Trends in Microbiology

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Review

Diagnosing arthropod-borne flaviviruses: non-structural protein 1 (NS1) as a biomarker

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In recent decades, the presence of flaviviruses of concern for human health in Europe has drastically increased,exacerbated by the effects of climate change – which has allowed the vectors of these viruses to expand into new territories. Co-circulation of West Nile virus (WNV), Usutu virus (USUV), and tick-borne encephalitis virus (TBEV) represents a threat to the European continent, and this is further complicated by the difficulty of obtaining an early and discriminating diagnosis of infection. Moreover, the possibility of introducing non-endemic pathogens, such as Japanese encephalitis virus (JEV), further complicates accurate diagnosis. Current flavivirus diagnosis is based mainly on RT-PCR and detection of virus-specific antibodies. Yet, both techniques suffer from limitations, and the development of new assays that can provide an early, rapid, low-cost, and discriminating diagnosis of viral infection is warranted. In the pursuit of ideal diagnostic assays, flavivirus non-structural protein 1 (NS1) serves as an excellent target for developing diagnostic assays based on both the antigen itself and the antibodies produced against it. This review describes the potential of such NS1-based diagnostic methods, focusing on the application of flaviviruses that co-circulate in Europe.

Highlights

The co-circulation of West Nile virus (WNV), Usutu virus (USUV), and tick-borne encephalitis virus (TBEV) in Europe is a public health concern, and the lack of active surveillance programs and high-quality specific serology tests make it difficult to estimate the true disease burden. The potential for introducing other pathogens, such as the related Japanese encephalitis virus (JEV), could further complicate this picture.

The standard method for diagnosing flavivirus infections involves RT-(q)PCR to detect the viral RNA during the acute phase of the disease, followed by antibody detection in the convalescence phase. Both methods suffer from several limitations that make flavivirus diagnosis challenging, especially in areas of co-circulation.

Non-structural protein 1 (NS1) represents a promising marker for discriminating between co-circulating flaviviruses. However, NS1 antigen-capture tests are lacking on the commercial market for WNV, USUV, TBEV, and JEV.

An introduction to flaviviruses

Flaviviruses are arthropod-borne, single-strand RNA viruses of the genus *Flavivirus*, family *Flaviviridae*, which consists of more than 70 genetically and antigenically related members [1–3]. Several of them can be defined as relevant human pathogens (Figure 1A,B) capable of generating high morbidity and mortality rates and characterized by unpredictable and heterogeneous disease severity and long-term persistence [4]. The infection can range from asymptomatic or influenza-like illness to life-threatening diseases such as hemorrhagic fever in the case of dengue virus (DENV) and yellow fever virus (YFV), or meningitis, encephalitis, and neurological disorders associated with JEV, WNV, and TBEV [5,6].

Flaviviruses can be divided into three groups according to their dominant vector [6,7] (Figure 1C): tick-borne viruses; mosquito-borne viruses; and viruses for which the vector is unknown

The mosquito-borne virus group can be subdivided into viruses transmitted predominantly by *Culex* or *Aedes* mosquitoes which have different vertebrate hosts and pathogenesis. The *Culex* species use birds as reservoirs and are the main ones responsible for spreading neurotropic flaviviruses, which can cause severe meningoencephalitis. Flaviviruses mainly transmitted by *Aedes* mosquitoes have primate reservoirs and do not show neurotropism (except for Zika virus) and cause acute fever with arthralgias, myalgias, and, in extreme cases, hemorrhagic fever (dengue and yellow fever). The tick-borne viruses also form two groups: one group circulates among seabirds, while the other, the tick-borne encephalitis group, is primarily associated with rodents [8–10].

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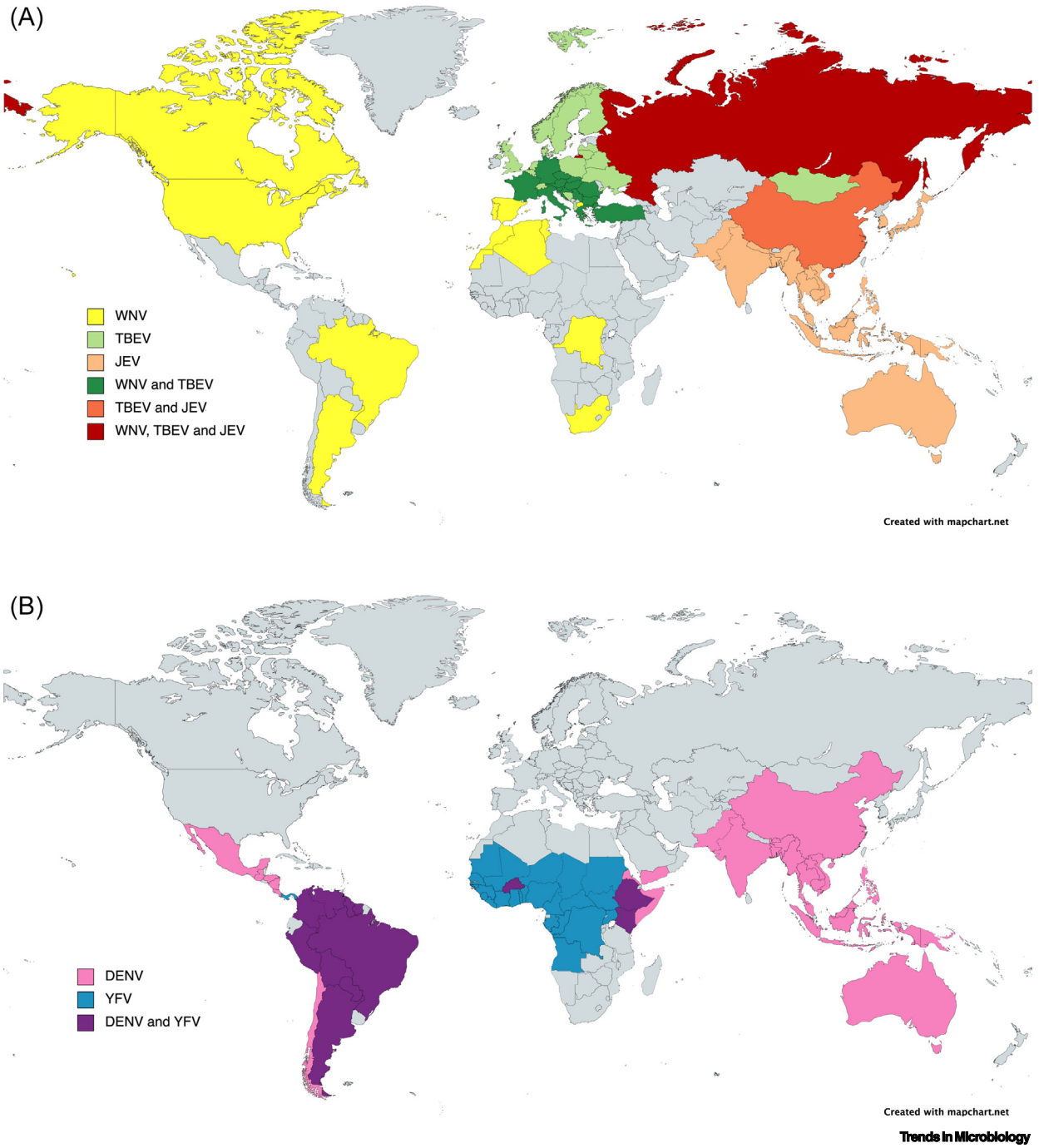

