# A rapid molecular detection protocol for Chikungunya virus directly performed on *Aedes albopictus* (tiger mosquito)

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**SUMMARY**

In the last few years tiger mosquitoes (*Aedes albopictus*), quickly and widely spread in Italy, represent ideal vectors for different Arboviruses, particularly Dengue virus (DenV) and Chikungunya virus (ChikV), who are causing millions of patients in the world per year. For ChikV, appeared for the first time in Italy in 2007, a Surveillance Plan was defined in the Marche Region, a neighbour county of the Italian outbreak site.

As a support for this surveillance, we decided to create a new multiplex RT-PCR protocol to detect ChikV directly in tiger mosquitoes.

All the mosquitoes were collected with BG-Sentinel® traps (Biogents AG, Regensburg, Germany). Total RNA extraction was carried with Helix RNA plus kit (Diatech srl, Jesi, Italy). For retro-transcription and amplification a Mastercycler® ep gradient S thermal cycler (Eppendorf AG, Hamburg, Germany) was used.

From the whole RNA extracted from captured mosquitoes, we developed a new end-point multiplex retro transcriptase polymerase chain reaction (RT-PCR), for both the detection and identification of *Aedes* spp. and ChikV.

This RT-PCR protocol is able to detect ChikV directly from adult insects, during alerts or emergencies. The entomological trapping associated with bio-molecular methods represents an effective strategy to detect ChikV directly from vectors. Moreover, after a specific evaluation, this RT-PCR protocol could be applied also for human blood samples in regions with the certain presence of this virus.

## INTRODUCTION

Chikungunya is an emerging Arbovirus of immense public health concern in Southeast Asian and African countries (6). Chikungunya virus (ChikV) is a single stranded positive sense enveloped RNA virus. It is a member of Alphavirus genus of the Togaviridae family and is transmitted to humans by *Aedes aegypti* (4). ChikV has two Open Reading Frames (ORFs), codifying for non structural proteins (nsP1, nsP2, nsP3 e nsP4) and for structural proteins (C, E2, E3, E1).

ChikV produces an illness in humans that is often characterized by sudden onset of fever, headache, fatigue, nausea, vomiting, rashes, myalgia and severe arthralgic pain (3, 5). Arthralgia may persist in a small proportion of cases even for months and has a great economic impact in many tropical countries.

Currently, the diagnosis of ChikV-infection is accomplished through either virus isolation or detection of virus-specific antibodies by ELISA or genomic detection by retro transcriptase polymerase chain reaction (RT-PCR).

In the last few years tiger mosquito, *Aedes albopictus* (*Diptera: Culicidae*), spread quickly and widely in the whole Italian territory. These insects are ideal vectors for different Arboviruses, particularly Dengue virus (DenV), and ChikV, causing millions of patients in the world per year. From ECDC data (European Centre for Diseases Control), several Member States have reported ChikV fever cases in travellers returning from affected areas, above all in South-East Asia.

ChikV appeared for the first time in Italy in 2007 and spread in the Provinces of Ravenna, Rimini, Forlì and Bologna, causing more than 300 clinical cases (2). The great alarm in the Italian population drove health authorities to improve the surveillance of Arboviruses.

This situation prompted regional public health authorities and the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche to begin a Surveillance Plan for this virus and a Vector Monitoring Program in the Marche Region, which is close to the area where the virus first appeared.

The aim of this plan was the reduction of vector density and the very early individualization of illness cases to prevent viral spread. Monitoring was carried out by ovitraps, while the capture of female adults was accomplished by BG-Sentinel® mosquito traps (Biogents AG, Regensburg, Germany).

In surveillance plans it is necessary to have the availability for both a rapid and effective capture system of vectors and a virus detection method. The last one is necessary to establish the real viral circulation in a limited area, during epidemic emergency. The typically daytime habits of *Aedes albopictus* make their capture more difficult as regards nocturnal *Culicidae*. For these reasons, in the cited Vector Monitoring Program, has been used BG-Sentinel® traps, with no carbon dioxide necessity, exploiting the only chemical attraction, verifying their efficiency in field conditions.

The aim of this study was to set up a new multiplex RT-PCR protocol detecting ChikV directly from *Aedes albopictus* pooled samples, exploiting new primers, which can be used together with Aa_rpL17-F and Aa_rpL17-R oligos for the simultaneous identification of the virus and the mosquito.

