DIAGNOSTIC CHALLENGES IN FLAVIVIRUS INFECTIONS

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ABSTRACT

Flaviviruses are arthropod-borne viruses which have been involved in most significant epidemics worldwide: dengue, West Nile fever, Japanese encephalitis, Zika, yellow fever, tick-borne encephalitis. Because of their close antigenic relatedness and of the fact that most often in a region more flaviviruses co-circulate, the laboratory diagnostic may be extremely difficult. We review the main diagnostic methods of both autochthonous and imported from endemic regions flaviviruses, and discuss them based on the experience of our laboratory.

Keywords: Flavivirus, diagnostic, dengue, West Nile virus, Zika infection, tick-borne encephalitis, yellow fever.

REZUMAT

Flavivirusurile sunt virusuri transmise de artropode vectoare care au fost implicate, pe plan mondial, în infecţii cu potential epidemic major: denga, febra West Nile, encefalita japoneză, infecţia Zika, febra galbenă, encefalita de capuşe. Din cauza strânsei înrudiri antigenice şi a faptului că, cel mai adesea, circulă concomitent mai multe flavivirusuri într-o anumită regiune, diagnosticul de laborator poate fi extrem de dificil. În prezentul articol, trecem în revistă principalele metode de diagnostic pentru infecţiile cu flavivirusuri autohtone sau importate din zone endemice şi le comentăm pe baza experienţei laboratorului nostru.

Cuvinte-cheie: Flavivirus, diagnostic, denga, virus West Nile, infecţia Zika, encefalita de căpuşe, febra galbenă.

INTRODUCTION

Flavivirus genus is the largest genus in the *Flaviviridae* family, which also contains *Pegivirus*, *Pestivirus* and *Hepacivirus* genera. The genus *Flavivirus* contains about 70 viruses [1], transmitted to vertebrates by infected arthropods (mosquitoes and ticks), many of them infecting humans and animals of economic importance. Flaviviruses produced some of most significant epidemic emergence (Zika virus infections) or re-emergence (dengue, yellow fever, West Nile fever, tick-borne encephalitis). Rapid detection of their circulation and early diagnosis of human cases, either locally infected or imported from endemic areas, are of crucial importance for clinical approach, early warning, surveillance and outbreak prevention and control.

Historically, the viruses in the *Flavivirus* genus were grouped based on antigenic relatedness in complement fixation tests [2], and later developed hemagglutination-inhibition tests [3] in the serogroup B of arboviruses (arthropod-borne viruses). *Flavivirus* genus in the *Flaviviridae* family, as defined by Westaway *et a.l* in 1985 [4], and recognized by the International Committee on the Taxonomy of Viruses (ICTV), are small (40 to 50nm in diameter), with icosahedral symmetry, with a single strand, positive sense RNA genome. This viral genome of 10-11kb encodes a polyprotein that contains three structural proteins: capsid (C), membrane associated protein (M) and envelope (E), and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), with multifunctional role and involved in RNA synthesis and virus replication [4].

Flaviviruses (Table 1) are distributed globally (Fig. 1). In Europe many flaviviruses