Figure 1. Geographic distribution of most relevant flaviviruses and their antigenic relationships. (A) The global distribution of the most prevalent neurotropic flaviviruses. Data are lacking for the gray areas. (B) The global distribution of the most prevalent hemorrhagic flaviviruses. Data are lacking for the gray areas. The figures were generated using an online tool (https://mapchart.net). (C) The phylogenetic tree shows the antigenic relationships among flaviviruses. The flavivirus names are colored based on their respective serocomplex (legend on the right), while the arced lines cover the viruses that share the same vector. The full-length polyprotein amino acid sequences from various flaviviruses were obtained from the NCBI database and pairwise aligned using Muscle. Phylogenetic analysis was inferred using the Neighbor-Joining method [88]. The evolutionary distances were computed using the p-distance method [89] and are in the units of the number of amino acid differences per site. The analyses were conducted in MEGA11 [90,91]. Abbreviations: DENV, dengue virus; JEV, Japanese encephalitis virus; TBEV, tick-borne encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus.

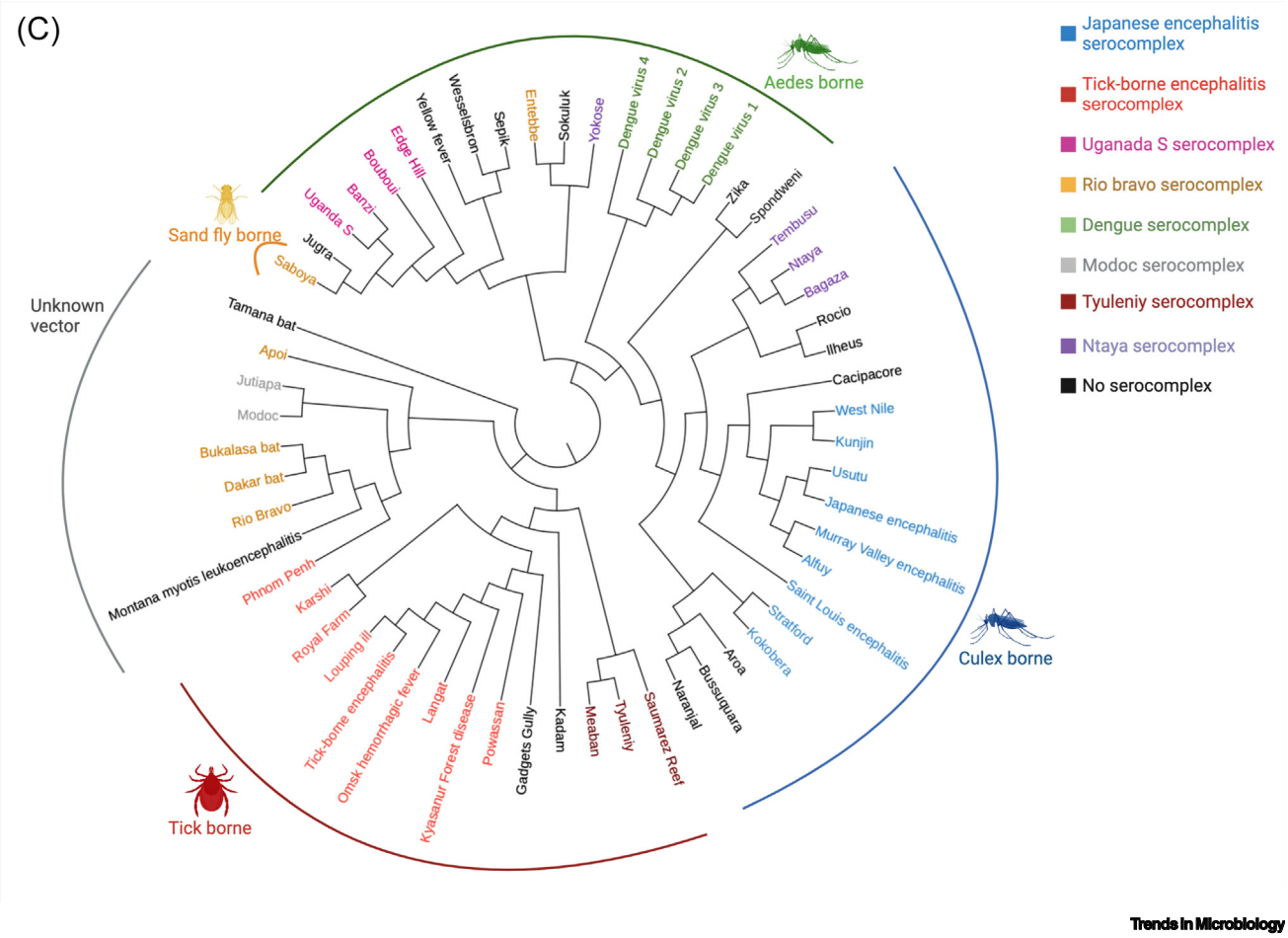

Figure 1 (*continued*).

Serological studies enable the definition of different serocomplexes based on antigenic cross-reactivity [9,10]. The majority of flaviviruses relevant to human health can be clustered into eight serocomplexes and seventeen independent viruses that are not antigenically related enough to be included in a serocomplex [11] (Figure 1C).

