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Diagnosis and Molecular Characterization of Chikungunya Virus Infections

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Abstract

In recent years, large-scale outbreaks of chikungunya arbovirus (CHIKV), which is transmitted by the *Aedes* mosquito, have enabled the rapid propagation of the virus across the world. After acute infection phase with commonly fever, joint pain, headache, or rash, chronic rheumatism (arthralgia or myalgia, anorexia, and concentration disorders) up to 40% of cases is observed. The chronic form is defined by symptoms persisting for more than 3 months, and up to years, after initial diagnosis. Chronic discomfort has been linked to one of the four genotypes described. These genotypes represent different geographic lineages (classification based on partial sequence of viral E1 glycoprotein): West African, East-Central-South-African (ECSA), ECSA-diverged or Indian Ocean Lineage (IOL), and Asian. The first marker detected in CHIK infection is the viral RNA, usually by reverse transcription-polymerase chain reaction (RT-PCR). This marker can be identified in samples within 8 days of symptom onset. The infection can also be diagnosed with serological testing to detect CHIKV-specific immunoglobulin IgG and/or IgM. Sequencing studies can determine the infecting genotype.

Keywords: chikungunya, genotype, chronic

1. Introduction

Chikungunya virus (CHIKV) is an emerging mosquito-borne alphavirus that causes severe acute febrile infection, often debilitating polyarthralgias and arthritis [1], and its symptoms are similar. The name chikungunya is derived from an African language *makonde* which means “that which bends up” or “stooped walk” because of the incapacitating arthralgia caused by the disease.

Chikungunya virus, the first reported case of which was in Tanzania in 1952 [2], is endemic in certain parts of West Africa, human serosurveys having identified antibodies to chikungunya virus in 35–50% of the population in some areas [3, 4].

1.1 Epidemiology, structure, and classification of chikungunya

Since 2004 CHIKV has spread into novel locations, with outbreaks having occurred in other parts of Africa as well as Asia, Europe, and the islands of the Indian

and Pacific Oceans, and more recently in the Americas, mostly during the tropical rainy season. The risk of CHIKV being imported into further new areas is ever present because of the high attack rates, which affect between one-third to three-quarters of the population in areas where the virus is circulating, associated with the recurrence of epidemics, along with the high levels of viremia in infected humans and the worldwide distribution of the vectors, *Aedes aegypti* and *Aedes albopictus* [4]. Infected travelers can import chikungunya into new areas [1, 5–7], and in areas with *A. aegypti* and/or *A. albopictus* mosquitoes, local transmission can follow.

CHIKV multiple outbreaks have been identified as a cause of dengue-like illness with arthralgia in Africa and febrile hemorrhagic disease in Asia [2]. Although outbreaks of CHIKV were limited from the 1970s onward, in 2004–2005 the virus was detected in Kenya and the islands of the Indian Ocean in 2004–2005 [4, 8–10], and in the last decade, there have been massive outbreaks in new areas as well as in those where the virus is endemic [11]. What is more, local transmission of CHIKV has also been reported for the first time in parts of Europe, Asia [12], Australia [13], and America [9, 14, 15].

In the Americas, autochthonous CHIKV transmission was first reported in the Caribbean, on the island of St. Martin, in December 2013 [16], from which it is known to have spread to 45 countries or regions in Central/South/North America, where, according to the Pan American Health Organization, there had been 2 million suspected cases by 2014 [17]. It was detected in Central and South America in 2014, where there have since been multiple widescale outbreaks [13, 17], and between June and November of that same year, cases were reported in people returning to Northwest Italy from the Caribbean and Central America, even though there had been no reported cases of CHIKV in either region for 3 years [18]. More recently, in 2016–2017, autochthonous CHIKV transmission was reported in India [19], Pakistan [20], and Italy [21]. It can be assumed that the combination of increased global travel and trade, the wide distribution of the mosquito vectors, and a lack of herd immunity have all contributed to the introduction and rapid spread of CHIKV in naïve populations.

Dengue and Zika viruses are transmitted by the same mosquito vectors as chikungunya so all three viruses can co-circulate in a geographic region, and coinfections have been documented [22, 23]. This clearly has implications for diagnosis in the laboratory.

CHIKV belongs to the *Togaviridae* family, genus *Alphavirus*, within the Semliki Forest antigenic complex. Among the other members of this antigenic complex are the Mayaro, O’Nyong-nyong, and Ross River viruses, all of which are capable of causing disease in humans [24].

Chikungunya is a positive-sense single-stranded RNA virus with approximately 12 kb. It is spherical in shape and has a diameter of about 70 nm. The viral particle comprises 240 copies of the capsid protein contained within a lipid bilayer envelope which has 80 trimer-shaped spikes formed by E1 and E2 glycoproteins protruding from it [25]. There are two open reading frames (ORFs) in the genome: the 5’ORF (genomic RNA) is responsible for encoding the nonstructural proteins nsP1, nsP2, nsP3, and nsP4, and the 3’ORF (subgenomic RNA) is responsible for encoding a polyprotein that is processed into the structural capsid and envelope proteins (E1 and E2) and two peptides (E3 and 6 K) [26].

Several CHIKV genotypes which have been described are shown below: East-Central-South-African (ECSA) isolates which comprise the East, Central and South African, and West Africa isolates (West Africa) and Asian isolates (Asian). The Indian Ocean Lineage (IOL) was identified in 2004 as a descendant of the ECSA lineage [27, 28].

