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Development of a TaqMan® RT-PCR assay without RNA extraction step for the detection and quantification of African Chikungunya viruses

Boris Pastorino[a,b,*], Maël Bessaud[a,b], Marc Grandadam[a,b], Severine Murri[c], Hugues J. Tolou[a,b], Christophe N. Peyrefitte[a,b]

[a] *Unité de virologie tropicale, Laboratoire associé au Centre national de référence pour les arbovirus, Institut de médecine tropicale du service de santé des armées, BP 46, Parc du Pharo, 13998 Marseille Armées, France*
[b] *Université de la Méditerranée, EA 3292, IFR 48 Marseille Cedex, France*
[c] *Institut Pasteur, Centre national de référence pour les arbovirus, 21 Avenue Tony Garnier, 69365 Lyon Cedex 07, France*

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Abstract

Chikungunya virus (CHIKV), a member of the alphavirus genus, is of considerable public health concern in Southeast Asian and African countries. However, despite serological evidence, the diagnosis of this arthropod-borne human disease is confirmed infrequently and needs to be improved. In fact, illness caused by CHIKV can be confused with diseases such as dengue or yellow fever, based on the similarity of the symptoms, and laboratory confirmation of suspected cases is required to launch control measures during an epidemic. Moreover, no quantitative molecular tool is described to study CHIKV replication or detection in clinical samples and cell culture supernatants. In this study, a specific and sensitive CHIKV one-step TaqMan® RT-PCR assay was developed as a tool for the diagnosis of African CHIKV as well as a rapid indicator of active infection by quantifying viral load. This study also showed that a simple heat viral RNA release during the reverse transcription step constituted an alternative to the conventional RNA extraction method.

Keywords: Alphavirus; Chikungunya virus; Heat-release; Direct one-step TaqMan® RT-PCR

1. Introduction

Chikungunya virus (CHIKV) is an arthropod-borne alphavirus disseminated widely throughout Africa (Saluzzo et al., 1983; Muyembe-Tamfum et al., 2003), South-East Asia, Western Pacific and India (Lam et al., 2001). CHIKV is single strand positive RNA enveloped virus member of Togaviridae family and is transmitted from primates to humans generally by *Aedes aegypti* but also various aedine mosquitoes species (Yadav et al., 2003). Molecular epidemiological studies have shown that CHIKV clustered into three major distinct lineages, Asian, Central East and West African (Powers et al., 2000) and the virus originated probably in tropical Africa then imported into southern Asia. CHIKV illness has an important economic impact in many tropical countries, and due to the lack of specific symptoms, this infection cannot be differentiate from diseases such as dengue or yellow fever. CHIKV is classified serologically as a member of the Semliki Forest antigenic complex (Karabatsos, 1975) closely related to O'nyong-nyong virus (ONNV) because of its cross-reactivity. Detection of most alphaviruses is dependent currently on virus isolation from the blood of viremic patients, infected tissues or blood-feeding arthropods which process is time-consuming. The alphavirus species can be characterized by hemagglutination inhibition, complement fixation and neutralization of viral infectivity using reference sera (Calisher et al., 1988). For genus-specific detection of alphaviruses, an ELISA has been established (Greiser-Wilke et al., 1991) as well as sensitive RT-PCR or nested RT-PCR

* Corresponding author. Tel.: +33 4 91 15 01 54; fax: +33 4 91 15 01 72.
E-mail address: publi.viro@laposte.net (B. Pastorino).

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assays (Pfeffer et al., 1997; Powers et al., 2000; Paz Sanchez-Seco et al., 2001; Hasebe et al., 2002; Pfeffer et al., 2002; Bronzoni et al., 2004). Although CHIKV could be considered as a re-emerging threat, a few specific serological or molecular diagnosis tools are available. To date, only conventional RT-PCR methods have been suggested for the study of CHIKV replication in supernatants, clinical samples or for epidemiological survey. The aim of this study was to develop a rapid, sensitive and specific real-time RT-PCR method to detect and quantify African CHIKV in serum samples and infected cell supernatants. In a second step, this tool was optimised for direct viral RNA detection in small volumes without a previous RNA extraction or purification step (Thompson and Dietzgen, 1995; Klebe et al., 1996), in order to reduce sample processing, time and cost.

