for 15 s. We then loaded $630\ \mu\text{L}$ of the solution obtained on a QIAamp Mini column, which we centrifuged at $6000 \times g$ for 1 min. The following steps of the extraction procedure were then carried out as described by the manufacturer (Qiagen, Hilden Germany). Finally, all 96 extracted RNA samples were analyzed by real-time RT-PCR targeting the NSP1 gene of CHIKV, as described by Panning et al. [20].

Similarly, 60 SFP samples were analyzed for CHIK IgM antibodies by antibody capture MAC-ELISA, with an “in-house” protocol. Sterile tubes containing one cut spot ($20\ \mu\text{L}$) were incubated for 2 h in $600\ \mu\text{L}$ of phosphate-buffered saline (PBS) 1X supplemented with 0.5% Tween 20 and 5% non-fat dried milk (PBS-T NDM). Each eluate obtained was used in the MAC-ELISA test, as described by Talarmin et al. [21]. In this serological method, the CHIKV antigens used were derived from a suckling mouse brain preparation.

3.4. Statistical analysis

The results of the molecular and serological assays performed with SFP were compared to those obtained with frozen serum, which was considered to be the gold standard. The sensitivity and specificity of the assays were determined with two-by-two tables, as widely used in the evaluation of diagnostic methods.

4. Results

During December 2013, 121 samples from patients with suspected CHIK from Saint-Martin Island were used to evaluate the performance of Chikungunya infection diagnosis from serum spotted onto filter paper, by molecular and/or serological techniques. The patients had presented at the biological laboratory of Saint-Martin with fever, headache, myalgia and severe joint pain. The Marseille-Lab used thawed frozen serum samples and Cayenne-Lab

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