

# Laboratory Diagnostic Options and Challenges for Chikungunya Viruses

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## Abstract

**Background:** Chikungunya infection is a *Aedes* mosquito transmitted viral disease caused by Chikungunya virus (CHIKV), a member of the Alphavirus genus. It is an important human pathogen that causes a syndrome characterized by fever, chills, headache and severe joint pain usually of the smaller joints, with or without swelling. Though the disease is almost self-limiting, during the recent outbreak CHIKV was also found to cause long-term arthralgia, neurological disease and even few fatalities. Despite the fact that CHIKV is associated with epidemics of unprecedented magnitude, only a few specific serological and molecular diagnostic tools are available. **Objective:** CHIKV diagnosis is essentially based on virus isolation, reverse transcription (RT)-PCR and ELISA assays. The gold standard of CHIKV diagnosis is culture, however, required facilities and skills are not available in any routine laboratory in the country. Highly sensitive and specific PCR assays for CHIKV have been developed and commercially available. **Conclusion:** Although the reagents and equipment are costly for widespread use RT-PCR is the method of choice for the early detection and confirmation of virus in clinical samples as most acutely infected patients seek medical attention within the first few days of illness when role of serology based diagnosis is minimum.

## Key words

Chikungunya virus, *Aedes* mosquito, RT-PCR

## Introduction

Chikungunya virus (CHIKV) is an enveloped, RNA positive-strand Alphavirus belonging to the *Togaviridae* family. It caused a considerable public health concern in Southeast Asian and African countries. First it was isolated from serum of a febrile human in Tanzania in 1953. The name came from the Makonde word meaning "that which bends up" in reference to the stooped posture developed as a result of arthritic symptoms of the disease.<sup>1</sup> CHIKV is geographically distributed throughout Africa and Southeast Asia, and its transmission to humans is mainly via the *Aedes* mosquito species. CHIKV

is most prevalent in urban areas and epidemics are sustained by the human-mosquito-human transmission cycle, since humans act as very efficient reservoirs for the virus.<sup>1</sup> Since 1953, CHIKV has caused numerous well-documented outbreaks and epidemics in both Africa and Southeast Asia, involving hundreds of thousands of people. A CHIKV outbreak of unprecedented magnitude swept the Indian Ocean territories principally involving Reunion Island, Comoros, Mauritius, Seychelles and Southwestern India in 2005-2006.<sup>2-5</sup> Recently, the virus has also crossed the tropics and its presence has been recorded for the first time in the Emilia Romagna

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Region of northeastern Italy and USA; the *Aedes albopictus* being implicated as the mosquito vector.<sup>6,7</sup> The exact reasons for the re-emergence of CHIKV in the Indian subcontinent as well as other small countries in the southern Indian Ocean are mysterious. However, the plausible explanations include increased tourism, the introduction of virus into a naive population, viral mutation and lack of vector control.<sup>8</sup>

The severity of the illness varies, tending to be less severe in children. Most patients recover after a few weeks but a small proportion of individuals may experience chronic joint pain for some years. Deaths related directly to infection with CHIKV have not been documented to date, but CHIKV may be a contributing factor in the death of some individuals with underlying health problems. Polyarthralgia, the typical clinical sign of CHIKV is very painful. Symptoms are generally self-limiting and last 1-10 days. However, arthralgia may persist for months or years. In some patients, minor hemorrhagic signs, such as epistaxis or gingivorrhagia, have also been described. The symptoms are most often clinically indistinguishable from those observed in dengue fever. Indeed, the simultaneous isolation of both dengue and CHIKV from the sera of the same patients has previously been reported, indicating the presence of dual infections.<sup>9</sup> It is, therefore, very important to clinically distinguish dengue from CHIKV

infection. In contrast to dengue, hemorrhagic manifestations are relatively rare in CHIKV infection and shock is not typically observed in CHIKV infection. Fever and polyarthralgia are the major symptoms that result in a positive clinical diagnosis. However, in 5% of cases the disease is asymptomatic.<sup>10</sup> Neurological complications, such as meningoencephalitis, hepatic cytolyses, severe lymphopenia, severe dermatological involvement, death and neonatal infections have also been reported in a small proportion of patients during the recent Indian, as well as the French Reunion Island outbreaks.<sup>8,11</sup>

Although not listed as a hemorrhagic fever virus, illness caused by CHIKV can be confused with diseases such as dengue or yellow fever, based on the similarity of the symptoms as discussed above. Thus, the differential diagnosis of these two infections is essential for clinical management and epidemiological study in the tropics. Despite the fact that CHIKV resurgence is associated with epidemics of unprecedented magnitude, only a few specific serological and molecular diagnostic tools are available. A definitive diagnosis of Chikungunya infection can be made only with the aid of laboratory support since clinically, symptoms may resemble those of dengue fever. Laboratory diagnosis is therefore critical to establish the differential diagnosis and enable the initiation of a specific public health response.