Because of the increasing global burden of flavivirus-associated diseases, the necessity of diagnostic methods for accurate, specific, and straightforward discrimination between the different flaviviruses, which can also be used during different stages of the disease, is crucial [3]. Developing a diagnostic test that detects a specific flavivirus remains challenging. This is especially the case in regions where antigenically related viruses co-circulate, as traditional serological assays routinely performed in hospitals and laboratories may suffer from cross-reactivity [12,13].

This review focuses on flaviviruses that co-circulate in Europe, highlighting the limits of the current diagnostic methods and the potential of NS1-based tests to improve the diagnosis of different flaviviruses that co-circulate in the same geographic areas.

Flavivirus epidemiology in Europe

Factors such as climate change, rapid urbanization, increased transportation, commerce, and travel increasingly contribute to changes in the distribution, spread, and seasonality of flaviviruses and their vectors in Europe [14,15]. Global warming further creates an environment conducive to the spread and establishment of ticks and mosquitos in more temperate countries [10,16]. Moreover, rising temperatures are extending the seasonal activity of insect and tick vectors. In this regard, mild winters can prolong tick activity, leading to increased human infections and changes in flavivirus seasonality [14]. Furthermore, low precipitation in winter and warmer springs can contribute to the enhanced spread of viruses by *Culex* mosquitos and the increased growth rate of the mosquito population, potentially leading to an earlier start of the transmission season [17,18].

In Europe, TBEV, WNV, and USUV, and to some extent also JEV, are of particular concern for public health.

WNV

WNV has the widest geographical distribution in Europe, with the largest number of vectors and nonhuman hosts [8,19]. It has circulated in Europe since the 1950s, where it causes sporadic outbreaks in humans. More recently, a large outbreak was described in Romania in 1996, when 390 cases of WNV were registered [20]. Two different lineages of WNV have been identified as the cause of different outbreaks: lineage 1 was predominant in the USA and was present in southern and central Europe until 2010 when it was gradually replaced by lineage 2. Lineage 2 has been primarily responsible for the outbreaks of WNV in Europe after 2010 [10,21]. Today, WNV is the most widespread flavivirus in Europe and poses one of the largest infectious disease threats to the region. Its presence has been detected in 27 European countries, mostly in the south of the continent, where the number of infections and virulence have increased in recent years [22]. As of 31 May 2023, the EU and the European Economic Area (EEA) countries have reported 1133 human cases of WNV infection through the European Surveillance System (TESSy), including 92 deaths in 2022, of which 1112 were locally acquired, 17 were travel-related, and four had an unknown importation status and unknown place of infection[i]. Around 30% of infected people develop West Nile fever, and the symptoms range from a flu-like syndrome to neuroinvasive diseases like encephalitis, meningitis, and acute flaccid paralysis in <1% of cases. The fatality rate associated with neurological symptoms is around 10% [20,21].

WNV is principally transmitted by *Culex pipiens s.l.* and *Culex modestus,* while different bird species act as reservoirs. Humans and equines can be accidentally infected by mosquitos and are considered dead-end hosts [20,23]. It is hypothesized that WNV has been introduced into Europe by birds migrating from Africa. According to this hypothesis, birds are responsible for long-distance WNV spread, while mosquitos mediate short-distance diffusion [21,24].

USUV

USUV was first identified in Europe in 1996 as the cause of death in common blackbirds found in the Tuscany region of Italy [8]. In 2001, the first large outbreak was registered in different bird species in Austria [22,25], and in 2009, the first cases of neurological symptoms associated with USUV infection were reported in two immunocompromised patients in Italy [25,26]. Since 2009, USUV has been detected in 16 European countries (Austria, Belgium, Croatia, Czech Republic, France, Germany, Greece, Hungary, Italy, The Netherlands, Poland, Serbia, Slovakia, Spain, Switzerland, and the UK) by virus isolation/detection or serologically [22].

As for WNV, USUV belongs to the JEV serocomplex of flaviviruses. These viruses mostly share the same vector and hosts and have a similar life cycle [10,20,22,23]. In contrast to WNV,

USUV seems more virulent in birds, causing significant blackbird mortality, especially in central Europe, while human infection seems less common [20,23]. Between 2012 and 2021, 105 cases of human USUV infection were reported in Europe, 12 of which showed neurological symptoms [27]. Most of the cases were found in Italy, but USUV infections were also detected in Croatia, Germany, Czechia, Austria, Hungary, and France [20,27,28].

TBEV

TBEV is considered the most medically significant arbovirus in Europe, infecting over 10000 humans every year [8,29]. Its presence in Europe was reported for the first time in 1931 in south-eastern Austria, and today it is considered endemic in 27 European countries, mostly East-European countries, with Slovenia showing the highest reported incidence[ii] [30]. In Europe, all three different subtypes of TBEV have been identified: the European subtype (TBEV-Eu), transmitted by *Ixodes ricinus* ticks and endemic in rural and forested areas of central, eastern, and northern Europe; the Siberian subtype (TBEV-Sib), transmitted by *Ixodes persulcatus* and endemic in the Urals region, Siberia, Russia and in some areas in north-eastern Europe; and the Far Eastern subtype (TBEV-FE), transmitted by *Ixodes persulcatus* and mainly typical of Asia but has been found in several Eastern European countries [30,31].

Approximately two-thirds of human TBEV infections are asymptomatic, but 10–30% of patients can develop nonspecific symptoms such as fever, fatigue, headache, and myalgia. In rare cases, the nervous system could be involved in patients showing meningitis (50%), meningoencephalitis (40%), meningoencephalomyelitis (10%), paralysis, and radiculitis[iii] [24]. TBEV-FE is associated with the most severe neurological manifestations and has a fatality rate of around 20%. By comparison, the European subtype shows milder disease and mortality rates below 1%, with severe neurological sequelae in up to 10% of patients [31–33].

Tick vectors are responsible for transmitting the virus to animals (mostly rodents and deer, which act as amplifying hosts) and humans, who act as dead-end hosts [24]. According to data published in February 2023 in the context of the VectorNet project, the presence of *Ixodes ricinus*, the main vector of TBEV, was detected in all European countries. This makes it possible, at least theoretically, for TBEV to spread even in areas where it is not yet considered endemic[iv]. In this regard, the first three autochthonous TBEV cases were reported in Belgium during the summer of 2020, but the common vector of TBEV was already widespread in the country, and TBEV antibodies were detected in animals such as dogs, cattle, roe deer, and wild boar before evidence of human infection [34].