1.1.1 Chikungunya genotypes

CHIKV can be genetically classified into three major lineages: West African (WA); East, Central, and South African (ECSA); and Asian. Prior to 2004, these were rarely found outside the geographic limits implied by their names. The epidemics subsequent to 2004 were mainly due to isolates forming a distinct clade within the ECSA lineage [29], likely originating in eastern Kenya before spreading to cause large outbreaks affecting millions in islands of the Indian Ocean, India, and Asia [30], as well as numerous imported cases in previously nonendemic regions, including Europe and the Americas. More recently, since 2013, the Asian genotype has also caused significant outbreaks in the Caribbean and the Americas.

Additionally, the Indian Ocean (IOL) sublineage emerged within the ECSA clade, and the Asian/American sublineage emerged within the Asian clade [31, 32].

While differences in epidemiological and pathological characteristics among outbreaks involving different CHIKV lineages and sublineages have been suggested, few targeted investigations comparing lineage virulence levels have been reported.

The most important clades in terms of public health impact are the Asian lineage (including Asian/American) and the IOL sublineage, as well as some other ECSA lineage strains responsible for African outbreaks. They are responsible for multiple CHIKV outbreaks over the last 15 years involving millions of people [29, 32, 33], which continue to arise in Asia and Africa [34, 35].

CHIKV phylogenetic analysis, based on previous work speculating on the existence of distinct lineages [36], has established that the three principal currently circulating genotypes emerged from a common ancestor less than 500 years ago and that the ECSA and Asian genotypes separated within the past 150 years [29].

West African genotype is particularly well adapted to sylvatic mosquitoes or to a certain vector-host combination within the sylvatic cycle.

The Asian genotype that once circulated in India during the 1950s and 1960s formed a clade that was phylogenetically distinct from the Southeast Asian (SEA) strains that continue to circulate today. There is a remarkable spatial and temporal pattern in the evolution of the SEA lineage, spreading from Thailand to Indonesia and then to the Philippines and the South Pacific.

2. Transmission, pathogenesis, clinical disease, and differential diagnosis

Chikungunya virus is transmitted to people, as mentioned above, primarily via mosquito bites (primarily during the day but also at night). Mosquitoes become infected when they feed on a person already infected with the virus and then spread the virus to other people via biting, after the virus reaches the mosquito salivary glands. Other less-frequent routes of transmission are through blood products, organ transplantation, and maternal-fetal.

Pregnant women infected with CHIKV are not at increased risk of atypical or severe disease, but maternal CHIKV infection has been associated with miscarriage in the first trimester [16, 37, 38]. However, when maternal infection occurs toward the end of pregnancy, only 12% of newborns are expected to be symptomatic, clinical manifestations—fever, poor feeding, tenderness, unexplained apnea, peripheral edema, thrombocytopenia, and rash—appearing 3–7 days after delivery [37, 39]. More severe symptoms have also been observed, such as encephalopathy and myocardial disease, and neurocognitive development was found to be poor in children with perinatal CHIKV infection [40].

2.1 Clinical disease

The “natural” history of symptomatic CHIKV infection has been classified into three phases: acute, post-acute, and chronic.

Acute phase is considered the first 3 weeks of clinical manifestations. The incubation period is 3–7 days (range 1–14 days), and clinical manifestations begin abruptly, usually with high fever ($>39^{\circ}\text{C}$), during 3–5 days (range 1–10 days), and malaise [41]. More than 85% of patients are symptomatic [1]. The duration of acute phase is usually 7–10 days.

Polyarthralgia, commonly bilateral and symmetric, begins 2–5 days after onset of fever and involves multiple joints: hands (50–76% of infected individuals), wrists (29–81%), ankles (41–68%), and axial skeleton (34–52%) [42, 43]. Pain may be intense and disabling, leading to immobilization.

Rash (maculopapular) has been reported in 40–75% of patients [43] and pruritus in 25–50% of patients.

Serious complications are not common, except in patients older than 65 years and patients with underlying pathologies, where the infection and symptoms can contribute to the cause of death. These severe forms usually involve the central nervous system, respiratory system, and urinary system.

Most patients recover fully, but in some cases joint pain may persist, or the patient experiences relapses of signs and symptoms for several months or even years [26].

In the *post-acute phase*, only a small proportion of patients remain completely asymptomatic 2–3 weeks after the onset of disease [44]. Generally, most patients exhibit only transitory improvements in their clinical condition, and relapses occur after a brief “healing” period. Most studies have indicated that, on average, clinical manifestations persist in 50–90% of patients after the second or third week, and the percentage of patients with persistent polyarthralgia after the acute phase of CHIKV infection is more frequent in those older than 40 years and in women [45].

Clinical manifestations observed during the post-acute phase, which indicate the persistence of the initial inflammatory process, include arthritis/arthralgia, edematous polyarthritides of fingers and toes, morning pain and stiffness, and severe tenosynovitis [42].

A set of nonspecific clinical manifestations that are not always associated with CHIKV usually occurs, such as chronic fatigue, changes in skin color, alopecia, decompensated endocrine and metabolic diseases, as well as the decompensation of other preexisting chronic diseases, depression, and anxiety [44].