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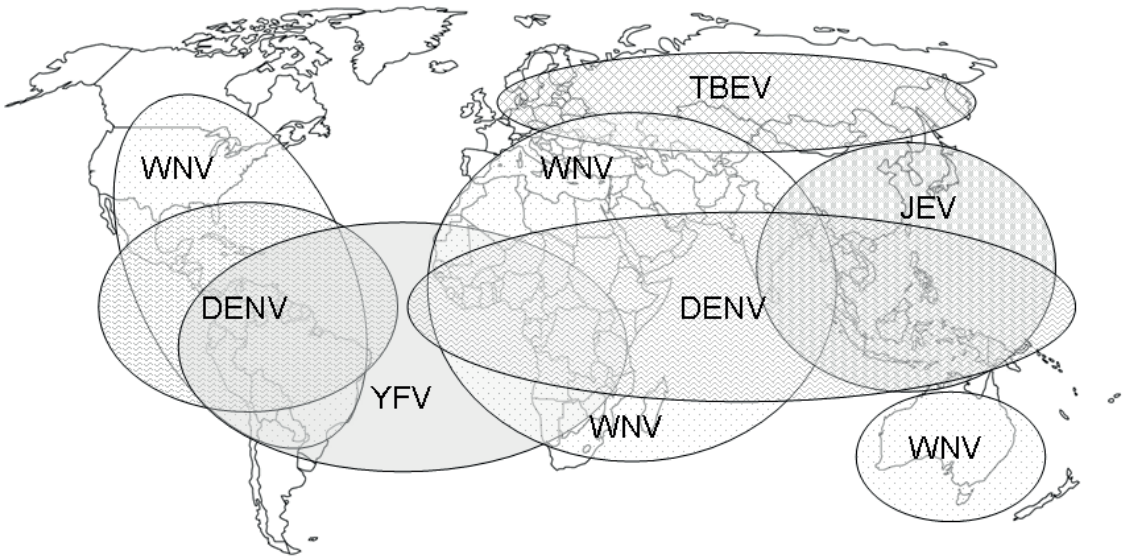

Fig. 1. Global distribution of most important flaviviruses pathogenic to humans.
DENV - dengue virus; JEV- Japanese encephalitis virus; TBEV: tick-borne encephalitis virus; WNV- West Nile virus; YFV – yellow fever virus.

have endemic circulation: tick borne encephalitis virus (TBEV), West Nile virus (WNV), Usutu virus (USUV), while others are imported occasionally such as Zika (ZIKV), dengue (DENV), yellow fever (YFV) viruses (Table 1), having the potential to cause locally transmitted disease, if arthropod vectors are present [5, 6].

Because different flaviviruses circulate concomitantly in Europe, their differentiation by diagnostic tests is of paramount importance. The laboratory diagnosis is necessary to confirm the etiology of flavivirus infections, since the clinical picture of the infection is not specific.

Serological diagnosis of flavivirus infections, an indirect diagnostic method, is complicated by the antigenic relatedness of flaviviruses which generates high antibodies cross-reactivity. Another challenge is the diagnostic of secondary flaviviral infection, as in one's life a person may be exposed to different flavivirus infections and the pattern of immune response in such cases may be misleading.

Most flavivirus antibodies are directed against the envelope protein which contains both epitopes highly cross-reactive, flavivirus genus specific, and more specific to virus species, which eventually might differentiate the different flaviviruses. The sero-neutralization tests by the reduction of the number of plaques formed by the virus after contact with serum containing neutralizing antibodies (PRNT) are the golden standard for serologic diagnostic of flaviviruses. Paired patient sera (acute and convalescent) are to be tested against a panel of flaviviruses which are co-circulating or suspected for the patient-case, in order to identify the causative viral agent. Sero-neutralization assays are laborious, require work in adequate biosafety facilities and take a longer time than it might be expected both in terms of clinical approach and operative epidemiological measures. As well, classic complement fixation (CF) tests and hemagglutination inhibition (HI) tests require testing against a panel of flaviviral antigens and provide a late diagnostic answer only. Therefore in most laboratories other serological methods like enzyme-linked immunosorbent assays (ELISA), indirect immunofluorescence (IFI), immunoblotting are commonly used to detect antibodies of different immunoglobulin classes: IgM, IgG, IgA. Commercial assays are available with good quality standards and good diagnostic accuracy. For some viruses, like DENV, "point of care" immuno-chromatographic tests are also available as rapid tests for clinicians. However the serological confirmation of flaviviral infections should be performed in laboratories with expertise in discriminating

Table 1. Flaviviruses pathogenic to humans
a) Hemorrhagic Fever; b) Fever; c) Dengue Hemorrhagic fever; d) Dengue Shock Syndrome; e) Neuroinvasive Disease; f) Non Specific Febrile Disease.