JEV

JEV is the prototype of the JEV serogroup that also includes WNV and USUV [35]. It is one of the leading causes of viral encephalitis, with an annual number of cases between 30000 and 50000[v]. It is considered endemic in at least 24 countries in Asia and Oceania, and it is estimated that around 3 billion people live in JEV-epidemic areas[vi] [35].

JEV infection generally causes mild febrile symptoms, while approximately 1% of patients can develop a severe neuroinvasive illness characterized by high fever, headache, neck stiffness, disorientation, coma, seizures, and spastic paralysis, with a mortality rate of around 30%[v] [42]. The neuroinvasive disease may also be responsible for lifelong disabilities or cognitive impairments in approximately 30% of patients who recover after JEV infection [36,37].

JEV is spread mainly by *Culex* mosquitos and circulates in various species of birds that are the natural reservoir, while pigs are considered the main maintenance or amplifying host [19]. As

for WNV and USUV, humans are dead-end hosts because viremia is insufficient to transmit the infection to another vector [38].

The introduction of the JEV in Europe is a potential risk due to international travel and commerce with Asia and Oceania. This could lead to the introduction of infected mosquitoes. If the virus is introduced, it could become established in Europe due to the presence of susceptible mosquito vectors and vertebrate hosts[v]. In 1996–1997, the first evidence of JEV antibodies and RNA in Italian birds was reported [39,40]. Afterwards, JEV-like sequences were detected in *C. pipiens* specimens in northern Italy in the summer of 2010 [40]. JEV gene amplification was detected in bird specimens collected in Tuscany, where JEV-positive mosquitoes were also found. According to the epidemiological analysis, no JE patients were found where the bird specimens were collected [35]. This suggests that there was a limited epidemic cycle of JEV between birds and mosquitoes in southern Italy. The lack of pigs, the primary hosts of JEV, may have prevented the virus from spreading on a larger scale. The detection of JEV in both birds and mosquitoes indicates that the virus has spread to Europe, specifically to Italy, from traditional JEV epidemic areas in Asia [35,39].

Flavivirus co-circulation in Europe

The era of global change has brought significant modifications to the distribution of flaviviruses in the European continent, and in the coming years, the increasingly tangible global warming is expected to cause further alterations. In this context, the tick species *Ixodes ricinus*, which is the primary vector of the European variant of TBEV, has been discovered at higher altitudes where it was previously absent, and in greater numbers in areas where it was originally present. Similar behavior has been observed for vectors of WNV and USUV in Europe, such as mosquito species *C. pipiens s.l.* and *C. modestus,* which are considered the main bridge vectors of WNV from avian reservoirs to dead-end hosts, including humans. In the past decade, this thermophilic species has expanded its territory northward and has been reported in several European countries for the first time [14,20].

In recent decades, WNV, TBEV, and USUV have been detected in most European countries, showing a significant co-circulation in the same geographic areas [8] (Figure 2). Co-circulation of WNV and TBEV has already been observed in central and eastern Europe. Since its first report in Europe, USUV spread in European countries has significantly overlapped with the circulation of WNV. Both viruses share similar vectors and amplifying hosts, as well as geographic distribution. This means that there is potential for WNV to spread to areas where only USUV has been observed so far, and vice versa. This is especially true considering that both WNV and USUV have been shown to infect several bird species that are at least partially migratory [20,22].

The presence of these viruses on European territory, as well as their possible co-circulation, could be underestimated by the fact that most countries have no active surveillance programs to detect flavivirus circulation both in humans and animals [22]. Furthermore, specific serology tests are lacking to study seroprevalence and do systematic serosurveillance [41]. Increased travel and transportation from endemic areas raise the risk of introducing other neurotropic flaviviruses in Europe, such as JEV [42], causing further problems in the diagnosis and surveillance of this family of viruses [24,43].

Diagnostic methods to detect flavivirus infection

Given the increasing public health risk posed by the spread of various flaviviruses in Europe, it is now more crucial than ever to be able to accurately diagnose the virus responsible for the infection. The early and precise diagnosis of the infectious agent is necessary for appropriate clinical care before symptoms exacerbate (i.e., patients can rapidly progress to life-threatening neurological