Chronic phase: It is estimated that the percentage of patients infected with chikungunya virus who progress to chronic phase (more than 3 months) varies from 40 to 80% [45–47], and they may endure clinical manifestations for a few months or even years [44]. Although no clear evidence exists to explain the pathogenesis of persistent symptoms following infection, two hypotheses have been proposed: (a) That viral and/or antigenic debris remains in the tissues of joints and muscles. Unfortunately, to date, the virus has not been isolated from such tissue. However, CHIKV proteins have been found in macrophages and muscle cell tissue of relapsing CHIKV patients, supporting the notion that there may be low-grade replication of the virus or non-replicative viral debris present. (b) That infection triggers a persistent immune response. Studies are underway with mouse models to determine whether, and in what way, immunological mechanisms might be altered in patients with persistent symptoms [48, 49].

Chronic phase sufferers can be divided into three groups in terms of disease progression: those whose symptoms disappear either spontaneously or following treatment and who have no long-term complications, which accounts for the majority of such cases; a group who experiences prolonged and persistence generalized

clinical symptoms, with or without joint problems; and another set of patients in whom the degenerative or inflammatory process is exacerbated and their condition becomes serious [44].

Twenty-five percent of CHIKV cases have been estimated to result in chronic inflammatory rheumatism, although this study did not address the issue of differences between genotypes [50]. Other symptoms, such as chronic pain, mental health issues, and nonspecific manifestations, are also common in this phase [51–54], with arthralgia and arthritis with pain accompanied by articular edema or morning joint stiffness being the most frequent clinical manifestations [55]. Tenosynovitis is also frequent (with two or more tendons affected), and less common symptoms are fatigue and neuritis [44]. Clearly the daily life of patients with long-term chronic CHIKV symptoms is considerably impacted [54], and interestingly there is some evidence that the incidence of chronic symptoms is greater in high-income than low-income countries, albeit that the confidence intervals overlapped [54]. These same authors did consider the relationship between virus genotype and self-reported chronic discomfort and found this symptom to be less prevalent in those infected by the ECSA group, followed by those carrying the Asian strain. The highest prevalence was linked to the ECSA-diverged/IOL genotypes, though there was overlap with the Asian strain.

There is no commercial vaccine against CHIKV, although development is underway [56–58] and there is no specific antiviral therapy for acute infection. Treatment in this phase consists of supportive care for as long as the infection is confirmed. It is, however, known that systemic glucocorticoids and other immunosuppressive medications should be avoided in patients during acute infection [44].

The management of persistent or relapse symptoms, particularly joint disease, depends upon the duration of the symptoms. Treatment with anti-inflammatory drugs and analgesics is appropriate in the acute phase. On the other hand, for symptoms persisting for more than 3 months after infection, the use of disease-modifying antirheumatic drug (DMARD) therapy, such as methotrexate (MTX) and hydroxychloroquine [44], or, as an alternative, physiotherapy is indicated [59].

2.2 Differential diagnosis

Fever and polyarthralgia are key symptoms of CHIKV infection but are far from being reliable diagnostic markers since they have a sensitivity of only 84%, a positive predictive value (PPV) of 71%, and an 83% negative predictive value (NPV) [25]. Thus differential diagnosis of both the acute and the chronic manifestations of the disease can be complex and take time due to the nonspecific nature of the typical symptoms of arthralgia, high fever, and rash. The shared vectors, as well as symptoms, of CHIKV and the DENV, ZIKV, O'nyong-nyong, and Mayaro viruses, as well as the fact that the viruses all co-exist and are known to co-infect, mean that they must all be ruled out in the differential diagnosis.

Therefore, it is difficult to differentiate from those of other febrile illnesses or be misdiagnosed in areas where dengue occurs. Other infections that should be considered include malaria, yellow fever, leptospirosis, measles, mononucleosis, or African tick bite fever.

An additional diagnostic complication is the similarity of chronic arthralgia caused by chikungunya fever with other more common causes, and it has been demonstrated that patients with underlying joint disease prior to CHIKV infection have worse prognosis and increased morbidity [60]. Differential serological diagnosis includes the exclusion of rheumatoid and systemic arthritis, lupus erythematosus, Reiter arthritis, rheumatoid arthritis, and hepatitis C.

It is thus clear that differential diagnosis is essential to establish CHIKV infection and ensure the appropriate public health response as well as the optimal treatment regime for the patient. Employing a single PCR which targets all three infectious agents (CHIKV, DENV, ZV), available from US Centers for Disease Control and Prevention (CDC) and approved laboratories, would expediate diagnosis [61–64].

3. Diagnosis

Chikungunya virus infection should be suspected in patients with acute onset of fever and polyarthralgia and who meet the relevant epidemiologic exposure criteria (residence in or travel to an area where mosquito-borne transmission of chikungunya virus infection has been reported).

The laboratory diagnosis of CHIKV infection can be achieved in the majority of cases by following two different strategies: the detection of viral RNA (virological diagnosis) and the identification of the specific immune response (serological diagnosis). To this end, choosing the appropriate timing of specimen collection and of the use of the most suitable diagnostic methodologies is crucial for accurate diagnosis. The algorithm developed by the US Center for Disease Control and Prevention (CDC) to diagnose CHIKV infections is based on the characteristics of CHIKV infection and the timing of specimen collection (**Figure 1**).