Flavivirus antigenic group	Virus species	Vector	Distribution	Syndrome	Vaccine available
Yellow fever virus (YFV)	YFV -Type species for genus Flavivirus	Mosquito (*Aedes*)	Africa, Central and South America	HF[a] with hepatitis	yes
Dengue virus serogroup	Dengue (DENV)1-4	Mosquito (*Aedes*)	Africa, Asia, Central and South America, North Australia	F[b], rash, DHF[c], DSS[d]	yes
Japanese encephalitis virus (JEV) serogroup	Japanese encephalitis virus type virus (JEV)	Mosquito (*Culex*)	Asia, North Australia Oceania	ND[e]	yes
	Murray Valley encephalitis virus (MVEV)	Mosquito	Australia	ND	no
	West Nile virus (WNV)	Mosquito (*Culex*) Soft ticks (*Argasidae*) *Hyalomma* ticks	Africa, Europe, Asia, Australia, America	NSFD[f], ND	no
	Saint Louis Encephalitis virus SLEV	Mosquito (*Culex*)	America	ND	no
	Usutu virus (USUV)	Mosquito (*Culex*)	Africa, Europe, Middle East	F, rash, ND	no
Spondweni virus serogroup	Zika virus (ZIKV)	Mosquito (*Aedes*)	Africa, Asia, Oceania, Central and South America	F, rash, congenital microcephaly	no
Tick-Borne encephalitis sero-group	Tick Borne Encephalitis virus TBEV, with three subtypes: European, Far Eastern, Siberian	Ticks (*Ixodes*)	Europe, Asia	F, ND	yes
	Powassan virus (POWV)	Ticks (*Ixodes*)	North America	ND	no
	Kyasanur Forest Disease Virus (KFDV)	Ticks	South India	HF	no
	Omsk hemorrhagic fever OHFV	Ticks	Southern Russia (Asian part)	HF	no
	Alkhurma hemorhagic fever virus	Ticks	Saudi Arabia, Egypt	HF	no

flaviviruses sero-diagnostic. To interpret the results of the serological tests one has to take into account the immune status of the patient, any possible immune deficiency, vaccination status (previous YFV, JEV or TBE vaccination), previous exposure to flavivirus infection in endemic area. Serological investigations require testing at least a pair of serum samples taken at two -three weeks interval, in the acute and the convalescent phases of disease. In primary flavivirus infection, the detection of specific IgM and the sero-conversion for IgG, or four fold increase of specific antibody titers in paired sera, support acute/recent infection. In general, in primary flavivirus infections, the virus can be found in serum or plasma (viremic phase) 2–7 days post onset, and an IgM immune response builds up after 5-7 days post onset with a peak on day 15; while IgG appears at 8-10 days post onset, and such antibody dynamics is demonstrable, for example, in primary dengue fever. There are variations from this antibody response pattern in neuroinvasive flavivirus infections. We review the main diagnostic methods of both autochthonous and imported from endemic regions flaviviruses, and discuss them based on the experience of our laboratory.

West Nile virus

West Nile virus (WNV) is transmitted in nature in birds-*Culex* mosquito cycles, and

accidentally may infect humans causing either a nonspecific febrile diseases or a neuroinvasive disease (meningitis, encephalitis, acute flaccid paralysis), which may be severe in elderly [7]. At least 90% of patients with WNV encephalitis or meningitis present IgM antibodies in cerebro-spinal fluid (CSF) within 8 days from symptom onset [7]. However, if taking into account also patients with non-neuroinvasive WNV infection, only 58% of patients had a positive IgM-ELISA result at clinical presentation [8]. Nucleic acid testing (NAT) of plasma may be used early post onset in patients with WNV non-specific febrile disease [8]. In the experience of our laboratory in patients who develop WNV neurological infection, most often elderly, high titer of IgM antibodies may be detected in serum and CSF at hospital presentation, starting even with first or second day post onset. In the case of neuroinvasive WNV infection the detection of specific IgM in cerebrospinal fluid (CSF) using the IgM capture ELISA with recombinant WNV antigen represents confirmation criterion for WNV meningitis or encephalitis. Because IgM antibodies do not cross the blood-brain barrier, their detection in CSF indicates CNS infection. In non-neurological WNV disease, which we usually diagnosed in younger persons, antibodies to WNV could be detected late, in convalescent phase only.

