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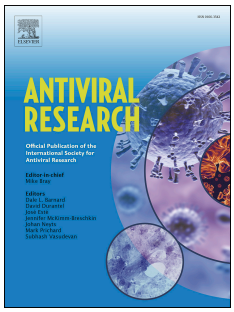

GloPID-R report on Chikungunya, O'nyong-nyong and Mayaro virus, part I: Biological diagnostics

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PII: S0166-3542(19)30124-X

DOI: https://doi.org/10.1016/j.antiviral.2019.03.009

Reference: AVR 4491

To appear in: *Antiviral Research*

Received Date: 5 March 2019

Accepted Date: 17 March 2019

Please cite this article as: Pezzi, L., Reusken, C.B., Weaver, S.C., Drexler, J.F., Busch, M., LaBeaud, A.D., Diamond, M.S., Vasilakis, N., Drebot, M.A., Siqueira, A.M., Ribeiro, G.S., Kohl, A., Lecuit, M., Ng, L.F.P., Gallian, P., de Lamballerie, X., on behalf of GloPID-R Chikungunya, O'nyong-nyong and Mayaro virus Working Group, Boyer, S., Brasil, P., Diallo, M., Failloux, A.B., Jaenisch, T., Lourenço-de-Oliveira, R., Neyts, J., Rios, M., Rodriguez-Morales, A.J., Rosa-Freitas, M.G., Sall, A., Simmons, G., Simon, F., Vega Rua, A., GloPID-R report on Chikungunya, O'nyong-nyong and Mayaro virus, part I: Biological diagnostics, *Antiviral Research* (2019), doi: https://doi.org/10.1016/j.antiviral.2019.03.009.

GloPID-R report on Chikungunya, O'nyong-nyong and Mayaro virus, part I: biological diagnostics

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Abstract

The GloPID-R (Global Research Collaboration for Infectious Disease Preparedness) Chikungunya (CHIKV), O'nyong-nyong (ONNV) and Mayaro virus (MAYV) Working Group is investigating the natural history, epidemiology and medical management of infection by these viruses, to identify knowledge gaps and to propose recommendations for direct future investigations and rectification measures. Here, we present the first report dedicated to diagnostic aspects of CHIKV, ONNV and MAYV. Regarding diagnosis of the disease at the acute phase, molecular assays previously described for the three viruses require further evaluation, standardized protocols and the availability of international standards representing the genetic diversity of the viruses. Detection of specific IgM would benefit from further investigations to clarify the extent of cross-reactivity among the three viruses, the sensitivity of the assays, and the possible interfering role of cryoglobulinaemia. Implementation of reference panels and external quality assessments for both molecular and serological assays is necessary. Regarding sero-epidemiological studies, there is no reported high- throughput assay that can distinguish among these different viruses in areas of potential co-circulation. New specific tools and/or improved standardized protocols are needed to enable large-scale epidemiological studies of public health relevance to be performed. Considering the high risk of future CHIKV, MAYV and ONNV outbreaks, the Working Group recommends that a major investigation should be initiated to fill the existing diagnostic gaps.

Keywords:

Alphavirus; Chikungunya virus; O'nyong-nyong; Mayaro virus; diagnostics

1. Introduction

The GloPID-R (Global Research Collaboration for Infectious Disease Preparedness) Chikungunya (CHIKV), O'nyong-nyong (ONNV) and Mayaro virus (MAYV) Working Group presents a report dedicated to diagnostic aspects of these pathogens. Overlapping clinical presentations, wide geographic spread and the possible selection of viruses capable of transmission by new vectors highlight the need for laboratory diagnostic support to unambiguously identify etiological agents in cases of undefined febrile illness with arthralgia and/or rash. This is important to ensure an early detection of cases and to support a clinical and public health response.

In order to assess the currently available molecular and serological tools to diagnose infections by CHIKV, ONNV and MAYV, the experts of the GloPID-R have performed a systematic review of English literature on the diagnostic aspects of the three viruses present on PubMed until September 2018. Diagnosis of the diseases at the acute phase mostly relies on molecular detection of the virus genomes: we discussed about kinetics of viral loads and biological sampling, choice of molecular tool,

available in-house and commercial molecular tests, international standards and external quality assessments organized to investigate laboratories' capability for viral detection. Alternatively, diagnosis can be provided by the detection of specific antibodies, so we focused on kinetics of immune response, choice of serological tool, commercially available tests, international standards and external quality assessments; moreover, cryoglobulinaemia and cross-reactivity, that can interfere with the correct identification of antibodies, were discussed, as well as cross-protection and cross-neutralization among the three pathogens.

Viral isolation by culture has not been taken into account because its turnaround time hardly fits with the need for an early diagnosis. Although in some cases (*i.e.* 2005-2006 CHIKV outbreak in Mauritius (1)) virus isolation has been performed for laboratory diagnosis with good sensitivity, providing results 2-3 days after inoculation, this diagnostic modality is being replaced by molecular techniques including RT-PCR, that are faster, more sensitive and safer and require less laboratory infrastructure. However, virus isolation remains a crucial tool in reference laboratories to characterize circulating strains to support control and development of diagnostic tools.

In this report, the experts identified knowledge gaps and provided adapted recommendations. The objective was to suggest relevant research priorities in the field, in order to improve individual patient care and outbreak management.

2. Background

2.1. Epidemiology

At least 5 million cases over the last 15 years (2) are reason enough to recognize *Chikungunya virus* (CHIKV) as an emerging global health threat. Data from the two largest CHIKV epidemics so far highlight the ability of the virus to spread rapidly over great distances and in multiple locations. During the 2004-2006 outbreak in the Indian Ocean region, attack rates peaked at 63% in the Comoro Islands; La Réunion Island registered 47,000 cases in a single week (January 30th to February 5th, 2006), with almost 40% of the population estimated to have experienced CHIKV disease; India

recorded at least 1.4 million cases at the end of 2006. In late 2013, CHIKV emerged in the New World, when in the island of Saint Martin CHIKV infection was identified in patients with no history of travel abroad. The virus spread to 45 countries and territories in North, Central, and South America and the Caribbean Islands; by the end of 2017, 544 deaths directly or indirectly related to the outbreak in the Americas were recorded, together with 2.5 million cases of infections. Transmission is ongoing in some locations (3).

CHIKV is a mosquito-transmitted alphavirus belonging to the family *Togaviridae* (4). The two major vectors of the disease are *Aedes (Stegomyia) aegypti* and *Ae. (Steg.) albopictus* (5), the latter identified during the 2004-2006 outbreak in the Indian Ocean, when the first in a series of adaptive mutations in the glycoprotein genes of the virus increased its replication in this specific vector (6,7). Two transmission cycles have been described. A sylvatic cycle maintains CHIKV in Africa involving forest-dwelling mosquito species (such as *Ae. africanus* and *Ae. furcifer–taylori*) and mainly nonhuman primates (8); in this context, humans are incidental hosts and become infected when they enter the forest or when infected vectors invade adjacent villages from the forest. Several wild animal species, especially nonhuman primates, have been investigated as possible virus reservoirs sustaining virus circulation in the environment in the absence of human cases (9). CHIKV can also be maintained through an urban human-mosquito-human transmission cycle, that involves *Ae. aegypti* and *Ae. Albopictus* mosquitoes; this cycle has been observed in the Americas, Indian Ocean, Asia and, more recently, Europe. In this urban context, humans can serve as reservoir and amplification hosts (10).

Several factors contribute to massive CHIKV circulation: e.g. high mosquito densities, including following recent invasions of the main urban vectors, *Aedes* species; vectorial capacity in transmitting the virus (high susceptibility to CHIKV as well as preference for feeding on human beings); high viremia level in infected humans; large population of susceptible individuals exposed for the first time in several countries; increasing travel from countries with ongoing circulation that enables

importation of viremic cases into virus-naïve regions, allowing the virus to extend its geographic distribution if conditions for local transmission are present.

CHIKV forms, with seven other alphaviruses, the Semliki Forest serocomplex, in which E1 envelope glycoprotein gene amino acid sequence divergence is below 40% (11). Among this serocomplex, viruses from the species *Mayaro virus* (MAYV) and *O'nyong-nyong virus* (ONNV) are of particular interest because they have potential to emerge in the human population (12,13).

ONNV is transmitted by anopheline mosquitoes; it is probably maintained through an enzootic cycle that has not been characterized yet, and humans can serve as amplification hosts during epidemics (14). MAYV is transmitted by *Haemagogus* species mosquitoes in an enzootic cycle in which nonhuman primates seem to be the main amplifier vertebrate hosts, with limited spillover to humans who frequently enter forest habitats (15).

CHIKV, ONNV and MAYV are phylogenetically related: they share some features that make differential diagnosis among the three viruses a challenging task.