CHIKV replicates rapidly to high titers in the host, and viral RNA generally can be detected by real-time RT-PCR in the first week after onset of clinical illness, while immunoglobulin M (IgM) antibodies are normally detectable in serum by days 5–7 after onset of illness [65]. Consequently, the kinetic replication and pathogenesis of CHIKV infection, including the duration of viremia and the response of the host immune response, should be considered when selecting the appropriate diagnostic tests [66]. Molecular assays (TaqMan real-time PCR, RT-LAMP assay, and reverse transcription PCR) are more sensitive in the early stage of chikungunya fever (2–5 days p.o.i.) when CHIKV-specific IgM is not yet detectable. In the later stages of chikungunya fever (>5 days p.o.i.), CHIKV-specific IgM is a more reliable indicator.

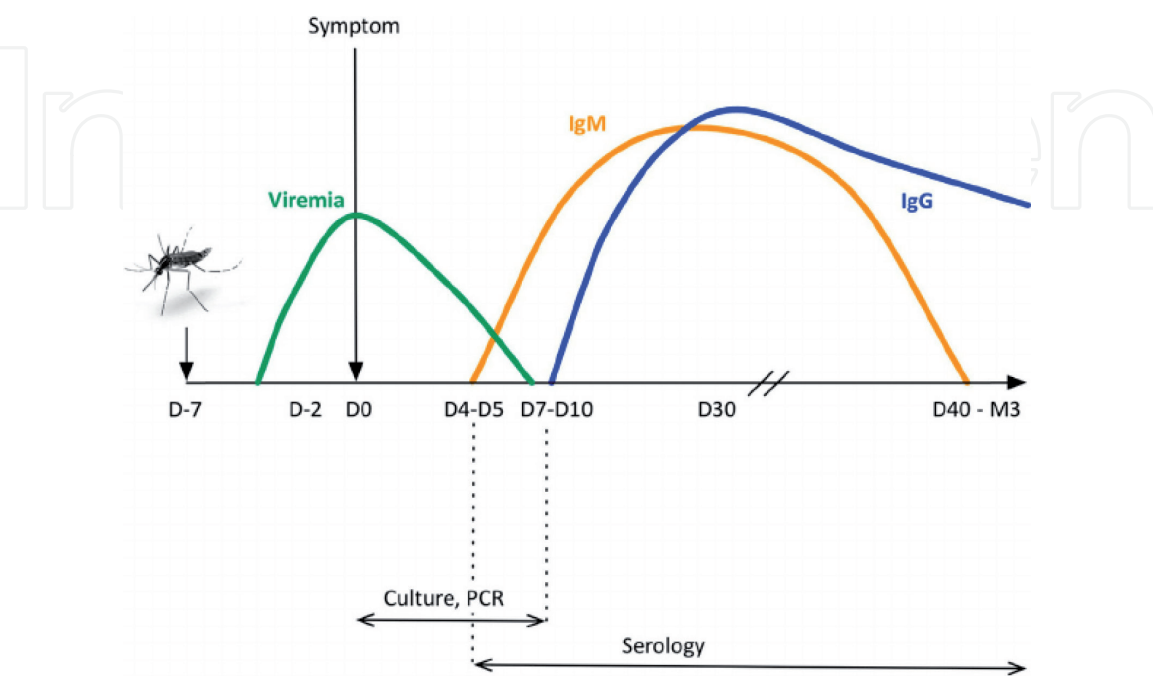


Figure 1.
Time course of chikungunya virus viremia and immune response. Source of CDC.