The IgM antibodies to WNV may last for more than a year [9]. Such long persistence of IgM antibodies to WNV may hamper diagnostic and surveillance of WNV infections, as current season infections are difficult to differentiate from previous infections. Detection of four fold increase in neutralizing antibodies titer or HI titer in paired serum samples would confirm acute infection. However diagnostic is requested in a timely manner. IgG avidity tests (IFI, ELISA) may be used to differentiate old from actual infections. Avidity measures the strength of antibody-antigen interactions, it increases with their binding affinity and reflects the maturity of antibodies: low avidity antibodies are synthetized during the primary infection, but in time avidity gradually increases. During secondary infection, or infection reactivation, high avidity IgG antibodies are produced. Low avidity may occur also in immunosupression. Low avidity IgG to WNV was demonstrated in 86% (ELISA) and 95% (IFI) IgG positive samples taken 2-43 days post onset, while high avidity IgG antibodies were detected in both ELISA and IFI in 100% sera obtained six months or more after the onset of symptoms [10].

Secondary infections with West Nile virus may occur, mainly in endemic areas in which different WNV genetic lineages circulated over a period of time. In Israel, a territory of circulation of multiple WNV lineages and strains [11], patients with a clinical picture consistent with WNV infection were defined as having non primary WNV infections if they demonstrated high IgG antibody titers with high avidity index (≥55%), absent or low titers of serum and CSF IgM, and positive WNV specific real-time RT-PCR of CSF or blood, the confirmatory diagnostic in this case, relying on direct detection of virus genome [11]. Since the last epidemic of WNV in Israel, during 2000, the number of patients with non primary WNV infection has increased dramatically, perhaps due to a relatively high prevalence of previous WNV seropositivity [11]. In our laboratory we have confirmed by WNV real-time RT-PCR a similar case, in which the serological picture suggested non-primary infection with WNV. An 81 years old resident in a locality of the Danube Plain in southern Romania, an area endemic for WNV and in which TBEV has not been detected to date, presented in 2016 with clear CSF meningitis. On the day 8 post onset the CSF was negative for WNV IgM and the serum had low, close to borderline IgM value (1.37, as compared to 1.1 cut off value), but high IgG (index value 3.84, as compared to cut off 1.1), and positive real-time PCR for WNV genome (Ct 32). With increased sensitivity and the finding that urine may be a proper sample for testing, real-time PCR (NAT) have gained value in diagnosing WNV infections. If viraemia is transient and viral load is low in plasma, serum or CSF, the urine samples proved suitable for virus detection much longer time: we have found that the urine of a patient with encephalitis of 2017 WNV outbreak, was positive for WNV real time RT-PCR for one month post onset. NAT may prove useful for testing immuno-compromised patients when

antibody development is delayed, when WNV is a secondary flavivirus infection, when cross-reactivity with other flaviviruses of the antibodies induced by WNV infection does not allow the virus species identification. As well NAT is used to screen blood donors in order to eliminate the risk of WNV transfusion transmission.

Usutu virus (USUV)

Usutu virus (USUV), another virus from the Japanese encephalitis sero-complex, transmitted in mosquito-bird cycles, is circulating in Europe and is affecting mainly black birds (*Turdus merula*) populations. Human neuroinvasive infections with USUV were detected in Europe [12-14]. The USUV was detected in cerebrospinal fluid (CSF) of patients and also in blood donors in Europe [15, 16]. Cross-reactivity of antibodies to USUV with WNV antigens is important, and laboratory differential diagnosis has to be taken into account.