## MATERIALS AND METHODS

### Insect and nucleic acid collections

All the mosquitoes were collected with BG-Sentinel® traps (Biogents AG, Regensburg, Germany), in the surveillance plan of Pesaro, a Province in the Centre of Italy. For this protocol evaluation, we used a ChikV cDNA kindly offered by Dr. Paolo Bonilauri (Istituto Zooprofilatico Sperimentale della Lombardia e dell'Emilia Romagna, Italy), coming from the 2007 Italian outbreak.

### *In silico* studies and primer design

The study started from the collection from GenBank (NCBI, USA) and the alignment (BioEdit v7.0.9) of selected sequences of *E1* gene (Figure I).

The primer design was carried out on the *consensus* sequence using Primer3 v0.4.0 (http://frodo.wi.mit.edu/primer3/), followed by some modifications of the reverse-primer length to accomplish the best annealing temperature for amplification.

### RNA extraction

Total RNA extraction was carried out from 50 pooled insects, after homogenization in 5 mL of PBS 1X sterile solution

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(NaCl 137 mM, KCl 0.27 mM, $Na_2HPO_4$ 0.81 mM, $KH_2PO_4$ 0.19 mM, Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 1000 *x g* for 10 minutes, we collected 200 µl of the supernatant and extracted the RNA with Helix RNA plus® kit (Diatech srl, Jesi, Italy). Qualitative and quantitative yields were evaluated with a BioPhotometer 6131 (Eppendorf AG, Hamburg, Germany).

**Retrotranscription (RT) and amplification (PCR)**

Retrotranscription was carried out with a Mastercycler® ep gradient S thermal cycler (Eppendorf AG, Hamburg, Germany) in a volume of 20 µL with the following components (final concentrations): Buffer SuperScript® II (Life Technologies-Invitrogen, Carlsbad, CA, US) (1X), dNTPs (0.5 mM), Dithiothreitol (DTT) SuperScript® II (Life Technologies-Invitrogen, Carlsbad, CA, US) (10 mM), RNasin® Ribonuclease Inhibitor (Promega, Madison, WI, US) (20 U), random hexamers (2.5 µM), RT SuperScript® II (Life Technologies-Invitrogen, Carlsbad, CA, US) (100 U) and 5 µL of extracted RNA. RT protocol had the following temperatures: 20°C for 10 min, 42°C for 60 sec, 95°C for 10 min.

PCR products were run on a 2% agarose gel and visualized with a GelDoc2000™ apparatus (Bio-Rad), through ethidium bromide staining. The molecular marker used was a 100 bp DNA ladder (New England Biolabs, Ipswich, MA, US).

## RESULTS

After the collection and the alignment of 117 selected sequences of *E1* gene (Figure I). we designed two specific primers for ChikV (ChikBaro1: 5'-TCC gAA TCA TgC AAA ACA gA-3' and ChiKBaro2: 5'-AAg CCA gAT ggT gCC TgA-3').

In the second phase of the study we set up a multiplex PCR protocol amplifying at the same time the cited fragment of the *E1* gene (389 bp) and one (123 bp) of the gene *rpL17* (Accession number AY826144) of *A. albopictus* (1).

After the optimization, the multiplex PCR reaction was carried out in a total volume of 25 µL with the following components (final concentrations): Buffer GoTaq® Flexi (Promega, Promega, Madison, WI, US) (1X), $MgCl_2$ (2 mM), dNTPs (200 µM), primers ChikBaro1 and ChikBaro2 (0.4 µM), primers Aa_rpL17-F and Aa_rpL17-R (0.2 µM), GoTaq® Flexi (Promega, Promega, Madison, WI, US) (1 U) and 5 µL of cDNA (1 for ChikV and 4 for *A. albopictus*). The amplification protocol had a first step of denaturation at 92°C for 2 min, followed by 35 cycles at 92°C for 20 sec, 56°C for 20 sec, 72°C for 30 sec and a final extension at 72°C for 4 min.

All molecular protocols were carried out using a Mastercycler® ep gradient S thermal cycler (Eppendorf AG, Hamburg, Germany).

The multiplex PCR has been optimized, through an annealing temperature gradient (50-60°C), and testing different combinations of both $MgCl_2$ and primers concentrations, to guarantee a better result in amplifying cDNA of ChikV compared to *A. albopictus* cDNA (Figure II).