2.2. Clinical presentation

Infections by CHIKV, ONNV and MAYV share an apparent similar clinical picture, with a "dengue-like" syndrome (10,14,15). This typically includes fever, headache and a papular or maculopapular rash during the acute stage, together with a more specifically remarkable incapacitating polyarthralgia, which is the hallmark of these so-called "alphavirus arthritogenic diseases". Although disease is generally self-limiting, severe joint pain can persist for months or even years in some individuals (16).

2.3. Co-circulation

CHIKV impacts human health globally, with local transmission reported in Asia, Africa, Europa, Americas, Pacific region and, transiently, in Europe (10,13). MAYV circulation has mostly been limited to South America with sporadic outbreaks next to forest environments (17–19). Recently detected MAYV infections in Haiti in a patient with no history of travel abroad living in a non-forest area, and

in Panama suggest (I) the spread of the virus towards Central America and the Caribbean and (II) a possible human-to-human transmission (20,21). ONNV is endemic in sub-Saharan Africa and it has been associated with sporadic large-scale epidemics. The first one, in 1959-1962, accounted for more than 2 million cases in eastern Africa alone (22); then, after an apparent absence of about 35 years, ONNV re-emerged in Uganda in 1996 (23), showing again its potential to cause massive outbreaks. Moreover, the actual distribution of ONNV in Africa is likely to be broader. Limited availability of diagnostic tools may have led to confusion of ONNV and CHIKV outbreaks (24).

Co-circulation of CHIKV-MAYV in Southern and Central America and CHIKV-ONNV in the African continent, do not allow to make a reliable diagnosis based on the geographic area where the infection is contracted; moreover, the wide circulation of other pathogens causing febrile illness (such as dengue virus and malaria-causing *Plasmodium* species) makes a travel history of the patient insufficient to narrow the differential diagnosis (25).

2.4. Transmission vectors

The three viruses do not share the same main mosquito vectors: CHIKV is primarily transmitted by *Aedes* spp; ONNV is unique among alphaviruses in its adaptation to *Anopheles* spp; MAYV enzootic vectors are mostly *Haemagogus* spp (26–28). However, experimental evidence that *Aedes* mosquitoes can transmit ONNV and MAYV exists (29–31), with a productive viral replication both *in vitro* and *in vivo*. As CHIKV has taught us, a single amino-acid change in the virus can result in increased vector competence from a mosquito species not normally considered a primary vector (32). The adaptation of CHIKV leading to higher competence of *Ae. albopictus* for virus transmission among humans illustrates that virus evolution might drive the involvement of other, more anthropophilic mosquito species.

3. Chikungunya virus (CHIKV)

3.1. Molecular diagnosis

3.1.1.Kinetics of viral load and dedicated biological sampling

CHIKV infections are characterised by high viremia levels (range: 10^4-10^8 RNA copies/mL (33,34)) during the acute phase, which includes the first 3-4 days after illness onset (35,36). This stage is followed by a rapid decrease of viremia (37): in 2008, Panning *et al*. (38) showed that real-time RT-PCR was 100% positive for all sera collected up to day 4 after illness onset, with sensitivity subsequently decreasing to 40% at day 7. Similarly, CHIKV loads have been proved to decrease significantly in serum from 5 days after the onset of symptoms onwards (39). Even if some persisting cases of viremia have been observed (up to 17 days) (40–42), molecular diagnosis should typically be performed within the first 7 days on an acute-phase specimen to confirm CHIKV infection (41,43,44). The preferred samples for diagnosis are serum or plasma (34,38,41,44,45), but other clinical samples have been evaluated for use in diagnosis.

3.1.1.1. Saliva

Saliva showed a lower sensitivity compared to blood for CHIKV: it has been observed that for confirmed CHIKV cases (by IgM or PCR in blood (46)) presenting during the 1st week after illness onset, detection rate in saliva was 58,3% compared to 86,1% in blood samples (44). Accordingly, testing saliva can be relevant during the 1st week post-symptoms onset if blood samples are difficult or impossible to collect, but is associated with decreased detection rate and lower negative predictive value.

3.1.1.2. Semen

Interestingly, semen has been found positive for a prolonged period of time after symptom onset (30 days), providing an expanded window for the molecular diagnosis of a recent infection with CHIKV (47). This finding needs to be further investigated, in order to assess the risk of sexual transmission of CHIKV and how this risk compares to the recently observed sexual transmission of other mosquito-borne viruses such as Zika virus (48).

3.1.1.3. Urine

The same study showed CHIKV RNA 30 days after initial symptoms in urine from a patient with a positive semen sample (47). Similarly, viral genomes were present up to day 30 post-infection in the urine of infected mice, long after viral clearance from the plasma (49). Recently, a case report described a patient with meningoencephalitis and ocular lesions having a positive urine sample 40 days after illness onset (50). However, Musso et al. (44) did not observe a prolonged window of detection of CHIKV in urine, with samples testing positive by PCR only if collected during the first week after symptoms onset. For confirmed CHIKV patients presenting during the first 7 days after illness onset, the detection rate in the urine was 8.3% compared to 86.1% in blood; after the 1st week after symptom onset, it decreased to 0%, with no positive urine samples that could confirm serological diagnosis of CHIKV infection (IgM+). Bozza *et al* observed low viral loads in the urine of patients with confirmed CHIKV infection (39). Considering the contradictory results, virus shedding kinetics in urine deserve a systematic re-assessment with enough power in the cohort size.

3.1.1.4. Breast milk

Recently, the presence of CHIKV RNA in breast milk was reported 23 days after the inception of symptoms in one patient (51); however, another study could not detect viral RNA in maternal milk in 8 samples collected during viremia (although plasma samples from the same patients were RT-PCR positive) (52).

3.1.1.5. Cerebrospinal fluid

In cases of CHIKV infections with neurological involvement, cerebrospinal fluid (CSF) samples can be tested with both serological and virological assays, with several studies describing detection of viral RNA in CSF (50,52–57). Case reports suggest that genomes can persist in the CSF for 7-10 days after illness onset (57,58), with viral loads in the CSF generally lower than in plasma (52).

3.1.1.6. Other fluids and tissues

The virus has been detected also in amniotic fluid, brain and liver biopsies, neonatal gastric fluids and placenta (52). Windows of detection of CHIKV genomes in different samples are presented in Figure 1.

3.1.2.Choice of test

Several diagnostic methods are available for viral genome detection. Molecular assays targeting nucleic acids are sensitive methods in the early stages of infection, before the rise of antiviral IgM antibodies (34). Isothermal amplification methods have been described for CHIKV, *i.e.* loop-mediated isothermal amplification (LAMP) and transcription-mediated amplification (TMA) (59–61). However, real-time RT-PCR remains the most frequently used technique for routine diagnosis at the acute stage of infection, because of its sensitivity, specificity and ease of performance (43). A positive result by real-time RT-PCR is enough to make a reliable diagnosis of CHIKV, ONNV or MAYV in areas where virus circulation has been previously documented. However, in case the virus is detected for the first time in a new geographic area, it is good practice to confirm a positive result by RT-PCR with a different test, to rule out a false-positive result caused by laboratory contamination. Conventional RT-PCR is still employed for research purposes (*i.e.* to identify to which lineage a viral strain belongs (20,62,63)) but is considered to be outdated for routine diagnostics.

3.1.3.In-house real-time RT-PCR

Several real-time RT-PCR systems have been published (34,38,64–68). Some of them have been evaluated in the External Quality Assessment (EQA) organized in 2014 by Jacobsen *et al* (69); their performances are discussed in the paragraph 3.1.6. An alignment of 50 CHIKV sequences available in Genbank representing main lineages was made to assess published in-house developed primer sets; GenBank accession numbers and results of this *in silico* analysis are presented in Table 1. Most PCR systems show one or more mismatches with CHIKV strains in different proportion and positions; particular attention was given to mismatches concerning the five 3' terminal nucleotides of a primer, because it is generally admitted that a single mutation in these positions can significantly compromise detection capability of a primer. The table highlights the fact that a number of available PCR systems are expected to detect viruses from the WA lineage less efficiently than viruses from the other lineages.