Tick-borne encephalitis virus (TBEV)

Tick-borne encephalitis (TBE) is divided into European, Siberian and Far Eastern subtypes, being produced by different TBEV genotypes and transmitted by specific *Ixodes* tick species.

Tick-borne encephalitis (TBE) endemic to Europe also known as Central European TBE or Spring-Summer Central European TBE, has a biphasic course: during the first phase (2-7 days with fever, flu-like symptoms) the diagnostic may be performed only by direct detection of virus using molecular techniques or virus isolation. The febrile phase is followed by an afebrile period of 2-10 days after which some patients may enter the second phase, neurological phase. Usually patients present to hospital in the neurological phase (meningitis, encephalitis with or without myelitis) when they may be diagnosed only by serological methods: IgM, IgG ELISA or IFI. In TBE endemic areas of Central Europe people may be vaccinated for TBEV, but vaccination is also recommended for travelers who may enter endemic areas [17]. Besides a primary series of vaccines, booster doses at certain intervals are necessary. TBEV clinical disease in vaccinated persons is rare but may occur, mainly in elderly. In persons over 50 years regular booster vaccinations according to the recommended vaccination intervals are important [18]. The serology of TBEV vaccinated persons who however develop TBE has the pattern of a secondary flavivirus infection: low or no IgM presence, and high titer of IgG with high avidity index, starting with the early acute serum sample.

Although in TBE IgM antibodies are less cross-reactive than IgG antibodies, differential laboratory diagnosis between TBEV and WNV neuroinvasive infections may be challenging. Acute serum and CSF of a patient with TBE may present low IgM antibody index when tested against WNV antigens. In such cases the clinical presentation (biphasic evolution in case of TBE) and the epidemiological context (exposure in endemic areas) play an important role for interpretation of laboratory results. In June 2017 we received in our laboratory serum and CSF samples of a 35 year old, immuno-competent male patient, recently returned from Austria, diagnosed with acute meningitis, to be tested for WNV infection [19]. IgM-capture ELISA specific for WNV had the following results expressed as antibody indexes (cut-off 1.1): serum 0.784 (negative); CSF 1.216 (positive). As compared to IgM antibody values in case of neuroinvasive WNV disease, the IgM antibody index in CSF was low, still suggestive for a flaviviral infection. Taking into account the recent travel into a highly endemic for TBE region, the samples were tested in indirect ELISA against TBEV antigen with the following results (cut off 1.1): CSF test showed high specific IgM (6.13) thus indicating neurological infection with TBEV; as well the serum was positive in end-point titration in ELISA till the dilution of 1:800 [19]. The serum was also positive for IgG specific to TBEV (index value 1.499), a result which is quite specific for TBE infection since neuroinvasive phase of infection occurs late after onset, and IgG response has time to build up.

TBE may occur in patients who previously had WNV fever, the diagnosis of such cases being extremely challenging. As well the clinical course of TBE may be very severe and antibody-dependent enhancement is considered by some authors [20].

Dengue virus

Dengue virus (DENV) is subdivided into four serotypes, DENV 1-4, which have high significance for human health. Dengue is now a global threat (Fig. 1), the most important virus infection by arthropods around the world, being endemic or epidemic in almost all countries from tropical areas. Over 50 million infections are estimated to occur every year, including 500,000 hospitalized dengue hemorrhagic fever cases, especially among children, with a mortality rate that may exceed 5% [21].