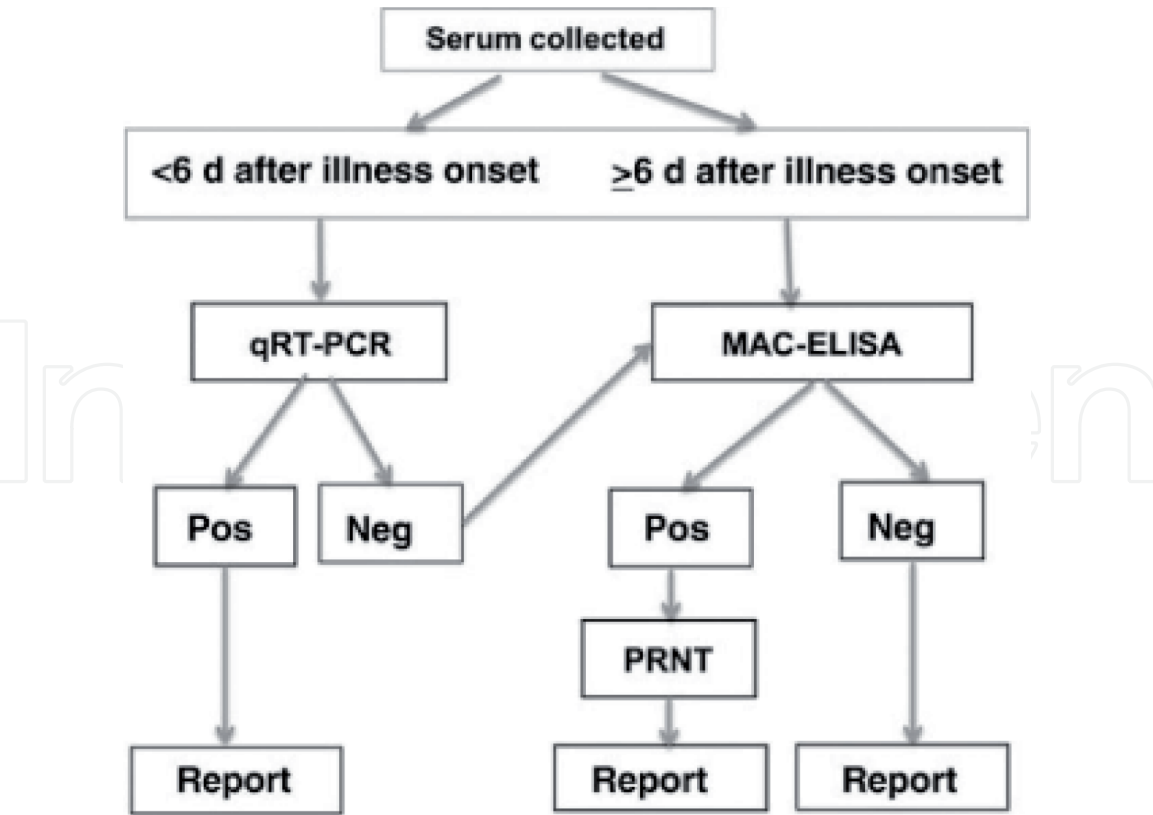


Figure 2.
The Centers for Disease Control and Prevention diagnostic testing algorithm for detection of chikungunya virus infection. Source of CDC.

In the CHIKV testing algorithm developed by the CDC arbovirus diagnostic laboratory, samples collected <6 days after onset of illness are first tested by virological methods (CHIKV real-time RT-PCR using two different set primers/probe that were developed to detect different genotypes (**Figure 2**)).

The CDC guidelines indicate that samples collected on or after day 6 of illness and samples with negative real-time RT-PCR results are tested by serological methods (the CHIKV IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) or an indirect fluorescent antibody assay (IFA) and plaque reduction hemagglutination test as confirmation) [67–70].

3.1 Types of samples for chikungunya infection diagnosis

Samples for chikungunya diagnosis have to be collected as soon as possible. Two different types of sample are suitable for virological and/or serological diagnosis.

Viral diagnosis depends on the characteristics of CHIKV infection and the time elapsed since the viral infection, the type of patient (e.g., fetus, pregnant, etc.), and clinical manifestation of infection. Virological diagnosis can be made using samples of blood (whole blood, plasma, or serum), urine, blister fluid, cerebrospinal fluid, amniotic fluid, or tissues such as the placenta and brain, among others.

Samples must be collected in strictly aseptic conditions and in the right container: sterile recipients for fluids and with transport viral medium for the others (blisters, biopsies, swabs, etc.).

Samples need to be sent to the laboratory quickly.

The best type of tube for serological diagnosis is serum separator. Heparin and EDTA are unsuitable for antibodies CHIK testing [71]. Serum used for serological test can be used for virological diagnosis, but plasma or whole blood (leukocytes) is preferable.

3.2 Virological diagnosis

As was described above, for virological diagnosis it is important to know the pathogenesis and the infection kinetics. The RNA viruses are found in plasma or serum specimens between 2 and 6 days following onset of fever, which corresponds to the period of incubation [41]. Viral load can subsequently rapidly reach up to $10^8/10^9$ genome copies/ml of blood [1, 26, 37].

Complete virions as well as viral fragments (antigen or genome) are frequent in this phase, although replication later declines. In line with this kinetic replication, three methods can be used in the laboratory: viral isolation and antigen or genomic detection.

3.2.1 Viral isolation

Viral isolation is generally a research tool [71–73]. For viral culture it is essential to preserve the complete virion. Culture sensitivity for chikungunya virus is high only in early infection (during the viremic phase) but drops 5 days after onset of illness. As a result, virus isolation is rarely used in the diagnosis of CHIKV infection because low sensitivity occurs after only a few days post-infection, and it is time-consuming and laborious procedure. However, it does allow for the identification of the viral strain and can be important for epidemiologic and research purposes. Samples where chikungunya virus is suspected should be handled under Biosafety Level (BSL) 3 conditions.

A wide variety of cells are used for in vitro CHIKV cultures in order to assess the full scope of the disease, including primary human skeletal muscle myoblasts [74], human blood monocytes [75], African green monkey kidney (Vero-E6) cells [76], and C6/36 (*Aedes albopictus* clone cells). Of particular interest, given the known tropism of chronic CHIKV for bone and synovial tissues, is the use of primary human fibroblast-like synoviocytes [77] and human osteoblasts.

Culture on HEL cells is more sensitive, and the presence of a cytopathic effect can be observed earlier than in Vero cells [78].

These cultures all need to be maintained under standard conditions.

3.2.2 Antigen detection

There are commercial immunochromatography antigen detection kits which detect CHIKV with high sensitivity in the early phase (up to 4–5 days after the onset of fever, when blood-enveloping proteins are still present). These tests also have high specificity since no cross-reactions with dengue virus are known to exist, and the method is straightforward and simple and does not require specially trained laboratory personnel [79].

However, a very important issue to consider is what genotype/s the tests are capable of detecting since not all detect all the known genotypes. Thus the geographical area from which the patient comes, meaning they are more likely to have one genotype or another, needs to be taken into account for accurate diagnosis (e.g., Okabayashi's immunochromatography only detects the Asian genotype) [80].

These methods are therefore, generally, sensitivity compromised.

3.2.3 Genomic detection (RT-PCR)

The diagnosis of CHIKV infection in the acute phase of infection is typically performed by the detection of viral RNA in plasma or serum (or other sample

types) by RT-PCR. The viral RNA can be detected by various molecular methods, such as nested and real-time PCRs [37].