2. Materials and methods

2.1. One-step real-time RT-PCR development

2.1.1. Virus preparation, titration and RNA extraction

Sindbis virus (SINV), Semliki Forest virus (SFV), Mayaro virus (MAYV), CHIKV strains 1720, 1728 and RCA (Central African genotypes with respective GenBank accession numbers AY549580, AY549582 and AY549584), Dengue virus (DENV), Yellow fever virus (YFV) were isolated in our laboratory. ONNV and CHIKV strain 37997 (West African genotype, GenBank accession number AF192892) were respectively kindly provided by Prof. H. Zeller (Institut Pasteur, Lyon, France) and the Institut Pasteur of Dakar.

All work with infectious virus was carried out in a biosafety-level 3 laboratory. The batches of viral inocula used in this study were prepared by two passages in *Aedes albopictus* cells (C6/36, ATCC clone CRL 1660) grown at 27 °C in Leibowitz's L15 medium (Biowhittaker, Verniers, Belgium) supplemented with 1% final L-glutamin (Biowhittaker), 5% final foetal calf serum (FCS) (Biowhittaker) and 2% final tryptose phosphate broth (Eurobio, Les Ulis, France). Briefly, cells were incubated for 1 h at 27 °C in the presence of the initial viral isolate at a multiplicity of infection (MOI) of 1 in complete Leibowitz's medium without FCS. Foetal calf serum was then added to a final concentration of 5% and cells were maintained infected for 5 days. Cell culture supernatants were collected and titrated by reduction plaque assay on Vero cells (E6 clone kindly provided by Dr. M. Bouloy, Institut Pasteur, Paris) seeded in flat-bottom 96-well plates, according to the dilution method reported previously (Luria et al., 1978). Viral titers (viral infectious doses or ID per ml) were estimated by solving the equation of maximum likelihood (Kleczkowski, 1968). Alphavirus and flavivirus RNAs were extracted from 200 μl of cell supernatants using the High Pure Viral RNA kit (Roche Diagnostics, Meylan, France) following the manufacturor's instructions.

2.1.2. CHIKV primers and TaqMan® probe

Sequences retrieved from the GenBank database (Fig. 1) were used to design primers and probe. CHIKV conserved regions were identified by generating multiple alignment

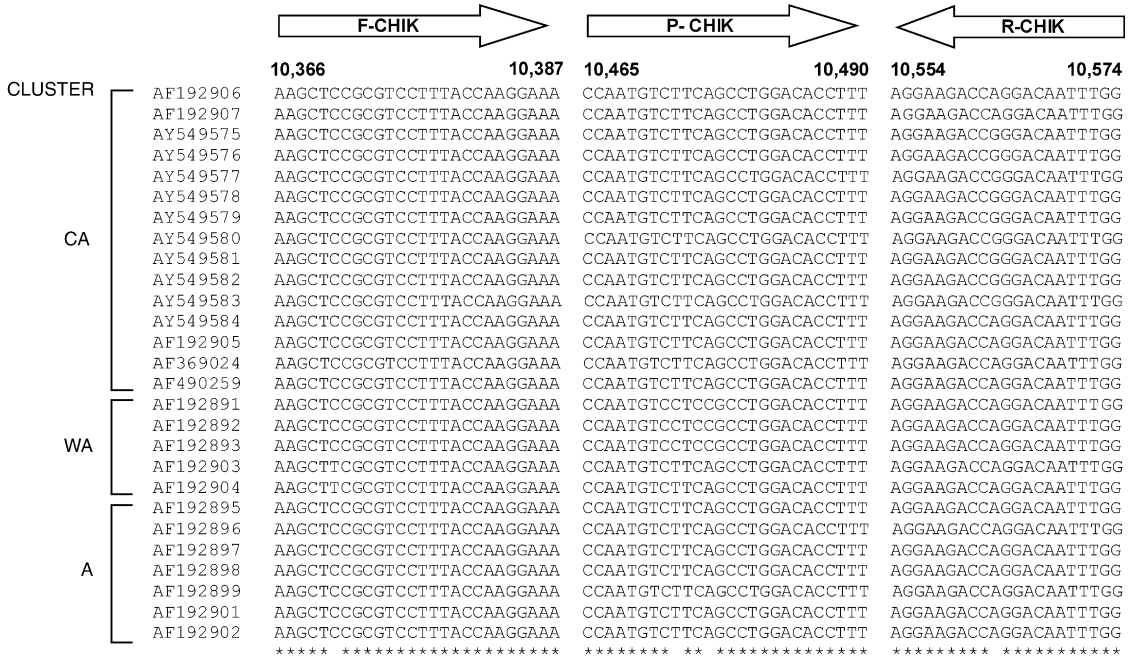

Fig. 1. Multiple alignment of the E1-target regions (genome position 10366–10574 of Ross CHIKV reference strain). Asterisks (*) indicate conserved nucleotides. GenBank database accession numbers of representative sequences are specified on the left. The CHIKV strains used for sequence alignments belong respectively to the Central Africa (CA), West Africa (WA) or Asia (A) clusters.