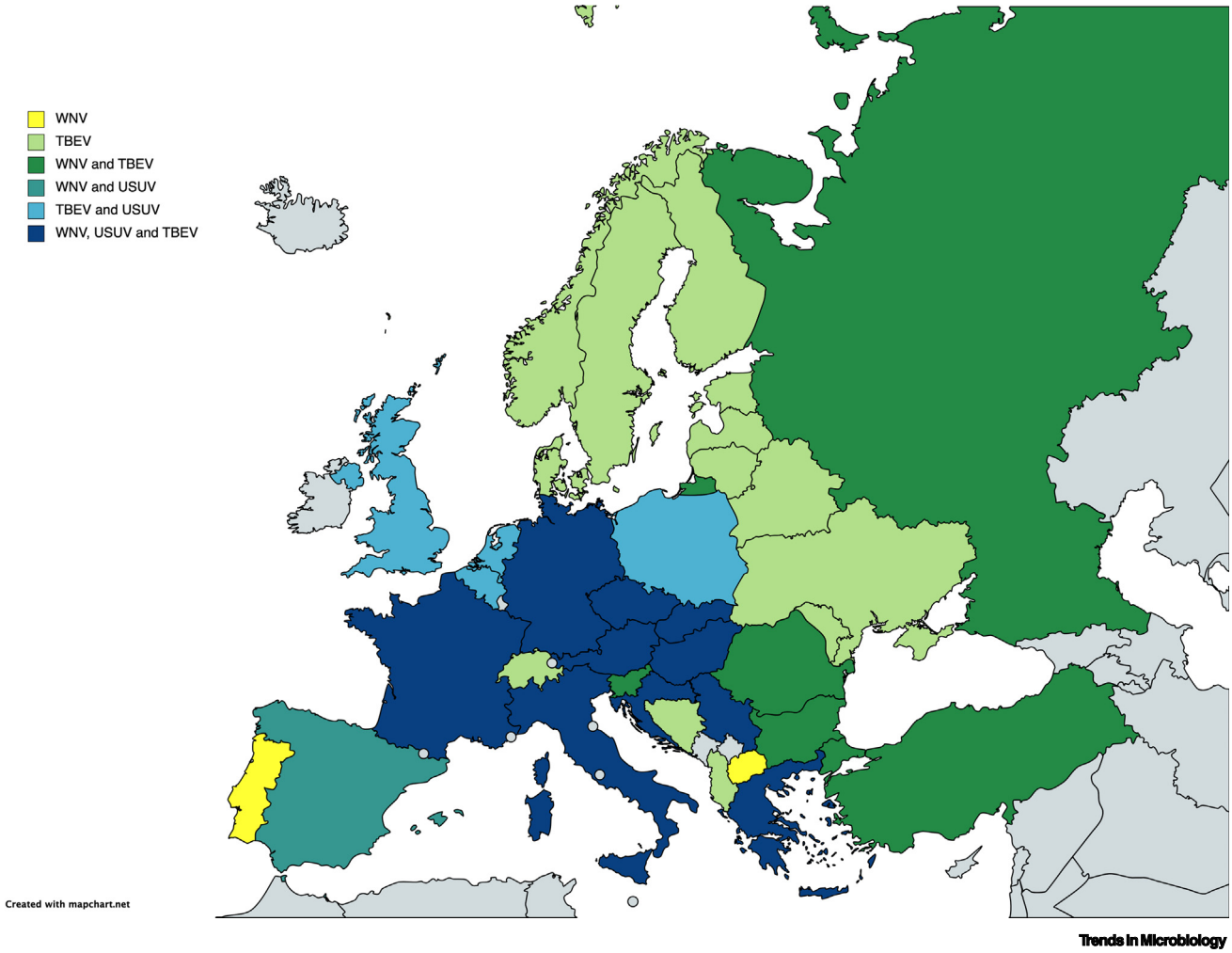

Figure 2. Geographic distribution of flaviviruses in Europe showing the countries in which WNV, USUV, and TBEV have been detected and their pattern of co-circulation. The figure was generated using an online tool (https://mapchart.net). Abbreviations: TBEV, tick-borne encephalitis virus; USUV, Usutu virus; WNV, West Nile virus.

complications), but also for surveillance and epidemiology [3,12,44]. The ability to discriminate between different members of this family, especially when they are part of the same serocomplex, is essential for understanding which viruses circulate in a given region and for time to take appropriate precautions, such as vector control and One Health surveillance. Furthermore, the implementation of a surveillance system and the possibility of timely detection of autochthonous and imported infections is becoming a priority in non-endemic areas to avoid new outbreaks caused by the spread of these viruses in new areas [45].

The standard method for diagnosing flavivirus infections involves detecting the pathogen, its nucleic acids, or specific viral antigens during the acute phase of the disease, followed by measuring specific antibodies present in the patient during the convalescence phase [45,46].

Molecular diagnostics

Flavivirus infection can be confirmed by the detection of the viruses in body fluids (usually blood, serum, or plasma, but they can also be detected in urine and cerebrospinal fluids). Viral nucleic

acids can be detected by performing an RT-PCR or an RT-qPCR [45]. This is the most specific and sensitive technique available because it can distinguish between two different flaviviruses at the RNA level. RT-PCR allows the detection of the presence of infection from the onset of symptoms until 7–10 days post-infection, allowing for rapid diagnosis of suspected cases. It is also highly standardized and allows for a high degree of repeatability and reproducibility [15,21].

The acute viremic phase of flavivirus infection lasts 5–7 days on average (Figure 3) and is often missed due to the generic flu-like symptoms that the patients develop during this phase, which can be confused with those of more common infections [1]. Usually, patients visit a doctor only when their symptoms persist or worsen, and, at that point, viral nucleic acids are often no longer detectable in the blood, excluding RT-PCR for diagnosis [8,15]. WNV, USUV, and TBEV can persist in the kidneys for extended periods and be excreted in the urine. In cases of neuroinvasive WNV infection, the diagnosis from urine samples can be more reliable and effective than from cerebrospinal fluid. However, urine samples do not yet seem to be routinely collected as standard sample material [14]. Furthermore, RT-PCR is complex, expensive, and requires specialized equipment and trained personnel to be performed [12].

Serological diagnosis

Serological assays to detect antibodies are the preferred diagnostic method in most laboratories or hospitals. IgM is detectable from the first week post-infection, and titers start to decline in the following 2–3 months (Figure 3). However, in the case of WNV infection, IgM has been reported to persist even for 1 year [15]. IgG levels, instead, can be measured with a few days delay relative to IgM but usually remain detectable for several months or years after exposure to the antigen [45]

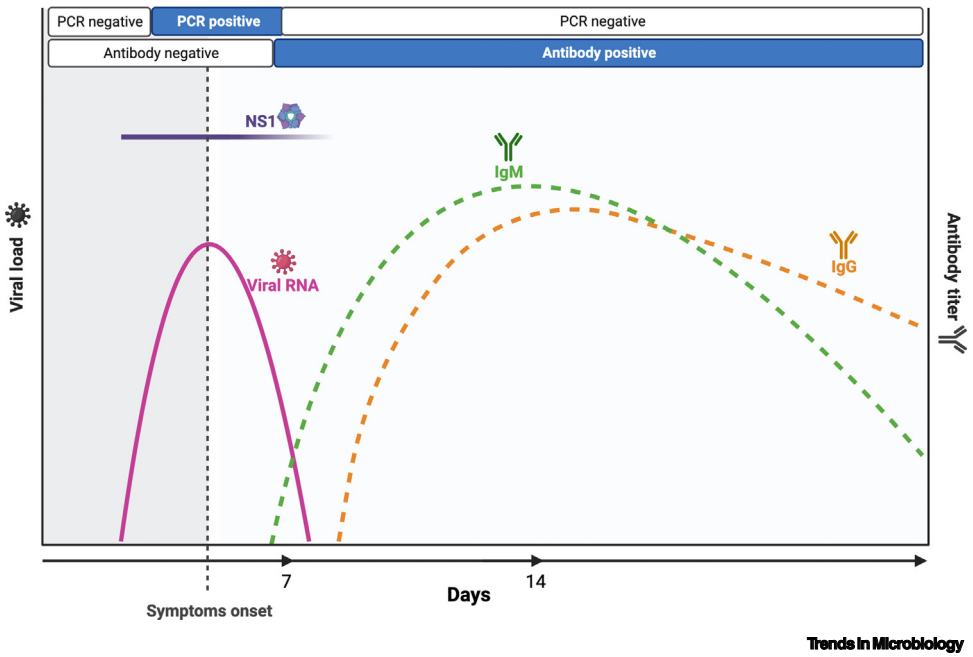

Figure 3. Flavivirus antibody, NS1, and viremia levels detectable in blood during the phases of the infection. The viral RNA (pink line) can be detected even before the onset of the symptoms and approximately 7–10 days post-infection. IgM antibodies (yellow broken line) are detectable from the first week post-infection, and titers start to decline in the following 2–3 months, even if, in some cases, IgM has been reported to persist even for a year. IgG (green broken line) levels can be measured with a few days delay relative to IgM but usually remain detectable for several months or years after exposure to the antigen. NS1 (purple line) can be detected as early as the detection of viral RNA, and it lasts until 9–12 days post-infection. The image was created using BioRender.