### Laboratory Diagnosis

Three main laboratory tests are used for diagnosing CHIKV infection: virus isolation, genomic detection by PCR-based methods and serological tests for demonstration of virus-specific antibodies.

### Virus Culture and Isolation

Virus isolation is the most definitive test and is considered to be the gold standard, unfortunately, it is not undergoing in the country. Detection of virus is dependent on virus isolation from the blood of viremia patients or infected tissues. The isolation of CHIKV is comparatively more simple and effective owing to the highly cytopathic and fast-growing nature of the virus, which will grow to very high titers.<sup>12</sup> CHIKV replicates in various cell lines, including insect cells, for example, C6/36, nonhuman viz, Vero, chick embryo fibroblast-like cells, BHK21, L929 and Hep-2 cells, and human cell lines, for example, HeLa, MRC5 in which it will often induce a significant cytopathic effect. Human epithelial and endothelial cells, primary fibroblasts and, to a lesser extent, monocyte-derived macrophages, are susceptible to infection and permit viral production. By contrast, CHIKV does not replicate in lymphoid and monocytoid cell lines, primary lymphocytes and monocytes, or monocyte-derived dendritic cells.<sup>12</sup> However, the isolation process is time-consuming and the degree of success is dependent on a number of complicating factors, for example, time of collection, transportation, maintenance of cold chain, storage and

processing of samples. Further, for virus culture BSL-3 laboratory is required to reduce risk of virus transmission.

### Molecular diagnosis

RT-PCR technique for diagnosing CHIKV has been developed using primer pairs amplifying specific components of three structural gene regions, Capsid (C), Envelope E1 and E2, and part of nonstructural protein (NSP).<sup>13,14</sup> Both gel-based conventional PCR and one step real time PCR kits are now available to detect CHIKV in the serum or plasma. Conventional PCR test systems are time consuming and labor-intensive, with a very high risk of contamination primarily due to post-PCR handling leading to carry over. All real-time PCR systems rely upon the detection and quantitation of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. The real-time RT-PCR technique has been used to detect an amplicon that is amplified during the PCR cycling, in real time. The development of fluorogenic PCR utilizing 5'-3' nuclease activity of Taq DNA polymerase made it possible to eliminate post-PCR processing, such as visualization in agarose gels. All of these advantages are due to the nature of the amplification reaction and the availability of instruments able to perform both thermocycling and fluorescence detection.<sup>15</sup> Real - time loop-mediated isothermal amplification (RT-LAMP) assay has also been found to be a useful molecular tool for rapid diagnosis.<sup>16,17</sup>

### **Serological Diagnosis**

Serodiagnosis of CHIKV relies on the demonstration of a four-fold increase in Chikungunya IgG titer between the acute and convalescent phase sera. However, obtaining paired sera is usually impractical. Alternatively, the demonstration of IgM antibodies specific for CHIKV in acute-phase sera is used in instances where paired sera cannot be collected. The most commonly used test is the IgM capture (MAC)-ELISA.<sup>18,19</sup> Cross-reaction with other Alphavirus antibodies such as ONNV and SFV usually limits the application of MAC-ELISA as a confirmatory test. A positive result supplemented with neutralization is taken as definitive proof for the presence of CHIKV. However, as already discussed, it is not always practical to adapt these approaches for routine and early detection. Therefore, a molecular approach based on reverse transcription (RT)-PCR technologies is useful for early confirmatory diagnosis prior to the appearance of IgM antibody.

### **Laboratories working on CHIKV in Bangladesh**

Molecular lab of Apollo Hospitals Dhaka first started simultaneous detection and differentiation of chikungunya and dengue by RT-PCR at time of clinical presentation and soon received huge number of requests from clinicians. Out of 1500 susceptible cases with fever during June 29 to October 31 this year 601 cases of chikungunya and 236 cases of dengue was confirmed. Institute of Epidemiology, Disease Control and Research (IEDCR),

Bangladesh is also detecting chikungunya virus by RT-PCR with the help from CDC, USA. Later on Square Hospital, DNA solutions and ICDDRB also started RT-PCR to detect chikungunya virus from blood at time of clinical presentation.

### **Importance of CHIKV Diagnosis**

The appropriate laboratory test for diagnosing CHIKV depends on the duration of patient's symptoms. Symptoms generally last for 3 to 5 days, during which viremia is present and can be confirmed by virus culture and isolation, polymerase chain reaction (PCR) and antigen detection tests. RNA and antigen from nonviable virus are also detectable for few days thereafter. CHIKV IgM antibodies become detectable around 5 days' post fever, and persist for 3 to 6 months. By 10-14 days, IgG is present. Most laboratories in developed countries quickly diagnose CHIKV by using molecular methods, mainly for travelers to the endemic countries. However, within endemic regions, the resource-limited countries where most of the people at risk reside, diagnostic capacity for CHIKV remains unsatisfactory.