3.1.4.Commercial molecular tests

Several commercial tests from different companies were identified for CHIKV molecular detection by real-time RT-PCR (Supplementary data-Table 1). Some of them are formulated in monoplex format, whereas others are multiplex assays targeting dengue virus (DENV) and Zika virus (ZIKV) as well as in some cases yellow fever virus (YFV) and usutu virus (USUV). RealStar from Altona Diagnostics (Hamburg, Germany) is the only ready-to-use kit for real time RT-PCR evaluated in the literature through the comparison with a published real-time RT-PCR protocol (38,70). The target region is within the non-structural protein 1 (nsP1) gene and the manufacturer claims that the kit allows detection of all three CHIKV genotypes; however, lack of data on primers and probe sequences does not allow for an *in silico* analysis to confirm this information, and only the ECSA genotype was tested in the evaluation study. The 95% limit of detection (LOD) of the kit is 3.2 genome copies per reaction

using a quantified RNA from CHIKV ECSA strain (71). The evaluation study defined the LOD using two different thermocyclers, Lightcycler 2.0 and Lightcycler 480. Using *in vitro*-transcribed RNA copies of a fragment of an ECSA strain, 95% LOD was 5.3 copies per reaction with Lightcycler 2.0 and 3.8 copies per reaction with Lightcycler 480. Using a plaque-purified and plaque-quantified CHIKV ECSA strain, LOD was 0.51 PFU/mL with Lightcycler 2.0 and 0.34 PFU/mL with Lightcycler 480. High specificity was established by the absence of cross-reactivity against a large panel of non-CHIKV alphaviruses (including MAYV and ONNV) and non-alphaviruses. The RealStar assay had 100% sensitivity and specificity when compared with the previously published real-time RT-PCR of Panning et al. (38), which has shown good sensitivity (95%) and specificity (87,5%) values in the context of a multi-partner External Quality Assessment (69).

The RealStar kit has been used for testing blood donations prior to transfusion as well as for CHIKV diagnosis in patients. The French Blood Agency used it during the 2014 CHIKV Caribbean outbreak for individual NAT (nucleic acid testing) screening of blood products as a part of implemented strategy to prevent CHIKV transfusion-transmitted infections (TTIs) (72).

According to the datasheet of the company, the FTD DENV/CHIKV real time PCR kit from Fast-track Diagnostics has been evaluated with EQA panels from Quality Control for Molecular Diagnostics (QCMD) (73); all samples (19) were detected correctly except one.

During the 2014 CHIKV outbreak in the Americas, a prototype of real-time CHIKV/DENV target-capture, transcription-mediated amplification (TC-TMA) assay by Hologic, Inc. (San Diego, CA, USA) was used for high-throughput screening of blood products (74) on the Panther system (Hologic, Inc.). Tests were conducted during and after the CHIKV epidemic in Puerto Rico in 2014 to detect Asian genotype viruses in plasma samples. The Panther system automates all aspects of NAT-based blood screening on a single, integrated platform; it is based on Procleix technology (developed by Hologic and Grifols), already adapted for transfusion screening for HIV, HBV and HCV. During the epidemic, high sensitivity was evidenced for both individually tested samples and 16-sample minipools, with a 95% LOD of 9.9 and 158 copies per reaction, respectively.

The same TC-TMA assay was used by another group in the same period (2014 CHIKV Caribbean epidemic) (61), with a LOD of 8.2 copies/reaction. Positive TMA results were confirmed by PCR, microarray and next-generation sequencing (NGS) analysis.

3.1.5.International Standard (IS)

The first CHIKV RNA World Health Organization (WHO) International Standard (IS) for Nucleic Acid Amplification Techniques (NAAT)-Based Assays was accepted in October 2017 and is distributed by the Paul Ehrlich Institute (Germany) (75). It has been prepared from strain R91064 of the East/South/Central African (ECSA) genotype (Indian Ocean lineage), isolated from a patient returning from India to the USA in 2006 (34). The accession number for the Genbank sequence is KJ941050 (76). The freeze-dried preparation contains heat-inactivated virus that has been diluted in human plasma negative for anti-CHIKV antibodies. This reagent has been assigned a unitage of 2,500,000 International Units/mL, when reconstituted as recommended in 0.5 mL of sterile nuclease-free water. The material has been evaluated in an international collaborative study involving 25 laboratories performing a wide range of CHIKV NAAT assays (77).

A CHIKV RNA reference reagent (RR) was produced in 2015 by the CBER/FDA (Center for Biologics for Evaluation and Research/US Food and Drug Administration) (78). It is now available from the Office of Blood Research and Review, CBER/FDA (79). The reference reagent consists of cell culture-grown, heat-inactivated CHIKV diluted in human plasma and frozen. The strain is also the above-mentioned R91064. In a collaborative study involving 8 laboratories, the RR showed an estimated overall mean of 7.56 $\log_{10}$ detectable units/mL, ranging from 6.2 $\log_{10}$ to 8.6 $\log_{10}$ (80).

International Standards based on West African and Asian lineages have not been developed. This is of specific importance, because Asian lineage of CHIKV has been broadly circulating in Asia, Latin America and the Caribbean, and there are still episodes of circulation of the West African lineage of CHIKV in Senegal and most probably in neighboring countries (81,82).

3.1.6.External Quality Assessment (EQA)

Four EQAs (Table 2) have been organised to investigate laboratories' capability for detection of CHIKV RNA by molecular tools, three of which were coordinated by the European Network for Diagnostics of Imported Viral Diseases (ENIVD) (now EVD-LabNet).

Donoso-Mantke *et al.* (83) organized in 2007 a study involving 24 European laboratories to assess both molecular and serological diagnostics for CHIKV. Testing a panel by molecular tools, a great variability in performances was observed; in particular, 4 of the 20 laboratories that participated used assays with low sensitivity and specificity.

In 2007, Panning *et al*. (84) organized an international proficiency study with 31 participants from Europe, Asia, Africa and South America, who were asked to test the material with any molecular assay routinely used for detecting CHIKV in human plasma or with a real-time PCR protocol previously distributed by the study coordinator. In total, 17/31 laboratories lacked adequate sensitivity; those who used the assay distributed by the consortium showed the highest performances in term of sensitivity, proving that *ad hoc* improvement of molecular diagnostics was possible. False-positive results obtained with nested RT-PCR, a technique often affected by risk of contamination, confirmed it as obsolete for routine diagnosis.

In 2014, a third EQA (by Jacobsen *et al*.) involved 56 international laboratories from Europe, Asia, Africa, Middle East, Americas, Caribbean, Oceania (69), allowing for worldwide performance evaluation. Conventional in-house RT-PCR tests had the highest rate of correct results, although only 5 laboratories used it; however, low detection rate was observed for the lowest viral load of the dilution series. In-house real-time RT-PCR systems were the most commonly used techniques; in particular, systems from Pastorino (68) and Panning (38) were used by 9 and 8 out of 42 labs, respectively. As other PCR systems used in the EQAs (by Lanciotti and Edwards, both used by 3 labs (34,67)), they showed heterogeneous results with laboratories using the same protocol especially in terms of sensitivity, suggesting problems associated with laboratory procedures; specificity and

genotyping were more often correctly evaluated. Some commercial real-time RT-PCRs (used by 13 labs) raised issues for their lack of sensitivity and specificity (with the closely related ONNV frequently found positive); however, the identification of these poorly performing kits was not provided.

The most recent EQA was conducted in 2015 by Soh *et al*. (85) among 24 national-level public health laboratories in the Asia Pacific region to assess both CHIKV and DENV diagnostics. The majority of them requested receipt of a CHIKV-positive control and/or real-time or conventional RT-PCR protocols to develop and validate their capacity for CHIKV diagnosis. High degrees of sensitivity and specificity were observed, with 19/20 laboratories (95%) having detected correctly CHIKV; the only one that detected CHIKV in a serum-only plasma used a real time RT-PCR protocol.

3.2. Serological diagnosis

3.2.1.Kinetics of the immune response

Virus-specific IgM antibodies appear in the serum within 4-6 days after the onset of illness (37,86). Accordingly, IgM may be absent at the initial consultation, at the acute stage of the disease. They generally can be detected up to 3-4 months after infection (37,86,87), but can persist for more than one year, especially for patients with chronic arthralgia (88,89). In case of CHIKV infections with neurological involvement, cerebrospinal fluid (CSF) can be tested for IgM antibodies (90,91). According to case reports, they would appear a little later than in serum (57); moreover, the higher the IgM titer in the serum, the higher it is in CSF (53).

IgG antibodies are typically found as early as 6-7 days after illness onset, a few days after IgM appearance (37,87); like some IgM antibodies, they can directly neutralize CHIKV multiplication and can persist in immune individuals for many years (92). In the case of a strong and long-lasting IgM response, it seems that IgG seroconversion can occur late, with no IgG detected in some patients 90 days after symptom onset (39). Among all four IgG isotypes, IgG3 antibodies dominate in the naturally-acquired IgG response, and they are mostly specific for the E2 protein (93,94). Their

appearance during the early convalescent phase has been associated with virus clearance, long-term clinical protection and better outcome (95).

Cross-reactivity of CHIKV antibodies has been observed with members of Semliki Forest serocomplex, especially with viruses having a close genetic and evolutionary relationship (ONNV, MAYV, ross river virus-RRV) (96–98). It will be discussed more in detail in the paragraph 3.2.8.