(Figure 3). During secondary infection, instead, the rise of IgM levels is often delayed compared to IgG, which can be rapidly detected within 2 days after the onset of the disease [15].

Detection of antibody levels against flaviviruses is the most widely used diagnostic method due to its rapidity, sensitivity, reproducibility, and affordability. It is cheaper than an RT-PCR and does not require complex equipment [8,21]. IgM Ab-capture immunoassay (MAC-ELISA) can be performed to detect an acute infection, while IgG indirect ELISA is more useful to diagnose a secondary infection [8]. The main problems with antibody detection as a diagnostic method are that the very early phase of infection might not be accurately detected as antibodies might not be produced yet, and the presence of high cross-reactivity found between different flaviviruses, particularly those belonging to the same serocomplex (e.g., WNV and USUV) [47,48]. These viruses share a high degree of structural and sequence homology, which results in a similar antibody response that causes extensive cross-reactivity [1,49]. This can lead to incorrect interpretation of diagnostic results and can also result in underestimating the presence of a flavivirus in a given geographic area, as may be happening in Europe for USUV, which, being less known and studied than WNV, could be confused with the latter [20]. TBEV and WNV show lower cross-reactivity compared to WNV and USUV, probably because they are not part of the same serocomplex, and they also have different vectors and amplification hosts. Despite this, cross-reactivity between WNV and TBEV was observed in Greece during the WNV outbreak in 2010, confirming that the possibility of cross-reaction also exists with more distantly related viruses [50]. Since both IgM and IgG antibodies circulate for multiple months following the onset of the infection, it can be challenging to determine if the positive antibody titer is the result of an acute infection or if it is the remnant of a previous infection or vaccination [8].

The diagnosis made by antibody detection can be further complicated by the fact that patients could have previously been vaccinated against one or more flaviviruses. In Europe, human vaccines are available only for TBEV, JEV, and YFV [51,52]. JEV and YFV vaccination is not routinely performed, and they are recommended only in case of travel to endemic areas, such as tropical and subtropical regions of Africa, South America, or Southeast Asia [8]. By contrast, TBEV vaccination is recommended and implemented in the countries in which TBEV is considered endemic. A cross-sectional study conducted in 2015 found that the average TBE vaccination rate of all the European countries evaluated was 25% of the total sample. Finland and Slovakia had the lowest vaccination rates (~10%) [53], while Austria is the European country with the highest vaccination coverage, corresponding to 88% [8]. In general, the vaccination rates in Europe are highly variable from country to country, with an overall low vaccination coverage [53]. Since the serological tools are not able to distinguish between naturally infected and vaccinated people, vaccination history and rates in a country should be investigated when interpreting diagnostic results [54].

When the samples are not distinguishable by antibody detection-based methods, it is recommended to perform a plaque-reduction neutralization assay (PRNT), which is considered the gold standard in flavivirus serological diagnosis [12]. This method requires the manipulation of live flaviviruses at a biosafety laboratory level (BSL) 2 for USUV or 3 for WNV, TBEV, and JEV, which can be performed routinely only in a few laboratories in Europe [8,20]. Moreover, the use of live viruses leads to high variability between assays and between laboratories due to the differences in cell lines used, the virus strain, other inter-laboratory variations, and overall lack of international standardization [12].

Viral antigen capture

Viral antigens can be used to diagnose viral infections in the early stages of the disease by detecting viral antigens directly in the clinical specimen [55]. Viral antigen detection by ELISA is a cost-

effective, rapid, and accurate diagnostic assay that could facilitate early viral detection [56]. However, viral antigen detection kits are commercially available only for dengue diagnosis [57], while for WNV, USUV, TBEV, or JEV, the kits are limited to research purposes and are not suitable for diagnosis in clinical settings. The potential and limitations of this methodology is discussed in detail in the next section.

Different flavivirus diagnostic methods have some advantages but also several limitations (listed in Table 1). It is evident that there is an urgent need to introduce on the market new diagnostic tests for flavivirus infection that can be easily implemented without expensive equipment, show high specificity and sensitivity, allow the diagnosis during the acute phase of the disease, and are not affected by the cross-reactivity of co-circulating flavivirus. In Europe, there is a clear necessity to have a diagnostic tool that can discriminate principally between WNV, TBEV, and USUV that co-circulate. Furthermore, other viruses that are antigenically related, such as JEV, could potentially be introduced into Europe. These viruses show cross-reactivity with WNV and USUV, and it is crucial to accurately differentiate between them.

NS1 antigen capture for West Nile, Usutu, tick-borne encephalitis, and Japanese encephalitis diagnosis

In the search for an ideal diagnostic assay that can discriminate between different flavivirus infections, NS1 is a key viral protein that can be used to develop new diagnostic assays for flavivirus infections [3].

The flavivirus genome is composed of single-strand RNA of positive polarity, which is non-segmented and around 10–11 kbp in length. The genome encodes a large polyprotein precursor, which is co- and post-translationally processed by viral and host-derived proteases into three structural proteins [Capsid, prM, and Envelope (E)] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [5,58]. The structural proteins are responsible for the assembly of the virion, while the non-structural proteins contribute to viral replication [1,51].