Many other common tropical infections, like dengue, malaria, typhus, and leptospirosis present as non-specific fever in a similar way to CHIKV. These may be difficult to differentiate without laboratory help. Although there are no antiviral or vaccines available for CHIKV, still there are several advantages to diagnose the infection. Patients usually remain undiagnosed, yet many are empirically and unnecessarily

given antimicrobials.<sup>20</sup> This leads to wastage of resources and may contribute to antimicrobial resistance. Secondly, early diagnosis of CHIKV would result in interventions for early vector control to prevent further spread. Further, effective surveillance of emerging diseases in developing countries, can alert the global community to potential threats, such as current network for avian influenza surveillance. Finally, if the exact burden of CHIKV is known, resources are likely to be appropriately allocated for developing therapies, vaccines and other control strategies.

#### **Problems with current diagnostic modalities for CHIKV**

The gold standards of CHIKV diagnosis is culture and isolation.<sup>21,22,23</sup> Unfortunately, virus isolation requires facilities and skills which is not available in the country. Highly sensitive and specific PCR assays for CHIKV diagnosis have been described, but the reagents and equipment are costly for widespread use.<sup>24,25</sup> Efforts are going on to develop sensitive and rapid antigen based assays comparable to the sensitivity and specificity of RT-PCR (Tatsuo Shioda personal communication).

Serological diagnosis by detecting IgM or IgG antibodies is more widely used as it is relatively cheaper and easier to perform. The disadvantage of antibody testing is the possibility of cross-reacting with other alpha viruses and it will be much problematic in our community as dengue is already endemic. Therefore, CDC, USA recommended to do plaque reduction

neutralization test to confirm positive IgM ELISA test. IgM antibodies may persist for 3 to 6 months; a single raised IgM may indicate recent past infection rather than acute infection. On the other hand, to see four-fold rise of IgG antibodies paired sample collection is needed which is not possible most of the time.

Commercial IgM assays using recombinant CHIKV protein in ELISA and ICTs have been developed.<sup>26</sup> However, most CHIKV patients present within a few days of symptom onset, before IgM is present. When some kits were tested in clinical settings, sensitivities for diagnosing acute CHIKV infection by IgM detection were just 4% to 22%, when compared with PCR.<sup>27,28</sup> Detection of IgM after 1 week rose to more than 80%, but this retrospective diagnosis is less useful for clinicians.<sup>27-29</sup> Therefore, these currently available assays are unsuited for diagnosing acute CHIKV in most cases. Moreover, the performance of commercially available IgM assays varies widely (Table 1).<sup>30</sup>

#### **The ideal test for acute CHIKV infection**

The ideal test should have the characteristics recommended by WHO: affordable, sensitive, specific, user-friendly (minimal training required), rapid and robust in different climates, equipment-free (referring to electricity-dependent machines), and delivered to those who need it.<sup>31</sup> In this respect PCR testing faces significant challenges as it is not cheap, technology needs equipment and special training. As most acutely infected patients seek medical attention within the first few days of illness, the ideal test should

**Table1: CDC, USA evaluation of commercially available chikungunya virus IgM antibody assays**

| Manufacturer     | Assay                  | Type  | Performance  |
|------------------|------------------------|-------|--------------|
| Euroimmun        | Anti-CHIKV IgM         | IFA   | High         |
| Euroimmun        | Anti-CHIKV IgM         | ELISA | High         |
| Inbios           | CHIKjj Detect MAC      | ELISA | High         |
| Abcam (NovaTec)  | Anti-CHIKV IgM human   | ELISA | Inconsistent |
| Genway (NovaTec) | CHIKV IgM u-capture    | ELISA | Low          |
| CTK Bioteck      | RecombiLISA CHIK IgM   | ELISA | Low          |
| SD Diagnosis     | CHIKa IgM              | ELISA | Low          |
| CTK Biotech      | On-site CHIK IgM Combo | Rapid | Low          |
| SD Diagnosis     | SD BIOLINE CHIK IgM    | Rapid | Low          |

medical attention within the first few days of illness, the ideal test should detect RNA or antigen. So, RT-PCR is the method of choice until availability of antigen test which is a more realistic aim.

**Conclusion**

The ongoing spread of CHIKV is an example of the globalization of infectious disease. Thus there is importance of understanding the dynamics of CHIKV infection, its immune response and the type and limitations of the assay used in the design of diagnostic assays. We hope further advances in diagnostics will bring benefit to all those at risk.

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