3.2.2.Choice of test

After the period of viremia has ended, diagnosis may rely on virus detection in other fluids or tissues (see above) or on serological assays. A variety of laboratory diagnostic methods have been developed over time, including immunofluorescence tests (IFT), haemagglutination-inhibition assays (HIA), enzyme-linked immunosorbent assays (ELISA) and neutralization tests (NT).

ELISAs are the most commonly used assays for detection of both IgG and IgM antibodies. ELISA and IFT, in contrast to HIA and NT, can make the distinction between IgM and IgG; this is useful because the detection of IgM, or detection of IgG seroconversion or a four-fold rise in antibody titers in paired specimens (collected during the acute and convalescent phases of the disease) are generally used as criteria to make a reliable diagnosis of infection.

3.2.3.Commercial serological tests (IgM)

A search for commercially available tests identified different kits dedicated to the detection of anti-CHIKV IgM, most of which have been evaluated in clinical or EQA studies (99–105) (Supplementary data-Table 2 and table 3). Immunochromatographic rapid tests (CTK and SD Diagnostics) are an attractive diagnostic option, but their performances are characterized by low sensitivity (99–102,105). IFT by EUROIMMUN proved to be highly sensitive and specific (96,9% and 98,3% respectively) (103), although a variation in sensitivity was observed in two independent outbreaks caused by different strains of CHIKV, possibly due to amino acid differences in the viral E1 and E2 envelope proteins (102). Immuno-enzymatic tests represent the most common commercially

available assays, although performances in terms of sensitivity and specificity are quite diversified. According to Johnson et *al*. (99), who evaluated most of them, IgM ELISA by EUROIMMUN and InBios have the highest sensibility and sensitivity (although in a follow-up study EUROIMMUN ELISA was affected by cross-reaction, with detection of anti-ONNV IgM (100)). The Abcam ELISA also gave reliable results, although a considerable batch-to-batch variability was observed; ELISA assays by CTK, Genway, SD and IBL lacked sensitivity and are not recommended in their current format.

3.2.4.Commercially available serological tests (IgG)

Some of the commercially available tests for IgG detection have been evaluated (Supplementary data-Table 3 and table 4). IFT (immunofluorescent test) by EUROIMMUN showed high sensitivity and specificity (95,4% and 100%, respectively) (103). ELISA by EUROIMMUN and IBL proved to be specific (95% and 96%), but had lower sensitivity (88% and 52%); both detected anti-ONNV IgG, and the EUROIMMUN ELISA also anti-MAYV IgG (100).

3.2.5.International Standard (IS)

No IS for serological test is currently distributed.

3.2.6.External Quality Assessment (EQA)

Three EQAs (Table 5) were organised by ENIVD to investigate laboratories' capacity for serological detection of CHIKV infections. Donoso-Mantke organised in 2007 an EQA analysis that included 24 laboratories from 15 European countries (83). Of 18 participants that performed serologic assays, 14 tested for both IgM and IgG and 4 tested only for IgG. Serology testing revealed greater differences amongst laboratories than molecular testing. Good performances were proved for 8 out of 14 laboratories testing for both IgM and IgG, as well as for 3 out of 4 laboratories testing only for IgG; all the other laboratories lacked in sensitivity. No false-positive reactions from cross-reactivity with antibodies against viruses other than CHIKV were observed.

In 2007, 30 expert laboratories from 23 countries in Europe, the Middle East, Asia, Africa, North America and the Caribbean were involved in the second EQA on diagnostic serological proficiency (Niedrig et *al.* (106)). Only 6/30 obtained the highest score: for the others, a lack of sensitivity, especially for IgM, was observed. Most laboratories used in-house tests; IFT IgM/IgG by EUROIMMUN was the most common commercial assay. No significant variation in performance was observed when comparing the assay type (immunoenzymatic *versus* immunofluorescence assays) or origin (in-house *versus* commercial assays); a strong variability in diagnostic accuracy was reported among laboratories using the same commercial assay, probably due to improper handling of samples and/or assays.

The most recent EQA for serological detection of CHIKV was organized in 2014 (Jacobsen *et al*, (69)) involving 56 laboratories from 40 countries in Europe, Asia, Africa, the Middle East, the Americas, the Caribbean and Oceania; 46 and 50 data sets were returned for anti-CHIKV IgG and IgM, respectively. A lack of sensitivity and, to a lesser extent, specificity, were more common for IgM detection than for IgG, with 1/50 and 20/46 laboratories achieving the highest score for IgM and IgG, respectively. The most widely used type of technology was a commercial IFT, followed by in-house ELISA, commercial ELISA and in-house IFT; other techniques such as virus neutralization test (VNT) and haemagglutination inhibition assays (HI) were rarely used. Commercial IFT assays were less capable of detecting low IgM titres, but not with IgG in the same dilution series; in-house ELISAs proved to be more sensitive than commercial ELISAs, but less sensitive than IFT and VNT.

3.2.7.Cryoglobulinaemia (IgM)

Cryoglobulins are single or mixed immunoglobulins that undergo reversible precipitation at low temperatures. Cryoglobulinaemia refers to a condition with cryoglobulins in the serum; it has been described for several infectious diseases, particularly hepatitis C infection. A high prevalence of CHIKV-mixed cryoglobulinaemia (MC) (with type II, II-III or III cryoglobulins) has been described by

Oliver *et al*. (107) in CHIKV-infected travellers coming back from the Western Indian Ocean. According to this study, CHIKV-MC can lead to misdiagnosis of the disease when ELISAs are performed on samples kept at 4°C: specific anti-CHIKV IgM could be trapped in the cryoprecipitate, causing unexpected seronegativity for patients with clinical suspicion of CHIKV infection. To circumvent the problem, it is suggested to manage blood samples as required for any cryoglobulin research: sampling and centrifugation at 37°C, decantation and serum pre-warming before the ELISA assays.

3.2.8.Cross-reactivity

Because of phylogenetic relationships among the three viruses, cross-reactivity, especially between anti-CHIKV and anti-ONNV antibodies, is a major concern when serological tests are performed to make a reliable diagnosis. Cross-reactivity between anti-ONNV and anti-CHIKV antibodies has been primarily investigated as MAYV had a distinct geographic distribution in the past; however, with the appearance of CHIKV in the Americas (108) and MAYV in the Caribbean (20), the antigenic relationship between the two viruses needs to be evaluated more carefully.

CHIKV is closer phylogenetically to ONNV than to MAYV; this explains the substantial cross-reactivity observed for both IgM and IgG using ELISA and IFT (82,96,100,109). However, there are no documented studies evaluating the exact incidence of serologic cross-reactivity between the two viruses. Even in seroneutralization, it is difficult to differentiate antibodies against CHIKV and ONNV unless consistent differences in reciprocal cross-neutralization occur, which is not typical.

CHIKV and MAYV are more distant phylogenetically, but cross-reactivity has been observed with both ELISA and IFT (96,100,110); presumably, it is less extensive than with ONNV, but no accurate estimation of cross-reactivity incidence between CHIKV and MAYV could be identified in the literature.

3.2.9.Cross-neutralization and cross-protection

CHIKV-ONNV-MAYV: Cross-neutralization and cross-protection are important aspects to consider, because of implications for disease spread, as well as from the perspective of vaccine development. Despite this, few studies have been performed, suggesting that anti-CHIKV antibodies can neutralize and protect against MAYV and ONNV infections better than how anti-ONNV and anti-MAYV antibodies can do against CHIKV infection.

One-way cross-neutralization has been demonstrated for CHIKV-ONNV, with anti-CHIKV immune serum inhibiting ONNV plaque formation, while antiserum to ONNV is less effective against CHIKV strains (111). Similar results were observed in studies showing that serum or monoclonal antibodies (mabs) derived from ONNV-infected animals or humans weakly neutralize CHIKV (112,113): Blackburn *et al*. observed that, using a immunofluorescent test (IFT), 86% of the mabs against CHIKV reacted with ONNV, whereas only 53% of the ONNV mabs reacted with CHIKV strain. A possible explanation could be that, during its evolution, ONNV has retained most of the CHIKV antigenic sites, whereas some of ONNV epitopes have undergone greater conformational change, so that mabs prepared against them neutralize weakly or not at all against CHIKV (113).

The ability of anti-CHIKV antibodies to neutralize and protect against ONNV infection has been recently investigated *in vivo*: a recombinant CHIKV candidate vaccine was demonstrated to elicit a strong cross-neutralizing antibody response in a mouse model, conferring protection also against ONNV infection (114).

As concerns cross-neutralization between CHIKV and MAYV, a plaque-neutralization test has showed that MAYV antiserum slightly neutralizes CHIKV, while no inhibition effect on MAYV has been caused by CHIKV antiserum (112).

