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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.

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 Chikungunya 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 μL). 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 (20–25 °C and 85–90% 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 μL 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 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 μL 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 μL) and those containing control samples were incubated in 560 μL of AVL buffer for 15 min for lysis. We added 560 μL of ethanol (96–100%) and mixed by pulse-vortexing for 15 s. We then loaded 630 μL of the solution obtained on a QIAamp Mini column, which we centrifuged at 6000 × *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 μL) were incubated for 2 h in 600 μL 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

Table 1
Overall sensitivity and specificity of tests performed on SFP specimens for the diagnosis of CHIKV infection.

Results with serum on filter paper	Results of standard methods with serum samples[a]		
	Positive	Negative	Total
Positive	55	4	59
Negative	0	59	59
Equivocal	1	2	3
Total	56	65	121

[a] RT-PCR or serology.

Table 2
Sensitivity and specificity of real-time RT-PCR diagnostic tests for CHIKV.

Results with serum on filter paper	Results of standard methods with serum samples		
	Positive	Negative	Total
Positive	38	2	40
Negative	3	53	56
Total	41	55	96

used dried serum spots on filter paper from the same corresponding patient and collection time, for all PCR and serology testing performed. Each laboratory used its own techniques for molecular and serological diagnosis. The participation of the two laboratories at a European quality control of CHIKV has previously shown the excellent concordant results between the two laboratories (data not shown). According to the time interval between disease onset and sample collection, 96 samples collected in the first seven days of the disease were tested by real-time RT-PCR, and 60 samples collected from day 5 onwards were tested by MAC-ELISA.

The Marseille-Lab reported that 56 of the 121 samples tested positive for CHIKV by at least one technique (real-time RT-PCR and/or serology), whereas 65 samples tested negative by both techniques. At the Cayenne-Lab, 59 samples tested positive and 59 tested negative. The other three samples were considered equivocal; 2.5% of the results were therefore inconclusive (Table 1). The concordance rate between the two laboratories was 96.6% (114/118). The overall sensitivity and specificity of this approach were 100% (55/55) and 93.6% (59/63; confidence interval [CI]: 0.87–0.99), respectively, with respect to the gold standard method.

4.1. Comparison of the molecular results obtained with frozen serum samples and SFP

In total, 96 patients were tested for Chikungunya infection by real-time RT-PCR: 38 tested positive and 53 tested negative, regardless of the nature of the biological material tested (Table 2). Frozen serum and SFP gave identical results for 91 patients (94.8%–91/96) and discordant results for the other five samples (5.2%–5/96). Three of the patients with discordant results tested positive with frozen serum samples and negative with the corresponding SFP. However, serological tests gave positive results for two of these three patients. The other two patients with discordant results tested negative with frozen serum and positive with filter paper. These samples were collected on days 0 and 1 of the disease and were therefore not investigated by serological techniques. The kappa coefficient was 0.89 (95% CI: 0.80–0.98). The sensitivity and specificity of molecular Chikungunya diagnosis from SFP were 92.6% (38/41, 95% CI: 0.84–1.00) and 96.3% (53/55, 95% CI: 0.91–1.00), respectively.

Table 3
Sensitivity and specificity of serological tests for the diagnosis of CHIKV infection.

IgM detection with serum on filter paper	Results of standard methods with serum samples		
	Positive	Negative	Total
Positive	15	1	16
Negative	0	42	42
Equivocal	1	1	2
Total	16	44	60