Conventional and real-time PCRs have been used to amplify nsP1, nsP2, or even envelope protein genes (E3, E2, or E1) [81, 82].

Molecular diagnostic tests used for the detection of CHIKV include reverse transcription (RT) and amplification (PCR) assay of fragments in the nsP1, nsP2, nsP3, nsP4, or E1 regions of the CHIKV genome, in general by real-time [65, 82–86]. Labeled probes or SYBR Green is used for real-time quantification of the amplified PCR products [87, 88]. In these assays, the limits of detection range between 0.5 and 1.5 log₁₀ RNA copies/reaction. Although the detection of positive-strand RNA is commonly used for diagnostic purposes, assays to detect negative-strand CHIKV RNA have also been developed. These include strand-specific quantitative RT-PCRs for nsP1 [89] and nsP3 [90], which use a tagged-primer system to improve PCR specificity and accuracy. In comparison to the detection of positive-strand RNA, the detection limit for negative-strand RNA assays is slightly decreased, 3 log₁₀ RNA copies [91].

While there is an ever-present possibility of CHIKV spreading further in developed countries in the Americas and Europe, CHIKV still predominantly occurs in resource-limited countries. *Loop-mediated isothermal amplification* (LAMP) amplifies nucleic acid under isothermal conditions without the use of a thermal cycler and is a fast, specific, and cost-effective technique [92]; LAMP represents a cheaper alternative amplification method, albeit with lower sensitivity than real-time RT-PCR. Monitoring by turbidity as well as observation of color change after adding SYBR Green has also been described for the detection of CHIKV [85, 92]. Visualization is made with the naked eye, meaning no sophisticated equipment is required, and thus LAMP is especially useful in developing countries or in field studies.

Another method, *microfluidic lab-on-chip* integrating multiplex molecular amplification and DNA microarray hybridization, has been developed for the simultaneous detection of 26 globally important tropical pathogens such as CHIKV, *Dengue virus* (DENV), and other arboviruses [93]. Such diagnostic capacity provides an effective and rapid means to establish the presence of specific potential pathogens.

The CDC protocol for chikungunya infection diagnosis uses two RT-PCRs. The nucleotide sequences of the two sets of primers and probes used are listed in **Table 1** [94, 95]. The 3855 primer/probe set is specific to the ECSA genotypes although it is

Primer/genome 5' position	Nucleotide sequence
CHIKV3855F	GAGCATACGGTTACGCAGATAG
CHIKV3957C	TACTGGTGATACATGGTGGTTTC + TGCTGGTGACACATGGTGGTTTC
CHIKV3886FAM (probe)	ACGAGTAATCTGCGTACTGGGACGTA + ACGAGTCATCTGCGTATTGGGACGCA
CHIK856F	ACCATCGGTGTTCCATCTAAAG
CHIK962C	GCCTGGGCTCATCGTTATT
CHIK908FAM (probe)	ACAGTGGTTTCGTGTGAGGGCTAC

Table 1.
Chikungunya virus oligonucleotide primers and probes used in the Centers for Disease Control and Prevention real-time reverse transcription-polymerase chain reaction assays designed to detect CHIKV Asian genotype strains.

Virus	Name	Sequence (5'- 3')	Amplicon size
Dengue	DEN.ZIK-TR-S	AAGGACTAGAGGTTAGAGGAGACCC	83 bp
	DEN-TR-A	GAGACAGCAGGATCTCTGGTCT	
	DEN-FAM	AACAGCATATTGACGCTGG	
Zika	DEN.ZIK-TR-S	AAGGACTAGAGGTTAGAGGAGACCC	96 bp
	ZIK-TR-A3	GGCCAGCGTGGTGGA	
	ZIK-FAM	AAACAGCATATTGACG	
Chikungunya	CHK-TR-S	CGGCGACCATTGTGATCA	127 bp
	CHK-TR-A	TTCGTATTCCGTTGCGTTCTG	
	CHK-FAM	CCGGCATCCTTGCTA	
Yellow fever	WN.FA-TR-S	GGCTGGGGCAAYGGCT	91 bp
	FA-TR-A	CCTCAAACAAACTCATGGATTG	
	WN-FA-FAM	CATGCGCCAAATT	
West Nile	WN.FA-TR-S	GGCTGGGGCAAYGGCT	96 bp
	WN-TR-A	CAAGATGGTTCTTCCTATTGCCT	
	WN-FA-FAM	CATGCGCCAAATT	

Table 2.
Primers and probes used to detect DENV, CHIKV, ZIKV, YFV, and WNV.

capable of detecting both Asian and ECSA genotypes [95]. The 856 primer/probe set targets the Asian genotype, which has a slightly higher sensitivity than the 3855 set, which is prevalent in the Caribbean. This elevated sensitivity makes it a valuable tool for confirming diagnosis in samples where CHIKV RNA levels are reduced. These characteristics should be taken into account for testing protocols (i.e., which sets to use and in what order).

In our experience “in-house” real-time PCR is a useful tool in the chikungunya diagnosis. As such, proprietary primers and probe have been designed (**Table 2**) for CHIKV. In addition, for the syndromic diagnosis of imported viral diseases, our in-house RT-PCR can also detect other arboviruses such as Zika, dengue, yellow fever, and West Nile (**Table 2**) with sensitivities/specificities comparable to, or even superior to, commercially available techniques [63].