NS1, a conserved glycoprotein with a molecular weight ranging from 46 kDa to 55 kDa, depending on the extent of glycosylation among the non-structural proteins of flavivirus, has been found in various intracellular compartments in infected cells as membrane-bound protein (mNS1) and secreted protein (sNS1) [3,13] (Box 1). After the NS1 protein is synthesized in cells, it forms a dimer that is transported to the plasma membrane with its C terminals in a head-to-head

Table 1. Methods for the diagnosis of flaviviruses circulating in Europe

Methods	Advantages	Limitations
RT-(q)PCR	• Provides an early diagnosis • Specific and sensitive • Highly standardized • Qualitative/quantitative	• Positivity limited to the acute phase (<10 days) • Requires expensive instruments and trained personnel
Antibody detection	• Rapid diagnostic test (RDT) kits • Low cost	• High cross-reactivity • Not suitable for early phase
PRNT	• Less cross-reactivity than antibody detection • Golden standard for serological diagnosis of flaviviruses	• Requires biosafety laboratories (levels 2–3) and trained personnel • International standardization lacking
Viral antigen capture	• Slightly extended time window in comparison with RT-(q)PCR (acute phase) • Less cross-reactivity compared to antibody detection • Low cost • Possibility to have rapid diagnostic test (RDT) kits	• Not available for flaviviruses circulating in Europe • Less sensitive than RT-PCR • Possible reduced sensitivity in secondary infections

Box 1. Structural characteristics of NS1

NS1 exists intracellularly as a membrane-associated dimer (mNS1) [60], while the soluble form is mainly described as a hexamer [3,13,59]. Although it was thought that the sNS1 structure was homogeneously hexameric, a recent study reported the existence of sNS1 in two different tetrameric structures (as a stable and loose conformation). This suggested that sNS1 comprises different particles, which are likely to have tetrameric and hexameric states [92] (Figure I). Moreover, sNS1 seems capable of dissociating into dimers that can bind to human high-density lipoproteins (HDL) and, with lower affinity, to low-density lipoprotein (LDL) [92,93].

The NS1 monomer is made up of three separate domains: the N-terminal β-roll domain (residues 1–29), the wing domain (residues 30–180), and the β-ladder domain (residues 181–352) [66] (Figure I). In the hexameric structure, three NS1 dimers are arranged side by side on the lipid bilayer. These are linked together by weak hydrophobic interactions and form a central core rich in lipids. The outer surface of NS1 features a 'spaghetti loop' in the β-ladder domain, a glycosylation site, and a disordered loop in the 'wing' domain [94].

NS1 is relatively well conserved among flaviviruses, exhibiting 20–40% identity and 60–80% similarity at amino acid level [95], especially in the case of flaviviruses that are part of the same serocomplex, such as WNV and JEV, with 71% similarity in NS1 [77]. Different studies have produced antibodies that cross-react between different flaviviruses, and it was observed that the epitopes that are most frequently recognized are primarily located in the easily accessible disordered loop of the 'wing' domain, the top of the C-terminal 'β-ladder' and the hydrophobic protrusion region. These epitopes correspond to the ones that ensure protection against a lethal flavivirus infection [63,65,66,96].

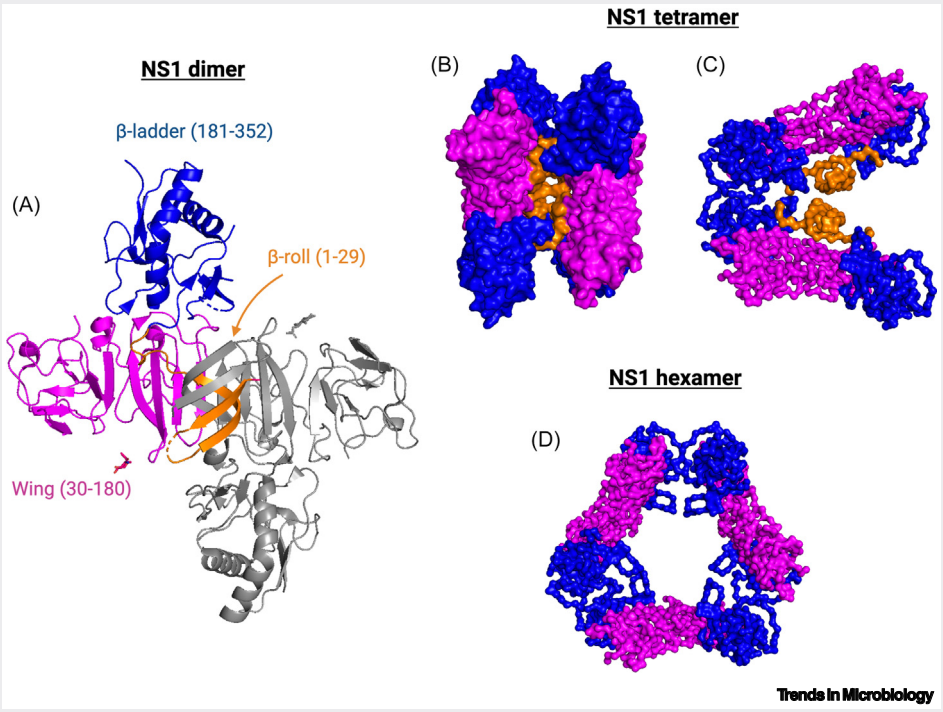

Figure I. Structures of non-structural protein 1 (NS1) in its different oligomerization states. (A) structure of dimeric NS1 (PDB: 4O6B). One monomer is represented in gray, while in the other one, the β-roll domain (residues 1–29) is orange, the wing domain (residues 30–180) is blue, and the β-platform domain (residues 181–352) is magenta. (B) Surface representation of NS1 stable tetramer (PDB7: WUT). (C) Surface representation of NS1 loose tetramer (PDB: 7WUU). (D) Surface representation of NS1 loose hexamer (PDB: 7WUV). In the hexameric structure, the density of the central core is weak. Therefore, it is not possible to distinguish the β-roll conformation [92]. In NS1 oligomeric structures, the domains of the protein are colored following the same color code used for the dimer.

configuration. In the Golgi apparatus of infected cells, the NS1 dimer is processed by glucosidase and glycosyltransferase to remove complex sugars. As a result, NS1 becomes soluble and is secreted [59].