Table 1
Primers and probe used for real-time amplification of CHIKV RNA

Name	5′ → 3′ sequence	Genome position[a]
R-CHIK	CCAAATTGTCCYGGTCTTCCT	10554–10574
F-CHIK	AAGCTYCGCGTCCTTTACCAAG	10366–10387
P-CHIK	[b]CCAATGTCYTCMGCCTGGACACCTTT[c]	10465–10490

[a] According to the CHIKV Ross strain sequence.
[b] Reporter dye (FAM; 6-carboxyfluorescein) labelled nucleotide.
[c] Quencher dye (TAMRA; 6-carboxy-tetramethyl-rhodamine) labelled nucleotide.

sequence using Clustal W1.7 software (Thompson et al., 1994). Primers and probe (Table 1) were selected in the E1 structural protein region according to specifications needed for TaqMan® detection system (Bustin, 2000). Primers were synthesized by standard phosphoramidite chemistry in our laboratory (3400 DNA Synthesizer, Applied Biosystems, Courtaboeuf, France). The TaqMan® probe (Eurogentec S.A., Herstal, France) was labelled at the 5′-end with the reporter molecule 6-carboxy-fluorescein (FAM) and at the 3′-end with the quencher 6-carboxytetrametyl-rhodamine (TAM).

2.1.3. Generation of CHIKV RNA synthetic transcript

A PCR product encompassing the targeted region was prepared using the CHIKV strain 1728 and cloned into the T7 polymerase expression vector pGEMT-easy (Promega, Lyon, France). The complete insert including the T7 promoter was amplified with vector-specific primers. The PCR product was gel purified (Qiaquick, Qiagen, Courtaboeuf, France) and in vitro transcribed using the RT-PCR Competitor Construction Kit (Ambion, Huntingdon, UK). Residual DNA has been eliminated by mean of several DNAse treatments. The RNA was then purified with Rneasy columns (Qiagen) and quantified in a UV spectrophotometer (Eppendorf, Hamburg, Germany).

2.1.4. PCR conditions

The one-step RT-PCR were performed in a final volume of 20 μl containing 2.5 μl of extracted RNA, 10 μl of 2× Thermoscript Reaction Mix buffer (Invitrogen SARL, Cergy Pontoise, France), 2 pmol of the TaqMan® probe, 9 pmol of each primers, 8 U of Rnase Inhibitor (Rnasin, Promega) and 0.8 μl of the Thermoscript Plus/Platinium *Taq* Enzyme Mix (Invitrogen). Real-time RT-PCR assays were carried out in a LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) with the following steps: reverse transcription at 50 °C for 20 min, initial denaturation at 95 °C for 2 min and 45 cycles with 95 °C for 5 s, 60 °C for 1 min.

2.1.5. Sensitivity, specificity and reproductibility of the CHIKV TaqMan® assays

The analytical sensitivity and reproductibility of the real-time TaqMan® RT-PCR was determined using a 10-fold dilutions of in vitro transcribed CHIKV strain 1728 RNA (2.7×10^1 to 2.7×10^8 molecules per reaction) in triplicate for both intra and inter-assays. In addition, viral RNAs from a titrated supernatant were extracted, serial diluted (10^{-1} to 10^{-7}) and tested in triplicate. Specificity of the CHIKV assay was evaluated by using infected cell supernatants with CHIKV strains 1720, RCA and 37997, SINV, SFV, MAYV, ONNV, DENV and YFV.

2.2. Evaluation of the CHIKV real-time RT-PCR assay without RNA extraction step

2.2.1. Reaction composition

Samples (2.5 μl) were incorporated directly into the reaction mixture without a previous RNA extraction step. Then, real-time RT-PCR was carried out as described in Section 2.1.4.

2.2.2. Thermal RNA-release effect

The same CHIKV strain 1728 supernatant was heating for 5, 20, 60 min at 50, 60, 70, 80 and 90 °C. Then, each sample was tested in duplicate with the direct CHIKV TaqMan® assay. The viral titer was also determined to evaluate the effect of the heat-treatment. Results were compared including non-heated CHIKV supernatants.