## DISCUSSION

With this work we have set up a new diagnostic method for the search of ChikV directly from *Aedes albopictus* pooled samples, for the simultaneous identification of the virus and the mosquito.

The amplification of *rpL17* gene demonstrates the correct execution of the upstream processes of PCR, so it can be considered as an internal control. This is strengthened from the difficulty of finding infected mosquitoes to use as positive controls.

This PCR protocol could represent an important instrument for the surveillance of viral diffusion and for epidemiological investigation of ChikV in case of epidemic to estimate and monitor the outbreak. Moreover, after a specific evaluation, this RT-PCR protocol could be applied also for human blood samples in regions in which is certified the presence of this virus. This bio-molecular technique, for its high sensitivity and its short protocol offers advantages compared to viral isolation, allowing shortening intervention strategies.

The use of BG-Sentinel® mosquito traps, in high infestation density areas, in case of suspected viral circulation, could help to investigate the presence of ChikV.

Frequent controls of the traps permit to obtain live and viable vectors, allowing performing bio-molecular investigations

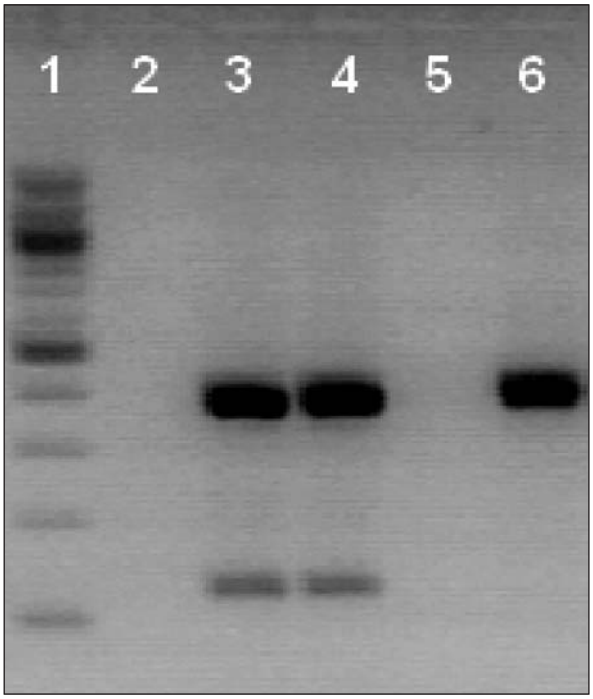


**Figura II.** ***Multiplex PCR results.*** *Lane 1: ladder 100 bp, Lane 2: negative control, Lane 3: ChikV* + A. albopictus, *Lane 4: ChikV* + A. albopictus, *Lane 5: H2O, Lane 6: positive control for ChikV.*

[…]

CGGCGCYTACTGCTTCTGCGACRCYGAAAAYACGCARTTGAGCGAAGCACAYGTGGAGAAG***TCCGAATCATGCAAAA***

***CAGA****ATTTGCATCAGCATAYAGGGCHCATACHGCATCBGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAAYAAYRTYACTG*

*TADCTGCYTATGCAAACGGCGACCATGCCGTCACAGTHAAGGACGCYAAATTCATNGTGGGGCCAATGTCTTCAGCCTGGACA*

*CCTTTYGACARYAAAATYGTGGTGTRCAAAGGYGRCGTYTAYAACATGGACTACCCRCCYTTYGGCGCAGGAAGACCDSSWSWW*

*WWWGGYGAYRTYCRARGYCGYACDCCKGRRAGYDWASRYVKHTATGSYAAYRCWCARYYRGTRCTRCAGAGRCCRKCHGYRGG*

*YACGGBRCAYGTRCCRTACTC****TCAGGCACCATCTGGCTT***YAAGTAYTGGYTRAARGAACGMGGRGCRTCRCTRCAVCAC

ACRGCACCRTTYGGYYGYCARATWGCRACAAACCCGGTAAGAGCKRTRAAYTGCGC

[…]

**Figura I. E1 sequence alignment.** Consensus *sequence of the ChikV E1 gene, after the alignment of 117 regions. The amplicon region is grey-colored (about 389 bp).*

for these RNA viruses.
In case of epidemic, this diagnostic method could have a predictive significance in showing the extent of this event, giving information about diffusion and infection risk for human health.

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