The most competent mosquito vector is *Aedes aegypti*, and the introduction of this invasive species in Europe (Madeira, Portugal) was followed in only five years by a significant dengue outbreak [22]. Another invasive mosquito species, the Asian tiger mosquito, *Aedes albopictus*, may transmit dengue virus, and limited number of cases, locally transmitted, have been detected in continental Europe: Croatia and France [23]. This invasive species in established in urban habitats in South of Romania also [24] Early detection of virus circulation in a new area is crucial for taking control measures in order to limit transmission, therefore in non-endemic areas in which the vector mosquitoes (*Ae. albopictus* or *Ae. aegypti*) are present, cases clinically compatible with dengue should be laboratory tested for dengue also.

All four dengue virus serotypes (DENV 1–4) are now circulating in Asia, Africa and the Americas, and this has an impact on the clinical pictures of dengue cases. In endemic regions children may experience a mild primary infection, but secondary infection may have a severe clinical picture (dengue shock syndrome, DSS), mainly if DENV 1 infection is followed by DENV 2 or 3 as secondary infection, or if DENV 3 is followed by DENV 2 [21]. This is caused by a mechanism called "antibody dependent enhancement": the existing heterologous antibodies form complexes with the new infecting virus, but do not neutralize it; the new virus and pre-existing antibodies complexes infect monocites and determine release of vaso-active mediators, resulting in increase vascular leakage and hemorrhagic manifestation typical for hemorrhagic dengue (DH) and dengue shock syndrome (DSS) [21]. Hemorrhagic dengue may also occur as severe primary infection in adults. Diagnostic in secondary dengue is challenging also, direct diagnosis being recommended in such cases; while virus isolation is laborious, direct viral genome detection genome by RT-PCR has a diagnostic window of 6-7 days post-onset. As well, the non-structural antigen NS1, which is secreted from infected cells, may be detected by ELISA very early, at onset, before IgM antibodies build up. The NS1 antigen also lasts longer in serum than the virus genome. In one of the dengue cases diagnosed in our laboratory, we detected NS1 antigen till day 16 post clinical onset. In secondary dengue infections, the rapid anamnestic rise of DENV serotype cross-reactive IgG antibodies form immune complexes which are rapidly cleared from circulation. As a consequence NS1 antigen is rarely positive in secondary infected patients beyond 5-7 days post symptoms onset [25]. Serological methods cannot discriminate between various dengue serotypes, have the limitation of cross-reactivity with other flaviviruses [26], and are of reduced relevance in secondary infection. Molecular detection is rapid, very specific and sensitive and allows identification of the DENV serotype involved. Viral load is expected to be high in the serum and plasma during the 1-7 days post-onset and virus genome shedding in urine may last longer. Molecular detection may be the only way to clarify a diagnosis in secondary dengue, and we had this experience in our laboratory. A patient with history of multiple travels and long periods of residence in India and other tropical areas presented to the hospital of infectious diseases with a non specific febrile syndrome. At three days post onset the molecular detection test detected Dengue 2 virus, and no IgM or IgG antibodies to DENV and WNV. Twelve days post onset of symptoms the serum was negative for DENV IgM, positive for DENV IgG (antibody index 3.66 / cut of 1.1) and positive for WNV specific IgM (high IgM antibody index: 6.16 / cut off 1.1) and IgG (IgG antibody index 3.98 / cut off 1.1). We did not receive any information on eventual previous YF, TBE or JEV vaccination. The DENV 2 acute infection has been

confirmed by sequencing [27]. These puzzling serological results may be interpreted taking into account the phenomenon of the "original antigenic sin": the patient most probably has had a previous primary flaviviral infection, or has been vaccinated for a flavivirus different from DENV but antigenically related to WNV, such as JEV which circulates throughout India. In the convalescent serum, IgG antibodies to both DENV and WNV were detected, but IgM antibodies were detectable only for WNV, a virus belonging to the JEV antigenic group of flaviviruses. During sequential flavivirus infections B cell –clones responding to the first infection synthesize antibodies with higher affinity for the first infecting virus, or viral antigens strongly related to the latter, than for the second infecting virus [28].