2.2.3. Sensitivity and reproductibility of the assay

Cell culture supernatants were diluted in human serum or RNAse-free H_2O and tested in triplicate with the CHIKV TaqMan® assay. The comparison between CHIKV real-time RT-PCR with or without an RNA extraction step was also evaluated as well as the detection limit of the direct assay (ID/reaction). CHIKV RNAs were extracted from 200 μl of supernatant as described previously. In order to compare values with the direct CHIKV TaqMan® detection assay, it was assumed that the yield of RNA recovery was 100%.

3. Results

3.1. TaqMan® RT-PCR development

3.1.1. Real-time RT-PCR detection

The quality of the synthetic CHIKV strain 1728 RNA transcript used for the development of the TaqMan® assay was estimated by different controls (data not shown): first the in vitro transcribed PCR product was sequenced; second the size and the purity of the RNA transcript was controlled by gel electrophoresis; third, direct amplification of the synthetic RNA with the Platinium Taq polymerase showed no DNA contamination. In pilot experiments, the Platinium Quantitative RT-PCR Thermoscript One-Step System (Invitrogen) was found to be more sensitive and robust than two other enzyme combinations tested in our laboratory for real-time RT-PCR detection of RNA viruses: the LightCycler-RNA Master Hybridization Probes kit (Roche) and the EZ-rTth RT-PCR system (Perkin-Elmer, Courtaboeuf, France).

The primers and probe were selected to potentially detect CHIKV isolates from Asia and Africa (Fig. 1) and optimal RT-PCR conditions were determined by the comparison of the Ct values obtained with CHIKV strain 1728 synthetic RNA transcript. Parameters such as time and temperature for each cycling step, as well as primers and probe concentrations in the reaction, were evaluated leading to the optimised CHIKV TaqMan® assay described in Section 2.1.4.

3.1.2. Analytical sensitivity and specificity

The results of the specificity and sensitivity experiments are summarized in Table 2. The correlation coefficient of the standard curve for CHIKV RNA transcript ranged from 0.96 to 1. All RT-PCR reaction components, with the exception of template RNA, were included in each run and no false-positive result was recorded. The limit of detection obtained was 27 synthetic RNA copies and 1.2×10^{-2} ID per reaction for the CHIKV strain 1728 tested. Similar results were obtained (data not shown) with two CHIKV strains belonging to the same Central African cluster (Chik RCA, Chik 1720, Pastorino et al., 2004) and with one West African genotype CHIKV strain 37997. Specificity of the assay was evaluated by testing alphavirus or flavivirus infected cell supernatants. No cross-reactivity was detected with SFV, MAYV, SINV, ONNV as well as DENV and YFV.

3.2. Direct CHIKV TaqMan® assay evaluation

3.2.1. Effect of thermal RNA-release

To assess that the viral RNA load was not under evaluated due to incomplete release, non-extracted CHIKV cell supernatants were heated before direct quantification with the real-time RT-PCR assay. Table 3 showed the variation of the CHIKV TaqMan® sensitivity depending on time and temperature exposition. The Ct comparison indicated clearly that the sensitivity of the direct TaqMan® assay decreased with the rise of the temperature and the increase of the heating time. The lower Ct value (17.50 ± 0.18) corresponding to the best sensitivity was obtained with the non-heated sample incorporated directly into the reaction mixture. This result showed

Table 2
Sensitivity and specificity of the CHIKV TaqMan® assay

Sample	CHIKV strain 1728				
	Quantity		TaqMan® assay		
	ID[a]	RNA transcript copies	Ct[b]	Ct[c]	Result
RNA transcript[d]					
Dilution					
10^{-2}		2.7×10^6	20.64 ± 0.06	20.8 ± 0.12	Det
10^{-3}		2.7×10^5	24.42 ± 0.09	24.3 ± 0.24	Det
10^{-4}		2.7×10^4	27.66 ± 0.25	27.44 ± 0.50	Det
10^{-5}		2.7×10^3	28.46 ± 0.47	28.71 ± 0.23	Det
10^{-6}		2.7×10^2	29.82 ± 0.15	30.21 ± 0.27	Det
10^{-7}		2.7×10^1	28.73 ± 1.72	30.27 ± 0.51	Det
10^{-8}		2.7			Ndet
Titrated stock virus[e]	1.2×10^5		12.30 ± 0.1		Det
Dilution					
10^{-4}	1.2×10^1		26.80 ± 0.21		Det
10^{-5}	1.2		29.88 ± 0.44		Det
10^{-6}	1.2×10^{-1}		31.82 ± 0.75		Det
10^{-7}	1.2×10^{-2}		31.57 ± 1.2		Det
10^{-8}	1.2×10^{-3}				Ndet
Alphaviruses					
CHIKV strain 1720	$>10^7$				Det
CHIKV strain RCA	$>10^7$				Det
CHIKV strain 37997	$1 \times 10^{5.75}$				Det
SFV	$>10^7$				Ndet
ONNV	$>10^6$				Ndet
MAYV	$>10^6$				Ndet
SINV	3×10^5				Ndet
Flaviviruses					
DENV	10^6				Ndet
YFV	5×10^6				Ndet