Considering overall findings about anti-CHIKV antibodies effect on MAYV and ONNV, they are diversified and need further assessment. Porterfield *et al.* (112) showed that CHIKV antiserum neutralizes efficiently ONNV but not MAYV. These results contrast with what observed by Fox *et al*. (115) using two murine mabs against epitopes on the B domain of the CHIKV E2 protein: *in vitro*, they

cross-neutralized MAYV more than ONNV; in a mouse model, they proved to be able to protect against MAYV infection, and to reduce disease caused by ONNV.

4. O'nyong-nyong and Mayaro virus

4.1. Molecular diagnosis

4.1.1.Kinetics of viral load and dedicated biological sampling

ONNV: Only few published studies have examined ONNV kinetics; similarly to CHIKV, ONNV viremia seems to last approximately one week or less (116).

MAYV: Case reports suggest a very narrow window during which molecular assays can detect circulating virus. Halsey *et al.* (117) observed negative results in two samples collected beyond day 3 of symptoms and tested by PCR; similarly, 4 days after the onset of illness Coimbra *et al.* could not detect viremia from two MAYV-case blood samples (118). However, an extended viremia (10 days) has been observed in an HIV-infected patient, possibly due to his immunocompromised status (119). For both ONNV and MAYV, molecular tests were performed only on sera collected at the acute stage of infection.

4.1.2.Choice of test

ONNV – MAYV: Suggestions about the choice of test for molecular diagnosis of infections are the same provided to detect CHIKV infection and have been discussed in the paragraph 3.1.2.

4.1.3.In house real-time RT-PCR

ONNV – MAYV: Few published studies have described PCR platforms for the detection of ONNV (66,120–122); they are listed in Table 6. As concerns MAYV, an alignment of 20 sequences available in GenBank, representing the main MAYV lineages, was made to assess primer sets developed in-house by various laboratories. The results of this *in silico* analysis are presented in Table 7; as explained for CHIKV, a single mutation in the five 3' terminal positions of a primer can significantly

compromise its detection capability, so mismatches concerning nucleotides in these positions are highlighted in the table. Most PCR systems use primers and probe showing one or more mismatches with MAYV strains.

4.1.4. Commercial molecular tests

ONNV – MAYV: They are not included as target in any commercial tests.

4.1.5.International Standard (IS)

ONNV – MAYV: No international standard for molecular detection of the two viruses has been developed.

4.1.6.External Quality Assessment (EQA)

ONNV – MAYV: No EQAs have ever been organised to evaluate laboratory' capacity for ONNV and MAYV detection.

4.2. Serological diagnosis

4.2.1.Kinetics of immune response

ONNV: Little is known about the kinetics of the antibody response to ONNV. IgM antibodies typically peak two weeks after the onset of illness and persist for about two months (62,116), although a few cases have been described with detectable IgM for 6 months or more (116). Information about anti-ONNV IgG comes only from two case reports: a traveller returning from Kenya experienced IgG seroconversion 26 days after disease onset (123); a report from Chad described a peak in a patient's IgG titre 68 days after the acute stage of illness (62).

MAYV: Anti-MAYV antibody kinetics are also poorly documented. IgM typically appears three days after the onset of illness (124) and lasts for three months or more, but not beyond six months (117,124). In contrast, IgG may persist for years (124). In a case report, the absence of IgG seroconversion was reported in a patient 3 months after onset of illness, probably because the time

between disease onset and the last blood sampling in this patient was too short to allow Ig class switching (125).

4.2.2.Choice of test

ONNV-MAYV: Suggestions about the choice of test for serological diagnosis of infections are the same provided to detect CHIKV infection and have been discussed in the paragraph 3.2.2.

4.2.3.Commercial serological tests

ONNV: There is no commercial kit for IgM or IgG detection.

MAYV: EUROIMMUN developed an anti-MAYV ELISA (IgM-IgG) but it has not been evaluated.

4.2.4.International Standard (IS)

ONNV-MAYV: No IS for serological tests is currently available.

4.2.5.External Quality Assessment (EQA)

ONNV-MAYV: No EQA has been organised to evaluate laboratory capacity for the serologic diagnosis of ONNV or MAYV infection.

4.2.6.Cryoglobulinaemia

ONNV-MAYV: The presence of cryoglobulins in sera from patients positive for CHIKV infection should have encouraged to assess it for ONNV and MAYV also. Actually, no study about cryoglobulinaemia in sera from patients positive for ONNV or MAYV has been performed.

4.2.7.Cross-reactivity, cross-neutralization and cross-protection

These items have been discussed in the paragraph 3.2.8 and 3.2.9.

5. Discussion

The development, validation and evaluation of diagnostic tools are crucial steps to developing accurate diagnostic methods of alphavirus infections. A considerable amount of financial resources and efforts are necessary to implement virus and virus-specific antibody detection. This justifies why diagnostic tools ideally should be developed before outbreaks, in order to ensure a rapid response, and it's valid especially for pathogens showing clear assumptions of large-scale dissemination.
The recent occurrence of extensive CHIKV epidemics has necessitated improved documentation of infection and has impelled laboratories and companies to develop specific molecular and serological assays. However, it has also highlighted the need for improved capacity for diagnostic surveillance, especially when the co-circulation of closely related viruses increases the chance of misdiagnosis. ONNV and MAYV have been discussed in parallel with CHIKV because their similar clinical presentations, serological cross-reactivity and geographic areas of co-circulation are significant barriers to specific diagnosis.

6. Knowledge gaps

6.1. Chikungunya virus

- Kinetics of viral load and dedicated biological sampling: different kinds of clinical samples have been used for the molecular detection of the virus. However, for some of them (i.e. urine and breast milk) the time window for detection of the viral genome remains unclear, with different

authors reporting contradictory results. Comparative studies to assess body fluids are missing: they should be performed to better document kinetics of viral loads and to identify the most appropriate samples for diagnostic use at the different steps of the disease.

- In-house real-time PCR protocols: several in-house real-time PCR protocols have been published, with different performances in the detection of the CHIKV lineages predicted from in silico analyses. An in vitro analytical evaluation would allow to better assess the adequacy of PCR systems to detect different lineages.
- Commercial real-time PCR assays: a large number of commercial tests are available for molecular diagnosis. The performances of only one kit have been reported under the form of a scientific article. Kits datasheets commonly provide insufficient information regarding the lineage(s) detected and how the performances of the assay were evaluated. Lack of information about primers and probes used does not allow assessment of adequacy for covering existing genetic variability. In the context of External Quality Assessments, some commercial tests are not clearly identified.
- Molecular International Standards: development of IS is essential for harmonisation of results among different laboratories; however, the only IS available for molecular diagnosis has been prepared with one ECSA strain, despite the significant genetic diversity between CHIKV lineages.
- Commercially available serological assays: a large number is available and several evaluations have been published; however, end-users would benefit from the guidance of a global and independent test evaluation.

6.2. O'nyong-nyong and Mayaro virus

- Kinetics of viral load and dedicated biological sampling: few cases are described in the literature and do not provide a clear picture of the kinetics of viremia. This could be problematic especially

for MAYV, which may have a shorter window of viremia compared to CHIKV and ONNV. To the best of our knowledge, no samples other than sera have been tested, so that kinetics of viral loads in different body fluids have never been described.

- Kinetics of the immune response: it is only documented from a few case reports; accordingly, it is not clear when antibodies would appear and how long they would persist in sera.
- In-house real-time PCR protocols: a few in-house real-time PCR protocols have been published for both pathogens. In silico analysis shows that most primers and probe sets have one or more mismatches with some MAYV evolutionary lineages. An in vitro analytical evaluation would allow to better assess the performances of the PCR systems and their adequacy to detect the different MAYV lineages.
- Commercial molecular and serological tests: none is available for ONNV; one serological assay (IgM) is available for MAYV, but it has not been evaluated.
- Molecular International Standards: none is available.
- External Quality Assessments (EQAs): they have never been organized to evaluate laboratories' capacity to diagnose ONNV and MAYV infection.

6.3. All three viruses

- Cryoglobulinaemia: false negative serological results due to cryoglobulinaemia have been reported for patients infected with CHIKV (only one study available). The presence and role of cryoglobulinaemia have not been investigated for ONNV and MAYV.
- Cross reactivity and virus co-circulation: substantial cross-reactivity between anti-CHIKV and anti-ONNV antibodies, as well as between anti-CHIKV and anti-MAYV has been reported, but poorly characterized. Differential diagnosis (CHIKV-ONNV and CHIKV-MAYV) in co-circulation areas is a

challenging task. Serological assays allowing differential identification of the infection by the different viruses are required.

- Cross-neutralization and cross-protection: few studies have been performed, which limits the ability to assess cross-neutralization and/or cross-protection.