Genomic analysis is also used to classify and characterize viruses usually by Sanger sequencing method.

The sequencing of envelope E1 (viral structural glycoprotein) is often used for phylogenetic analysis. The three principal strains of CHIKV (ECSA, West African, and Asian) have been typed by nucleotide sequencing of a portion of the E1 region using RT-PCR [96].

Using sequencing studies to examine genomic evolution, it has been determined that global chikungunya outbreaks since 2005 have occurred as a result of a mutation of the ECSA lineage: an alanine-to-valine substitution in the E1 envelope glycoprotein at position 226 (A226V), which enabled the virus to acquire a new mosquito vector, *A. albopictus*, commonly known as the Asian tiger mosquito, conferring increased virus adaptation and replication ability in *Aedes albopictus*.

There are also specific regions that showed nucleotide variability and novel mutations making them suitable for phylogenetic analysis in nsP2 and E2 CHIKV regions [97].

3.3 Serological diagnosis

A variety of serological methods (hemagglutination inhibition, ELISA, complement fixation, and neutralization of viral infectivity using reference serum samples) can be used to characterize the alphavirus species [15, 98]. A fourfold increase in levels of CHIK virus IgG antibody in serum samples taken during the acute and the recovery phase is required to serodiagnosis. However, it is often problematic to collect paired samples, and so the existence of CHIKV-specific IgM antibodies in acute-phase samples is used instead. Also, patient serum coupled with neutralization by reference serum can be taken as definitive proof of the presence of CHIKV.

Several serological assays have been developed, the large majority of which demonstrate high reliability and specificity. The most common first-line serological techniques used for CHIKV diagnosis are the enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFA) assays, and the most suitable sample type is serum, generally using 1/100 dilutions. Commercially available ELISA tests, for example, have been shown to demonstrate high sensitivity (ranging from 82 to 88%) and specificity in samples from recovering patients [99, 100].

The presence of IgM antibodies indicates recent CHIKV infection [41], generally occurring in samples taken 2 weeks prior to patients becoming symptomatic. Detection of specific IgG antibodies, on the other hand, indicates previous CHIKV infection, which can be recent or as long ago as several months or years, given the persistence of anti-CHIKV IgG antibodies [1].

3.3.1 Enzyme-linked immunosorbent assay

CHIKV-specific IgM and IgG can be detected in serum using enzyme-linked immunosorbent assay immunocapture. The first antibodies detected in patients with chikungunya fever is CHIKV-specific IgM, which commonly appears 3–8 days after the onset of infection and may persist for several months to 2 years. After 4–10 days since onset of infection, the IgG antibodies become detectable in serum and may persist for years (**Figure 1**) [1] and be potentially lifelong. The chances of cross-reactivity between IgG and other viruses of the Semliki antigenic serocomplex are less now than in the past due to improvements in test design and procedure, but IgG seroconversion (greater than fourfold increase) is still recommended for reliable diagnosis. Furthermore, the existence of closely related alphaviruses in the area where the patient was infected necessitates the use of plaque reduction neutralization testing to confirm CHIKV infection [101].

ELISA is a rapid and sensitive method largely used for the detection of anti-CHIKV antibodies. The most common tests used for the diagnosis of CHIKV infection are IgM antibody-capture ELISA (MAC-ELISA) and indirect ELISA (i-ELISA) for the detection of type M (IgM) and type G (IgG) immunoglobulin, respectively [102]. A list of different commercially available ELISA tests is shown in **Table 3**.

Enzyme-linked immunosorbent assays can be used to confirm the presence of anti-CHIKV antibodies, with IgM antibody levels highest 3–5 weeks post-infection and persisting for up to 2 months.

ELISA is not, however, without its disadvantages: false-positives because of cross-reactivity with other alphaviruses (Ross River virus, Barmah Forest virus, and Sindbis virus [103]) and its sensitivity being much reduced (4–20%) in serum samples taken during the acute phase [104].

Company	IgM	IgG	Test
Euroimmun	+	+	ELISA
Novatec	+	/	IgM-capture ELISA
Novatec	/	+	ELISA
IBL	+	/	IgM-capture ELISA
IBL	/	+	IgG-capture ELISA
Abcam	+	+	ELISA
DRG	+	/	IgM-capture ELISA
DRG	/	+	ELISA
GenWay	+	/	IgM-capture ELISA
GenWay	/	+	ELISA
Standard diagnostic	+	+	ELISA
Euroimmun	+	+	IFA

Table 3.
Commercial diagnostic test available for serological diagnosis of CHIKV infection.

For these reasons, commercially available rapid diagnostic kits often do not provide as reliable results as RT-PCR because they all tend to detect host-derived anti-CHIKV IgM antibodies. Furthermore, since IgM antibodies are produced later in the course of infection than the antigen, this is a less sensitive test and as such can delay diagnosis and reduce the effectiveness of disease management [105].

3.3.2 Indirect immunofluorescence

IFA is an accurate and reliable technique widely used for the detection of specific anti-CHIK antibodies. IFA reveals the presence of type-specific antibodies against CHIKV by detecting the presence of virus antigens in infected cells.

The specificity of commercial IFA tests has been found to range from 75 to 100% in serum collected 5–6 days after infection [106]. That said IFA has some shortcomings in that it is laborious and the personnel carrying out the procedure need to be specially trained. In addition interpretation of the microscope examination results can be rather subjective, and there is no standardization between labs.