Det: detected; Ndet: not detected.
[a] The quantity of virus expressed in viral infectious doses (ID) determined to be tested in the TaqMan® assay.
[b] Cycle threshold (Ct) value obtained from three replicates within each assay are represented as mean ± standard deviation if greater than zero.
[c] Cycle threshold (Ct) value obtained from three independent assays are represented as mean ± standard deviation if greater than zero.
[d] Ten-fold dilutions of the CHIKV RNA transcripts.
[e] Ten-fold dilutions of the CHIKV stock (strain 1728, GenBank accession number AY549582).

Table 3
Thermal RNA-release assay

Pre-treatment	Viral load ID/ml[a]	Direct CHIKV TaqMan® assay Ct[b]
No heating pre-treatment	6×10^6	17.50 ± 0.18
5 min at 50 °C	<10	17.68 ± 0.28
20 min at 50 °C	Ndet	18.72 ± 0.02
60 min at 50 °C	Ndet	19.25 ± 0.08
5 min at 60 °C	Ndet	18.50 ± 0.05
20 min at 60 °C	Ndet	19.08 ± 0.22
60 min at 60 °C	Ndet	21.01 ± 0.12
5 min at 70 °C	Ndet	18.61 ± 0.09
20 min at 70 °C	Ndet	19.63 ± 0.41
60 min at 70 °C	Ndet	21.91 ± 0.24
5 min at 80 °C	Ndet	19.73 ± 0.17
20 min at 80 °C	Ndet	20.24 ± 0.6
60 min at 80 °C	Ndet	26.3 ± 0.55
5 min at 90 °C	Ndet	22.03 ± 0.26
20 min at 90 °C	Ndet	25.51 ± 0.3
60 min at 90 °C	Ndet	30.4 ± 0.38

Ndet: not detected.
[a] The quantity of virus expressed in viral infectious doses (ID) per ml determined to be tested in the direct CHIKV TaqMan® assay.
[b] Cycle threshold (Ct) value obtained from two replicates within each assay are represented as mean ± standard deviation if greater than zero.

that a thermal pre-treatment for the direct CHIKV TaqMan® detection was not necessary and the simple RT step heating (20 min at 50 °C) used in our direct assay gave a highest sensitivity level. Moreover, the cell culture supernatant viral titer was very low (<10 ID/ml) after heating for 5 min at 60 °C and was not detected for all other thermal pre-treatment conditions. This result indicated that CHIKV was very sensitive to thermal inactivation.

3.2.2. Comparison of the CHIKV TaqMan® assay with or without the RNA extraction step

The comparison of the sensitivity of the direct TaqMan® assay with the conventional real-time method were presented in Table 4. Titrated CHIKV cell supernatants were 10-fold diluted in serum or H_2O, then successive dilutions were performed in H_2O. The samples were TaqMan® RT-PCR assayed with or without the RNA extraction step to determined the respective limit of detection. Table 4 showed that the detection limit of the direct CHIKV TaqMan® RT-PCR with cell supernatant sample diluted in H_2O or serum were comparable to those obtained with the RNA extraction step (approximately 1 ID/ml). However, when no RNA extraction step was performed, a 10-fold inhibition factor was observed for the serum sample until dilution 10^{-1} and for the pure cell supernatant sample (data not shown). This inhibitor effect disappeared in following dilutions.

Table 4
Comparison of direct and conventional CHIKV TaqMan® assay sensitivity

Sample, stock virus (2.6×10^7 ID/ml)	CHIKV TaqMan® assay sensitivity (ID/ml)[a]	
	Without RNA extraction	With RNA extraction
SN in serum[b]	1	1.1
SN in H_2O[b]	1.5	1.2

[a] Detection limit (ID/ml) obtained for three replicates within each assay with successive 10-fold dilutions of infected cell supernatants samples (SN).
[b] Two microliters of the CHIKV supernatant stock were diluted in 18 μl of serum or RNAse-free water, then successive dilutions in H_2O were performed.