7. Expert recommendations

7.1. Chikungunya virus

- In-house real-time PCR protocols: the numerous laboratory-developed protocols would benefit from a shared platform with in silico analysis of primers and probe sets; it could help to identify the most appropriate PCR for detection of circulating strains. A similar service has been previously proposed by the European Virus Archive website upon emergence of the CHIKV Asian genotype in the Caribbean. A host website governed by a non-commercial academic or public health organization, should be identified to implement this database and to contain results of laboratory comparative tests. After having defined mechanism of analysis and collection of results, this could be proposed as a model for other emerging viral diseases.
- Commercially available real-time PCR tests (i): analytical evaluation and comparative studies of commercial molecular assays should be performed to assess both sensitivity and specificity. Results of External Quality Assessments should be made available with a clear identification of the commercial assays used.
- Commercially available real-time PCR tests (ii): to assess the adequacy of commercial tests for genomic diversity of CHIKV, companies should make publicly available detailed information about primers and probes sets and/or provide updated performance analysis of their kits for detection of existing and newly identified variants.

- Serological tests: to better assess laboratory capacity for serological diagnosis, reference panels should be constituted and tested using operational tests. This could help to evaluate performances of both commercial and in-house tests.
- Commercially available serological tests: including commercial tests in EQAs and in comparative studies can help laboratories in their choice for the most reliable diagnostic assays.

7.2. O'nyong-nyong and Mayaro virus

- In general: both ONNV and MAYV require specific efforts for implementing clinical and epidemiological studies.
- Viremia: viremia kinetics, as well as viral loads in different body fluids, should be better documented.
- Immune response: the kinetics of the antiviral IgM and IgG response should be evaluated during the course of natural infection.
- Commercial molecular and serological tests: they should be developed and evaluated through comparative studies.
- International Standards (IS): they should be made available, taking into account the MAYV genetic heterogeneity.
- External Quality Assessments (EQAs): they should be organized to assess laboratory capacity of detecting ONNV and MAYV with molecular and serological tools.

7.3. All three viruses

- Kinetics of viral load and dedicated biological sampling: a detailed analysis of the presence of the viruses in different body fluids over the course of infection should be performed and lead to a

rational standardization of the process of clinical sampling according to the clinical presentation and stage of the disease.

- In-house real-time PCR protocols: the lack of information about assay performances could be filled by organizing comparative studies, with several laboratories testing reference panels by molecular methods; the evaluation should include other viruses at risk of cross-reactivity and different CHIKV and MAYV genotypes, in order to determine limit of detection (LOD) for each genotype. If necessary, new protocols for molecular diagnosis should be developed.
- Genomic sequence database: experts recommend that a genomic reference database should be made available similar to those existing for other viruses (e.g. see the sites of the Virus Variation Resource (126) or the Virus Pathogen Resource (127). This database would allow to store available sequence data, together with gene and protein annotations and information about isolation hosts and sources.
- International Standards (IS): it is highly recommended to develop IS for CHIKV Asian and West-African lineages, as well as for MAYV and ONNV.
- Cryoglobulinaemia: further studies should be implemented to confirm and better document the impact of cryoglobulinaemia in unexpected CHIKV seronegativity, as well as to extend investigations to ONNV and MAYV infections.
- Co-circulation: viral co-circulation requires the development of molecular and serological multiplex tools to differentiate CHIKV from ONNV in Africa and from MAYV in Southern and Central America.
- Cross-reactivity: multidirectional studies should be implemented to better define the exact extent of CHIKV cross-reactivity with ONNV and MAYV; this would be most probably required obtaining characterized samples for both naturally exposed humans and experimentally infected non-human primates.
- Cross-neutralization and cross-protection: cross-neutralization and cross-protection studies between the three viruses should be implemented, including studies to identify potential ONNV-

and MAYV- specific monoclonal antibodies; as for cross-reactivity, samples from both naturally exposed humans and experimentally infected non-human primates should prove useful for this purpose.

In summary, analysis of the currently available literature and consultation of experts indicate that inadequate diagnostics are currently available for ONNV and MAYV and, to a lesser degree, for CHIKV. Gaps in diagnostic tools and protocols allowing the identification of the etiological agent during the course of CHIKV, ONNV and MAYV infections have been identified for both diagnosis during the acute phase and for the long-term follow-up of patients, as well as for sero-epidemiological studies.

Diagnosis of the disease at the acute phase relies mostly on molecular detection of the virus genome. Molecular assays have been described for CHIKV, ONNV and MAYV but require further evaluation, standardized protocols and the availability of international standards representing the genetic diversity of the viruses. Alternatively, diagnosis can be provided by the detection of specific IgM, but the exact extent of cross-reactivity between the three viruses, the sensitivity of the assays, and the possible interfering role of cryoglobulinaemia require further investigation. Implementation of reference panels and EQAs for both molecular and serological assays is necessary.

Regarding sero-epidemiological studies, there is no reported high throughput assay that enables the different viruses to be distinguished in areas of potential co-circulation. Even neutralization assays can lead to ambiguous interpretation. New specific tools and/or improved standardized protocols are needed to enable large-scale epidemiological studies of public health relevance to be performed.

Regarding the long-term follow-up of patients infected by CHIKV (and potentially ONNV and MAYV) there is currently no biological marker of medical significance associated with disease progression and prognosis. This obviously requires a complete reassessment and specific investigation.

Finally, when pathogens emerge or re-emerge, there is little time for development of diagnostics, which should be designed and validated in advance to ensure a rapid response. Considering the high

risk of future CHIKV, MAYV and ONNV outbreaks, it is highly recommended that a major investigation should be initiated to fill existing diagnostic gaps.

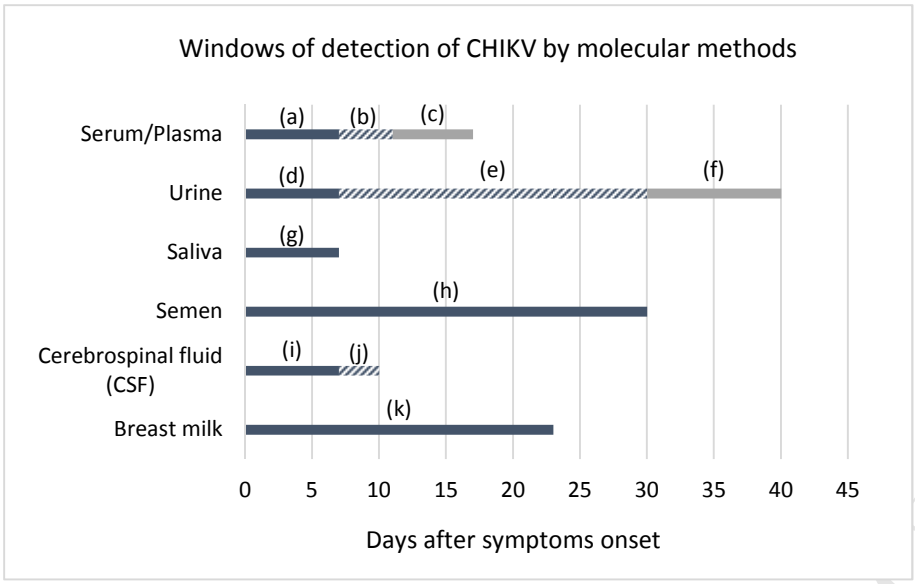

Fig. 1. Windows of detection of CHIKV genomes in different kinds of samples by molecular methods. The letters in brackets indicate the references. (a): (34,38,44); (b): (40,41); (c): (41); (d): (44); (e): (47,49); (f): (50); (g): (44); (h): (47); (i): (58); (j): (57); (k): (51).