3.3.3 Antibody neutralization assay

The neutralization assay is generally used following ELISA or IFA results to confirm CHIKV. Neutralization involves the interaction of viral antigens and specific antibodies to block infection. The neutralization test requires the mixing of virus and serum, and the resulting mixture is then inoculated into cell culture. Inhibition of the virus can then be tested, using a variety of methods, after a number of days [107]. The microneutralization assay (MNA) evaluates the neutralizing antibodies. Although both methods have high specificity and sensitivity, there are disadvantages, namely, they are labor-intensive since only a small number of samples can be processed in each run and a Biosafety Level 3 laboratory (BSL-3) is required because live virus is being used.

The drawbacks to antibody testing include cross-reactivity with other alpha viruses and the problem of it not being able to distinguish between recent past and acute infection, as well as the fact that its sensitivity varies between clinical settings.

3.4 Diagnosis in other patients

Diagnosis is clearly always important for appropriate patient management, but it becomes crucial in several specific circumstances.

3.4.1 Transplantation or transfusion

CHIKV, like other arboviruses, can be transmitted by blood transfusion or through organ transplant from infected donors who were asymptomatic at the time of donation. Transmission via organ transplantation could also occur since chikungunya viremia (which may exceed 10^9 RNA copies/ml plasma) is likely prior to onset of symptoms [86, 108, 109].

In a case series of Rosso et al., 80% of the patients presented high viral load ($>10^6$ copies/ml), and none of the solid organ transplant patients with CHIKV infection developed graft rejection or died [110].

In these patients, blood and/or tissues are essential in the diagnosis.

3.4.2 Maternal-fetal transmission

Vertical transmission of CHIKV is a possibility and can affect the fetus, both congenital and perinatal infection having been demonstrated.

Congenital and perinatal CHIKV infections were reported following delivery to mothers with documented viremia accompanied by symptoms of CHIKF [16, 111, 112].

Vertical transmission rate of CHIKV has been estimated to range from 27.7 to 48.29%, and the same paper found transmission to be increased in mothers experiencing viremia at the time of birth. Neonatal symptoms were found to begin within 3–9 days postpartum, although there is no evidence of any link between birth method and incidence of congenital disease [113].

No link between CHIKV exposure in the first trimester of gestation and higher miscarriage risk or congenital malformation has been found to date [114, 115]. However, infection in the second or third trimester has been linked with increased fetal mortality, although the mechanism of this is unclear [111, 116]. Furthermore, a change in the evolution of neonates vertically infected with CHIKV has been seen following the spread of the virus to Indian Oceanic regions and Latin America in terms of the previously minor, self-limiting symptoms having been replaced by multiple severe manifestations and sometimes death [117].

Of particular note is the serious outbreak of CHIKV in 2006 in the Reunion Islands where complications were reported, including seizures and abnormal MRI scans [118, 119] as well as cardiac defects in almost 50% of patients [119]. In infants, these conditions can lead to death in the first year of life if swift and targeted medical intervention is not carried out [120, 121]. All patients in this outbreak who exhibited symptoms of neuroinvasive disease were infected with the A226V variant strain [122].

Virus infection in fetuses can be documented by positive RT-PCR in the amniotic fluid, and placenta, and in the cases of miscarriage, the involvement of CHIKV can be investigated in the brain or other fetal tissues [40].

3.4.3 Children

The first descriptions of CHIKF in children were made in the 1960s during outbreaks in India and South Asia [123–127]. In these younger patients, the infection

presented as a febrile illness, with rash and joint pain or dengue-like illness, hemorrhagic fever, or CSF infection. Asian lineage infection of children resulted in a lower frequency of arthralgia than in those infected by the African lineages, although hemorrhagic fever was more frequent in children testing positive for the former [117].

Lately, in the regions surrounding the Indian Ocean and the expansion of CHIKV into the Western Hemisphere, CHIKV-WH infections in children and neonates are reported more frequently and with more severe syndromes and sequelae [117].

4. Summary

In recent years, large-scale outbreaks of the chikungunya arbovirus (CHIKV) have permitted rapid propagation of the virus across the globe. CHIKV is transmitted by the *Aedes* mosquito, and the acute infection phase typically results in fever, joint pain, headache or rash, and chronic rheumatism (arthralgia or myalgia, anorexia, and concentration disorders) in up to 40% of cases.

The chronic form is defined by symptoms which persist for more than 3 months, and up to years, after initial diagnosis. The negative impact of CHIKV infection on the patient's health-related quality of life is quite severe and is not limited to the acute disease, and the repercussions may last for several months after clinical recovery. The highest prevalence of chronic discomfort was linked to one of the four genotypes described. These genotypes represent different geographic lineages (classification based on partial sequence of viral E1 glycoprotein): West African, East-Central-South-African (ECSA), ECSA-diverged or Indian Ocean Lineage, and Asian lineage.

The first marker detected in CHIK infection is the viral RNA, usually by RT-PCR. This marker can be present in samples taken within 8 days of onset of the illness. After this initial acute phase, the infection can also be diagnosed by serological detection of CHIKV-IgG and/or IgM-specific immunoglobulin, and sequencing studies can determine the infecting genotype.

The correct diagnosis of chikungunya infection enables the disease progression to be better predicted and patients' treatment to be more effectively managed.

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