4. Discussion

CHIKV, a member of the alphavirus genus, is of considerable public health importance in southeast Asian and African countries (Kit, 2002; Lam et al., 2001; Mourya et al., 2001). Although Chikungunya infections are rarely fatal and generally do not require admission to hospital, it is important to identify and quantify this infection for epidemiological studies. In fact, viral load should be a useful marker of disease progression and a measure of the antiviral compounds efficiency. Moreover, as described for other arboviruses, a rapid, specific and sensitive test is necessary for effective surveillance of new CHIKV circulating strains.

CHIKV diagnosis is based essentially on virus isolation, but RT-PCR (Pfeffer et al., 2002; Hasebe et al., 2002) and ELISA assays (Hundekar et al., 2002) were already described for specific detection of CHIKV in mosquitoes or clinical samples. Nevertheless, to date, virus quantitation is not available and it was important to developed a one-step TaqMan® RT-PCR assay. The specificity of the assay was also of importance because the discrimination between CHIKV and particularly ONNV infections could be difficult (Porterfield, 1961; Karabatsos, 1975; Blackburn et al., 1995). Furthermore, by comparison with nested RT-PCR developed previously (Pfeffer et al., 2002), a sensitive CHIKV real-time detection system was more rapid (less than 2 h versus one working day) and minimize cross-contamination by using a one tube detection system. These tests were useful for extensive epidemiological survey.

Attempts were made to reduce sample time processing and to minimize viral loss during each processing step, a direct CHIKV TaqMan® assay was also tested. Several RNA-release methods were described (Shi and Liu, 1992; Klebe et al., 1996; Singh, 1999; Kundu, 2003) which generally employed thermal (heating, freezing–thawing) or chemical (solvent, detergent) pre-treatments of the sample. However, like traditional RNA extraction methods, these alternative methods constituted a time-consuming supplemental step. Heat-release of the viral RNA used in this method was described to be the most convenient method (Schwab et al., 1997; Richards et al., 2004). It was known that destabilization of the physical integrity of viruses by heating resulted in the release of viral RNA then potentially available for a RT-PCR detection. However, the temperature at which virus particules became disintegrated during heating differed significantly between virus types and physicochemical conditions (Walder and Liprandi, 1976; Siegl et al., 1984; Mutombo et al., 1993; Michels et al., 1999). To improve the real-time assay, a heat-RNA release step was evaluated. Even if 20 min at 50 °C led to partial

exposure of viral genome, the direct RT-PCR had a comparable sensitivity to column RNA extraction method, i.e. about 1 ID/ml in both cases, indicating that a thermal pre-treatment was not necessary. In our experiments, Ct values were found to increase with the temperature and the length of exposition. The highest Ct value was found at 95 °C for a few minutes, a condition commonly employed for direct RT-PCR in lieu of RNA extraction. This sub-optimal RT-PCR sensitivity was probably due to breakages of phophodiester bonds whithin the targeted sequence, as recently reported (Bhattacharya et al., 2004). The localisation of the CHIKV primers in a heat-sensitive genome region (Kim et al., 2003) could explain this results.

For sera samples, viral RNA extraction assays must be selected carefully for their efficiency to eliminate inhibitors such hemoglobin, heparin or lipids (Burgener et al., 2003; Salete de Paula et al., 2003). It was observed under these conditions that simple sera or cell supernatants dilutions were found to be effective in removing RT-PCR inhibitors. Moreover, the used of a one-step real-time RT-PCR was convenient for minimizing sampling requirements which reduced potential RT-PCR inhibition (Bisset et al., 2001).

In conclusion, a sensitive and specific one-step TaqMan® RT-PCR assay was developed to detect and quantify West and Central African genotype CHIKV RNAs in infected cell supernatants and sera. Moreover, considering the 100% matching rate of the designed primers with all published Asian strain sequences (Fig. 1), this method could also succeed in detecting CHIKV strains belonging to Asian genotype. This quantitative test was suitable to improved research settings and for application in viremic individuals.

It was also demonstrated that a direct TaqMan® RT-PCR detection of CHIKV RNA, i.e. without any pre-treatment for RNA extraction and purification, was as sensitive as the real-time RT-PCR with an RNA extraction step. However, the protocol allowed a very small volume template (2.5 μl) which might be advantageous for clinical analysis. A large number of samples could be processed in very short time period, minimizing potential viral RNA loss, risk of carry-over contamination and the use of costly reagents.

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