In house real-time PCR systems						CHIKV WA (nb seq=10)	CHIKV ECSA (nb seq=10)	CHIKV IOL (nb seq=10)	CHIKV ASIAN (nb seq=10)	CHIKV ASIAN/AMERICAN (nb seq=10)
Target(s)	Reference	Technique	Amplicon size (bp)			Mismatch positions in primers and probe; the 3' terminal position corresponds to position 1; mismatches in the five 3' terminal nucleotides are indicated in red and bold; (nb of CHIKV sequences with mismatch in the specific lineage considered)				
NSP2	Waggoner et *al*. (64)	Hydrolysis probe	96	FW primer 5'-3'	CATCTGCACYC AAGTGTACCA	**Pos 9: A>G (1)**				
				RV primer 5'-3'	GCGCATTTTGC CTTCGTAATG			**Pos 13: T>C (1)**		
				Probe	GCGGTGTACA CTGCCTGTGAC YGC					
E3	Cecilia et *al*. (65)	Hydrolysis probe	64	FW primer 5'-3'	CGAAAARGAR CCGGAGRAA	**Pos 1: A>C (10)** **Pos 2: A>G (10)** **Pos 4: G>A (10)**	**Pos 7: G>A (2)** **Pos 14: A>G (2)** **Pos 19: C>T (2)**			
				RV primer 5'-3'	GATAGTACCCR GGKCTCATGAC GTT	**Pos 6: G>C (10)** **Pos 18: C>T (10)** **Pos 24: A>G (10)**		**Pos 4: C>T (1)**	**Pos 3: G>A (3)**	**Pos 3: G>A (10)**
				Probe	CCCTRCGCATG CTTGA	**C>T (10)**			**C>T (3)**	**C>T (10)**
E1	Smith et *al*. (66)	Hydrolysis probe	253	FW primer 5'-3'	CAGTGATCCCG AACACGGTG					
				RV primer 5'-3'	CCACATAAATG GGTAGACGCC	**Pos 3: G>T (10)**			**Pos 9: G>A (1)** **Pos 15: A>G (10)**	**Pos 15: A>G (10)**
				Probe	CCGTCATCCCG TCTCCGTACGT GAA	**C>T (10)** **G>C (9)** **G>T (1)** **T>C (10)**	**C>T (3)**		**C>T (3)** **T>C (1)**	**C>T (10)**
NSP1	Smith et *al*. (66)	Hydrolysis probe	74	FW primer 5'-3'	TTTGTGATCAA ATGACCGGCAT C	**Pos 1: C>T (9)** **Pos 4: C>T (10)** **Pos 7: C>A (10)**				
				RV primer 5'-3'	TCAGCCCCACC AACAGCTTC	**Pos 8: A>G (10)** **Pos 16: C>T (10)** **Pos 17: G>A (1)**	**Pos 8: A>G (10)**			
				Probe	TTGCTACAGAA GTCAC	**T>C (10)** **A>G (10)** **A>G (10)** **C>T (10)**	**A>G (1)**			
NSP2	Smith (66)	Hydrolysis probe	70	FW primer 5'-3'	CCGAAAGGAA ACTTCAAAGCA ACT	**Pos 1: T>A (9)** **Pos 10: C>T (10)** **Pos 13: C>T (10)**	**Pos 10: C>T (10)**		**Pos 7: A>G (7)**	**Pos 7: A>G (10)**
				RV primer 5'-3'	CAGATGCCCG CCATTATTGAT G	**Pos 2: T>A (10)** **Pos 17: G>A (10)**			**Pos 5: T>C (10)** **Pos 21: A>G (1)**	**Pos 5: T>C (10)**

				Probe	GGGA**G**GTGGA **G**CA**T**G	**G>A (10)** **G>A (10)**			**T>C (10)**	**T>C (10)**
5'UTR	Smith et *al*. (66)	Hydrolysis probe	98	FW primer 5'-3'	ACACAC**G**TAGC **CTACCAG**TTTC		**Pos 5: G>T (1)** **Pos 6: A>G (1)** **Pos 11: C>T (1)**	**Pos 11: C>T (1)** **Pos 16: G>A (1)**	**Pos 8: C>T (1)** **Pos 16: G>A (1)**	**Pos 8: C>T (1)**
				RV primer 5'-3'	GCTGTC**A**GCGT CTAT**G**TCCAC	**Pos 15: A>G (10)**			**Pos 6: G>A (7)**	**Pos 6: G>A (7)**
				Probe	TA-CTGCTCTACTC TG		**- >A (1)**			
NSP1	Panning et *al*. (38)	Hydrolysis probe	82	FW primer 5'-3'	TGA**T**CCCGACT CAACCATCCT			**Pos 18: T>C (10)**		
				RV primer 5'-3'	GGCA**A**ACGCA GTG**G**TACTTCC T	**Pos 9: G>A (2)**	**Pos 18: A>G (4)**	**Pos 18: A>G (4)**		
				Probe	**G**CCAGCAAGG AGGATGATGT CGGA		**G>A (2)**			**G>A (1)**
NSP1	Lanciotti et *al*. (34)	Hydrolysis probe	87	FW primer 5'-3'	AAA**G**GG**C**AA**A** CT**C**AGCTTCAC	**Pos 12: A>G (10)** **Pos 15: C>G (10)** **Pos 18: G>A (10)**	**Pos 12: A>G (6)**		**Pos 9: C>T (9)** **Pos 12: A>G (9)**	**Pos 9: C>T (9)** **Pos 12: A>G (9)**
				RV primer 5'-3'	GCC**T**GG**GCT**CA TCGTTATTC	**Pos 11: C>A (10)** **Pos 17: T>C (10)**	**Pos 13: C>T (1)** **Pos 14: G>A (1)**		**Pos 2: T>C (1)** **Pos 17: T>C (1)**	
				Probe	CGCTGTGA**T**AC AGTGGTTTCGT G**T**G		**T>G (10)**		**T>C (9)**	
NSP4	Lanciotti et *al*. (34)	Hydrolysis probe	125	FW primer 5'-3'	TCA**CT**CC**C**TG**T** TGGAC**T**TGATA GA	**Pos 20: T>C (10)** **Pos 21: C>T (10)**	**Pos 14: T>C (2)**	**Pos 14: T>C (10)**	**Pos 8: T>C (3)** **Pos 17: C>T (2)**	**Pos 8: T>C (10)** **Pos 17: C>T (10)**
				RV primer 5'-3'	TTGACGAACA G**A**GTTA**GG**AA CATACC	**Pos 9: G>A (3)** **Pos 10: G>A (1)** **Pos 15: A>G (10)**				
				Probe	**A**GG**T**AC**G**C**G**C TT**C**AAGTT**C**GG **C**G	**A>G (10)** **T>C (10)** **G>T (1)** **C>T (10)** **C>T (10)**	**C>T (1)**		**C>T (1)**	
E1	Edwards et *al*. (67)	Hydrolysis probe	126	FW primer 5'-3'	TCGA**C**GC**G**CC**C** TC**T**TTAA	**Pos 4: T>G (10)** **Pos 11: G>A (10)** **Pos 14: C>T (10)**	**Pos 5: T>C (1)** **Pos 14: C>A (1)**		**Pos 8: C>A (10)**	**Pos 8: C>A (10)**
				RV primer 5'-3'	ATCGAATGCAC CGCACACT					
				Probe	A**C**CAGCCTGCA **C**CCA**T**TCCTC**A** GA**C**	**C>T (10)** **T>C (1)** **A>C (10)**	**C>T (2)**		**C>T (4)** **A>G (1)**	**C>T (10)**
E1	Pastorino et *al*. (68)	Hydrolysis probe	208	FW primer 5'-3'	AAGCTYCGCGT CCTTTA**C**CAAG		**Pos 5: C>T (1)**			
				RV primer 5'-3'	CCAAA**TT**GTCC YGGTCTTCCT				**Pos 16: T>C (1)**	

				Probe	CCAATGTCYTC MGCCTGGACA C**C**TTT		**C>G (1)**			

Table 1. Ten sequences were selected for each lineage (CHIKV WA, GenBank accession numbers: HM045815, HM045818, AY726732, HM045817, HM045785, HM045798, HM045786, HM045807, HM045819, HM045820; CHIKV ECSA: AF369024, AF490259, KJ679577, KP164570, KY704947, HM045822, HM045806, HM045795, HM045792; JQ067624; CHIKV IOL: GQ428211, FJ807896, FJ000062, FJ807899, GQ428212, EF027136, EU564334, EF012359, HQ456254, MG664850; CHIKV Asian FJ807897, HM045791, HM045787, HM045789, HM045814, HM045810, HM045788, EF027140, EU703759, KT308159; CHIKV Asian/American: KR559497, KR559496, KR559473, KR559492, KJ451624, KR046227, KR046231, LN898098, LN898093, KR559493). Mismatch positions between this reference sequence panel and the primers and probes are indicated in the table; mismatches concerning the five 3' terminal nucleotides of a primer are indicated in red and bold characters. In probes, 1 or 2 mutations generally do not compromise the hybridization potential, but this also depends on the length of the probe and its G+C content.

Year	Reference	Participants	Study coordinator	Objectives	Other viruses tested in the panels	Results of molecular assessment
2007	Donoso-Mantke *et al*. (83)	Total: 24 labs from 15 European countries // 20 labs for CHIKV PCR	European Network for Diagnostics of Imported Viral Diseases (ENIVD)	Assessing quality for CHIKV molecular and serological diagnostics	-	16/20 labs showed sufficient sensitivity and specificity
2007	Panning *et al*. (84)	Total: 31 labs from Europe, Asia, Africa, South America	European Network for Diagnostics of Imported Viral Diseases (ENIVD)	Coordinated Implementation of CHIKV RT-PCR	-	14/31 labs met proficiency criteria
2014	Jacobsen *et al*. (69)	Total: 56 labs from 40 countries (Europe, Asia, Africa, Middle East, Americas, Caribbean, Oceania)	European Network for Diagnostics of Imported Viral Diseases (ENIVD)	Assessing quality for CHIKV molecular and serological diagnostics	SINV, ONNV, DENV	23/60 data sets classified as "optimal", 7/60 "acceptable", 30/60 "need for improvement"
2015	Soho *et al*. (85)	Total: 24 labs from 22 countries and areas (South-East Asia, Western Pacific Regions) // 20 labs for CHIKV PCR	-	Assessing quality for CHIKV molecular diagnostic and for DENV molecular and serological diagnostics	DENV	19/20 labs reached the highest score

Table 2. EQAs for molecular detection of CHIKV RNA.