4.2. Comparison of the serological results obtained with frozen serum samples and SFP

As for molecular analyses, each FNCR used its specific "in-house" serological protocol. Equivocal results were obtained for only two of the 60 patients investigated by MAC-ELISA, corresponding to 3.3% inconclusive results (2/60) (Table 3). Inconclusive results aside, the results obtained with SFP and frozen serum samples were concordant for 57 of 58 (98.2%) samples and discordant for only one sample collected on day 5 after the onset of symptoms. This sample, which tested positive for IgM at the Cayenne-Lab and negative at the Marseille-Lab, gave a positive result in real-time RT-PCR performed at the Marseille-Lab. The kappa coefficient was 0.97 (95% CI: 0.93–1.00). The sensitivity and specificity of Chikungunya MAC-ELISA performed on SFP were 100% (15/15) and 97.6% (42/43; 95% CI: 0.93–1.00), respectively. Due to recent cases of Dengue in Saint-Martin, the two FNRCA laboratories also tested for the possible presence of dengue IgM antibodies in the 60 patients from whom samples were collected after day 5 of the disease. These investigations led to the detection of dengue IgM in 15 samples for which CHIKV infection had been ruled out (data not shown). These results were concordant for the two FNCR laboratories, regardless of the type of biological support and technique used.

5. Discussion

Since the emergence of CHIKV in the Americas, a number of outbreaks have been reported in some of the countries of this continent. Efficient diagnostic tools suitable for the monitoring and control of this arboviral disease in remote areas are lacking. The aim of this study was, therefore, to evaluate the performance of SFP for the diagnosis of CHIKV infection.

The overall sensitivity and specificity, for the combined technical approaches used on SFP, were 100% and 93.6%, respectively. The concordance rates between the two FNCR laboratories regardless of the biological material (SFP vs. frozen serum samples) and technical protocol used were 94.8% for molecular diagnosis and 98.2% for serological diagnosis. The Kappa coefficients were 0.89 and 0.97, respectively. The good concordance obtained by the two laboratories in the framework of a CHIKV Quality-Control organized by an European organization can ruled out the variability linked at the different technical protocols used by the two laboratories. The lowest concordance observed in particular for molecular diagnosis in comparison to serological diagnosis could be explained by the fact that the volume of specimen used for RNA extraction was different. The condition of transportation over ambient temperature could also have an impact on the concordance, due to the fragility of nucleic acids compared to antibodies.

Andriamandiny et al., who evaluated the usefulness of capillary samples applied to filter paper for the molecular detection of CHIKV reported similar results, with a concordance rate of 93.9% and a kappa coefficient of 0.87 [22]. Overall, these findings indicate that the collection of samples on filter paper is suitable for the molecular

diagnosis of CHIKV, regardless of the nature of the sample collected: serum or capillary blood samples.

Serological investigations for the 60 patients with suspected CHIKV infection also performed well, with a sensitivity of 100% and a specificity of 97.6%. However, as sensitivity was estimated with only a small number of samples, these results must be interpreted with caution. These performances and the concordant results obtained in this evaluation suggest that serological investigations are more efficient than molecular investigations using this approach. As advanced above, the better stability of the antibodies spotted in to filter paper compared to nucleic acids could explain this difference.

Overall, these results demonstrate that filter paper is a convenient format for the virological and serological diagnosis of CHIKV infection, particularly in regions lacking specialized laboratories capable of performing the biological investigations required. Indeed, this approach makes it possible to overcome the requirement for cold storage during transport, as already shown for other arboviruses [23–25]. This approach is thus cheaper than the shipment of frozen samples. In the epidemiological context of the Americas, where the Dengue and Chikungunya viruses are currently cocirculating, the simple collection of samples from patients with suspected arbovirus infections on filter paper could make it possible to identify the virus involved in the infection, and to enhance diagnosis and surveillance processes. The recent reports of Zika cases in Brazil also provide an opportunity to extend this approach to the diagnosis of a wider range of arboviruses [26,27].

Conflicts of interest

None.

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Competing interests

None declared.

Ethical approval

Not required.

Author contributions

All authors made substantial contributions to the following: the conception and design of the study (S.M., P.H., I.L.G.), data acquisition, analysis or interpretation (S.M., B.L., L.B., A.E., O.M., O.F., D.M., I.L.G.), the drafting of the article or its critical revision for important intellectual content (S.M., D.R., I.L.G.).

All the authors have approved the final article.

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