Zika virus (ZIKV)

Zika virus (ZIKV) member of Spondweni sero-group [29] got recent attention because of the unprecedented outbreak in Latin America. The distribution area of ZIKV largely overlaps that of DENV and this complicates enormously the serological diagnosis. Acute infection is either not clinically apparent or a simple fever with rash syndrome. Long period of virus shedding in different secretions, leading to multiple modes of inter-human transmission, the capacity to produce severe congenital effects (microcephaly) if occurring during pregnancy, and the potential to evolve epidemically in areas where the main vector (*Ae. aegypti* mosquito) is present, makes of this previously neglected flavivirus a hot topic: over 4000 papers were recorded by PubMed since the beginning of the Latin America outbreak. Molecular diagnosis is of certitude, and the virus genome may be detected, usually in low loads in serum, plasma and urine during the acute phase of illness [30]. In order to avoid high cross-reactivity with DENV and other flaviviruses, diagnostic tests detecting IgM and IgG antibodies to the nonstructural antigen NS1 were produced. Although they were more specific for ZIKV than the tests based on flaviviral envelope antigens, their sensitivity was lower. In some subjects with ZIKV infection confirmed by RT-PCR, antibodies could not be detected [31]. As it was found in our laboratory, previous yellow fever vaccination altered significantly the serological response [32].

Yellow fever virus (YFV)

Yellow fever virus (YFV) is the prototype virus for the genus *Flavivirus*. Yellow fever (YF) produces liver and kidney impairment, shock and hemorrhage which may lead to up to 50% lethal cases [33]. In endemic tropical areas of Africa and South America YFV is maintained by sylvatic cycles between monkeys and mosquitoes in jungle habitat. The virus is then introduced into human settlements where it produces outbreaks, as humans are amplifying hosts, and urban mosquitoes, mainly *Ae. aegypti* are vectors [33]. Vaccines using an attenuated live virus (strain 17D) are available, and vaccination together with control of urban vectors in the DDT era, have lead to some limitation of the YF outbreaks during XX century [33]. However YF is considered now a re-emerging disease as shown by the current outbreak in Brazil [34].

The serologic diagnostic for the detection of IgM antibodies or seroconversion for specific IgG may be performed in ELISA or IFI. However since the virus circulates in regions which are endemic for other flaviviruses and large segment of inhabitants may carry post-vaccination antibodies, the development of the molecular tools with high sensitivity and specificity was necessary and dramatically improved YF diagnosis [35].

Molecular detection of the YFV genome distinguishes between wild type virus and vaccine strain. The RT-qPCR assay is a powerful tool for reference or diagnostic laboratories and may be used to monitor viral load for disease prognostic [35]. Viraemia is high, but short, limited to a few days post symptoms onset, while virus shedding in urine offers a longer window for molecular diagnosis. In the experience of our laboratory the virus genome could be detected up to the 4^{th} day post onset in serum and plasma, however the RT-PCR test was positive in the urine sample of the 8^{th} day as well as in that of the 14^{th} day post-onset. The molecular detection may be performed on liver tissue biopsy, including on paraffin embedded samples for a post-mortem confirmation of infection [36]. A weakness of the molecular

methods in use for YFV detection represents the inability to detect different YFV strains (vaccinal, wild African or American strains [35], an obstacle we also met in our laboratory when trying to detect current circulating strain of the Brazilian lineage of YFV. Sequences data and molecular epidemiology of viruses circulating in different regions, must be used to continuously update the oligonucleotide sequences in molecular diagnosis protocols [26].

CONCLUSION

Flavivirus infections diagnosis is challenging and must be performed in laboratories with adequate expertise, periodically evaluated by participation in external quality assurance programs. Serological methods show high cross-reactivity and are of limited relevance in areas where different flavivirus species, subtypes, genetic lineages are endemically co-circulating. Protocols used for the molecular diagnosis of flaviviruses must be also tested for specificity and continuously adapted to keep the pace with the evolution and emergence of flaviviruses.

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