Kit	Company	Method	Performance (ref)
Human Anti-CHIKV IgM ELISA Kit	Abcam	IgM ELISA	Lot-to-lot variation: good concordance with CDC results vs low sensitivity (99)
CHIK IgM ELISA Test CE	CTK Biotech	IgM ELISA	CDC: Low sensitivity (99)
Chikungunya IgM Combo Rapid Test CE		IgM-rapid test	CDC: Low sensitivity (99–102)
Anti CHIKV IFT	EUROIMMUN	IFT	CDC: High accuracy and reproducibility; Good sensitivity and specificity; Variation in sensitivity for Ab against different CHIKV strains (99,102,103)
Anti-CHIKV ELISA (IgM)		IgM ELISA	CDC: High accuracy and reproducibility; Cross-reactivity with anti-ONNV Abs; High sensitivity; False neg and false pos results (99,100,104)
Chikungunya IgM μ-capture ELISA	GenWay	IgM ELISA	CDC: Low sensitivity (99)
Chikungunya IgM μ-capture ELISA	IBL International	IgM ELISA	Cross reactivity with anti-ONNV Abs; False neg and false pos results (100)
CHIKjj Detect™IgM ELISA Kit	InBios	IgM ELISA	CDC: High accuracy and reproducibility; High sensitivity (99,104)
SD Chikungunya IgM ELISA	SD Standard Diagnostics	IgM ELISA	CDC: Low sensitivity (99,105)
SD BIOLINE Chikungunya IgM		IgM - rapid test	CDC: Low sensitivity (99–101,105)

Table 3. Performances of commercial tests for the detection of anti-CHIKV IgM antibodies, according to published evaluations.

Kit	Company	Method	Performance (ref)
Anti CHIKV IFT	EUROIMMUN	IFT	Good sensitivity and specificity; Variation in sensitivity for Ab against different CHIKV strains (102,103)
Anti-CHIKV ELISA (IgG)		IgG ELISA	Quite good sensitivity and specificity; Cross-reactivity with anti-ONNV and anti-MAYV Abs; False neg and false pos results (100)
CHIKjj *Detect*™IgG ELISA Kit	InBios	IgG ELISA	Quite good sensitivity and specificity; Cross-reactivity with anti-ONNV Abs; False neg and false pos results (100)

Table 4. Performances of commercial tests for detection of anti-CHIKV IgG, according to published evaluations.

Year	Reference	Participants	Study coordinator	Objectives	Other viruses tested in the panels	Results of serological assessment
2007	Donoso-Mantke *et al*. (83)	Total: 24 labs from 15 European countries // 18 labs for CHIKV serology	European Network for Diagnostics of Imported Viral Diseases (ENIVD)	Assessing quality for CHIKV molecular and serological diagnostics	-	8/14 labs showed good results for both IgM and IgG
2007	Niedrig *et al*. (106)	Total: 30 labs from 23 countries (Europe, Middle East, Asia, Africa, North America, Caribbean)	European Network for Diagnostics of Imported Viral Diseases (ENIVD)	Assessing quality for CHIKV serological diagnostics	DENV, WNV	6/30 labs with 100% correct results, 13/30 labs with ≥ 85% correct results, 11/30 labs with ≤ 75% correct results
2014	Jacobsen *et al*. (69)	Total: 56 labs from 40 countries (Europe, Asia, Africa, Middle East, Americas, Caribbean, Oceania)	European Network for Diagnostics of Imported Viral Diseases (ENIVD)	Assessing quality for CHIKV molecular and serological diagnostics	RRV, WNV, DENV	IgM: 1/50 dataset classified as "optimal", 9/50 "acceptable", 40/50 "need for improvement". IgG: 20/46 "optimal", 2/46 "acceptable", 24/46 "need for improvement"

Table 5. EQAs for serological detection of CHIKV.

Year	Reference	Method	Target
2017	Waggoner *et al*. (120)	Monoplex real time RT-PCR	5'UTR-nsP1
2016	Liu *et al*. (121)	Real-time PCR-based TaqMan array card (TAC) (15 viruses)	E1
2013	Saxton Shaw *et al*. (122)	Monoplex real time RT-PCR	E1
2009	Smith *et al*. (66)	Monoplex real time RT-PCR	E1
			nsP1
			nsP2
		Multiplex (OONV-CHIKV) real time RT-PCR	5'UTR-nsP1

Table 6. Published reports of RT-PCR systems for the detection of ONNV, in decreasing order of the year of publication.

In house real-time PCR systems						MAYV D (nb seq=10)	MAYV N (nb seq=1)	MAYV L (nb seq=9)
Target(s)	Reference	Technique	Amplicon size (bp)			Mismatch positions in primers and probe; the 3' terminal position corresponds to position 1; mismatches in the five 3' terminal nucleotides are indicated in red and bold; (nb of CHIKV sequences with mismatch in the specific lineage considered)		
5'UTR-nsP1	Waggoner *et al.* (128)	Hydrolysis probe	109	FW primer 5'-3'	AAGCTC**T**TCC TCTGCATTGC	**Pos 14: T>A (1)**		
				RV primer 5'-3'	TGCTGGAAAC YGCTCTYTGTA			
				Probe	GCCGAGAGCC CGTTTT**T**TAAA ATCAC			**T>C (3)**
nsP1	Naveca *et al.* (129)	Hydrolysis probe	59	FW primer 5'-3'	CACGGACMTT TTGCCTTCA			
				RV primer 5'-3'	AGAC**T**GCCAC **C**TCTGCTKG**A** G	**Pos 2: A>G (1)** **Pos 8: T>1 (1)** **Pos 17: T>C (2)**	**Pos 11: C>T (1)**	**Pos 17: T>C (1)**
				Probe	ACAGATCAGA CATGCAGG			
E1	Llagonne-Barets *et al.* (130)	SYBR green	165	FW primer 5'-3'	TTCCRAAYCA AGTGGGATTC			
				RV primer 5'-3'	CACTTTACGT AYGGKGATGG	**Pos 3: T>C (1)**	**Pos 9: Y>A (1)**	
				Probe	NA			
nsP1	Friedrich-Jänicke *et al.* (131)	Hydrolysis probe	95	FW primer 5'-3'	CCTTCACACA GATCAGAC			
				RV primer 5'-3'	GCCTGGAAGT ACAAAGAA			
				Probe	**G**GTGGC**A**GTC TATCA**G**GATG **T**CTATG	**A>G (2)** **G>A (5)** **C>T (2)**	**G>A (1)**	**A>G (1)** **G>A (1)**
E2	Long *et al.* (30)	Hydrolysis probe	127	FW primer 5'-3'	CAAATGTCCA CC**A**GGCGAA **G**	**Pos 8: A>G (1)**		**Pos 1: G>C (2)** **Pos 8: A>T (3), A>C (5)** **Pos 13: T>C (4)**
				RV primer 5'-3'	GTGGTCG**C**AC **A**GT**G**AATCTT TC	**Pos 12: A>C (4)** **Pos 15: C>T (1)**	**Pos 9: G>A (1)**	**Pos 12: A>C (9)** **Pos 21: T>C (8)**
				Probe	GAC**C**TGT**C**GG ATAGCCT**A**CC A**C**CAT	**C>T (1)**	**C>T (1)**	**C>T (6)** **T>C (9)** **A>T (5)**

Table 7. We selected ten sequences for MAYV lineage D (GenBank accession numbers: KP842806, KP842795, KM400591, KJ013266, DQ001069, KP842807, KP842813, KP842800, KP842802, KP842809), one for MAYV lineage N (GenBank accession number: KP842812), nine for MAYV lineage L (GenBank accession numbers: KP842819, KP842820, KP842818, KT818520, KX496990, AF237947, NC_003417, KY618133, KY985361). Mismatch positions between this reference sequence panel and the primers and probes are indicated in the table; mismatches concerning the five 3' terminal nucleotides of a primer are indicated in red and bold

characters. In probes, 1 or 2 mutations do generally not compromise the hybridization potential but this also depends on the length of the probe and its G+C content. D: dispersed; L: limited; N: new.

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- Chikungunya, O'nyong-nyong and Mayaro virus represent emerging global health threats.
- Similar clinical presentations and geographic areas of co-circulation are significant barriers to specific diagnosis.
- Available molecular tests require further evaluation, standardized protocols and availability of International Standards.
- Low sensitivity of the assays and cross-reactivity may affect the correct identification of antibodies by serological methods.
- Experts recommend that a major effort should be done to implement available diagnostic tools.