The intracellular form of NS1 is central to viral replication, whereas the secreted one can be detected in serum and other body fluids and plays a role in immune evasion. A study identified a unique mutation in NS1 that causes the protein to lose its secretory capacity while retaining its role in viral genome replication, suggesting that sNS1 plays a role in the particle formation of flaviviruses through its interaction with the lipid membrane [60]. NS1 is also crucially involved in the pathogenesis of flaviviruses, directly causing endothelial dysfunction and stimulating immune cells to produce proinflammatory cytokines [61]. Soluble NS1 could re-attach itself to the surface of both infected and uninfected cells, which can affect the integrity of the endothelium and, hence, the permeability of blood–tissue barriers [62,63]. Moreover, the immune response to NS1 may harm endothelial cells due to the cross-reaction of antibodies and the formation of immune complexes [64]. This can trigger the production of autoantibodies that react with platelets and extracellular matrix proteins [59]. The presence of specific antibodies to mNS1 and sNS1 can further intensify the activation of the complement system [64]. While it is thought that extracellular NS1 plays a role in disease progression during infection, it also stimulates an immune response and triggers the production of antibodies. Monoclonal antibodies (mAbs) specifically targeting NS1 have been identified and have been shown to protect against lethal challenge models for viruses such as YFV, DENV, ZIKV, JEV, and WNV in mice. Moreover, mice vaccinated with NS1 were protected from lethal infection in multiple flavivirus models. NS1 thus represents a captivating target to develop new vaccinal or therapeutic strategies against flavivirus infection antagonizing NS1 pathogenic effects [61,63,65–67].

An extended version called NS1′ has a molecular weight of 52–53 kDa and was discovered during JEV, WNV, and DENV infection. Its presence seems to correlate with flavivirus neuroinvasiveness [3].

Detection of NS1 antigen

NS1 can be detected in the blood even before the onset of symptoms, generally as early as the detection of viral RNA (Figure 3). Thus, an NS1 antigen capture assay that evaluates the presence of NS1 in serum samples has the potential to be a valuable tool for early diagnosis of flavivirus infections [68] (Table 2).

While the detection of NS1 using an antigen capture assay seems to be less sensitive than detecting viral RNA using RT-PCR, the assay can be performed with a simple laboratory setup that can process hundreds of samples relatively quickly and with minimal effort [69,70]. Additionally, the NS1 antigen capture assay is a semi-quantitative test that is easier to perform and requires a lower level of complexity in laboratory infrastructure and staff training than RT-PCR quantifying viral RNA [69,71]. NS1 can be used as a biomarker to develop rapid tests according to the World Health Organization ‘ASSURED’ criteria. These criteria describe the ideal characteristics of a diagnostic test that can be used at all levels of the healthcare system: affordable, sensitive, specific, user-friendly, rapid, equipment-free, and delivered to those who need it. Rapid tests can serve as a cost-effective method for patient screening during regular medical check-ups, helping to detect asymptomatic infections in individuals residing in endemic regions. This plays a vital role in epidemiological monitoring, which is crucial for both patient treatment and epidemic preparedness [72].

However, previous studies have reported difficulty in detecting NS1 in secondary infections, which could represent a disadvantage compared to RT-PCR. In primary infections, NS1 can be found in infected serum or plasma samples until 9–12 days after disease onset, while during a secondary infection with the same virus, the IgG present in the blood could react with the protein forming immune complexes and making it impossible to detect NS1 in secondarily infected patients beyond 5–7 days after onset of symptoms [13]. To avoid the issue, NS1 detection can

Table 2. List of diagnostic assays based on NS1 and anti-NS1 antibody detection

Virus	Method	Refs/source
WNV	NS1 antigen capture ELISA	[76]
	NS1 antigen capture ELISA	[77]
	NS1 antigen capture ELISA	[78]
	Lateral flow immunoassay for NS1 detection	[79]
	NS1 Protein IgM ELISA kit (Cat. Number: 910-395-WNM) NS1 Protein IgG ELISA kit (Cat. Number: 910-390-WNG)	Alpha diagnostics
	NS1 Antigen ELISA Development Kit (Cat. Number: DEIAY10297)	Creative diagnostics®[a]
	NS1 IgG Antibody ELISA Kit (Cat. Number: VACY-1022-CY633) NS1 IgM Antibody ELISA Kit, Human (Cat. Number: VACY-1022-CY632)	Creative biolabs®[a]
USUV	rNS1-based ELISA for IgM/G	[80]
TBEV	Anti NS1 IgG ELISA	[81]
JEV	NS1 antigen capture ELISA	[71]
	NS1 antigen capture ELISA	[37]
	Lateral flow immunoassay for NS1 detection	[82]
	NS1 mAb-based blocking ELISA	[75]
	NS1 Protein IgM ELISA kit (Cat. Number: 910-175-JEM) NS1 Protein IgG ELISA kit (Cat. Number: 910-170-JEG)	Alpha diagnostics[a]

[a]All the kits and laboratory-based diagnostic tests listed in the table are intended for research use only, not for use in diagnostic procedures.

be supported by the measurement of specific IgM antibodies [13,69]. Furthermore, a study shows that acid treatment to dissociate immune complexes has been found to increase NS1 detection from 27% to 78% in secondary infections [73], while another study indicates no statistically significant difference in NS1 detection rates between primary and secondary infections [74]. NS1 can also be detected in urine at a later stage than in serum samples, remaining consistent until day 14. This extends the time window during which a flavivirus infection can be diagnosed using an NS1 antigen-capture assay [64]. In addition, using a urine sample could be a less invasive way to diagnose an infection without having to resort to a blood draw [14].

NS1 has been suggested as a marker for viremia because its concentration in the blood seems directly correlated with the viral titer [13,70]. Therefore, it may be used to predict the risk of developing severe symptoms and allow the clinicians to determine the most appropriate treatment for the patients, acting promptly towards patients with increased risk for severe disease and avoiding hospitalizations and unnecessary treatment for those at low risk. However, the correlation between NS1 levels in serum and disease severity has not yet been clearly demonstrated [64].

NS1 antigen-capture ELISA can limit the issue of cross-reactivity between antibodies of homologous and heterologous flavivirus antigens [70]. However, the effectiveness of immunoassays is largely determined by the quality of the antibodies employed and the distinctiveness of the epitopes that those antibodies target (Box 1). The specificity of these assays can be weakened by the presence of fewer unique epitopes, particularly when antibodies produced against antigenic domains of related pathogens show varying affinities towards the targeted epitope regions [68,69].

Detection of anti-NS1 antibodies

Not only can NS1 itself be used as a diagnostic tool, but the anti-NS1 antibodies can also be used to develop useful diagnostic tests (Table 2). The relative type-specificity of the antibody responses

has led to the creation of ELISA-based tests that can determine the infecting serotype, whether the infection is primary or secondary, and differentiate between different flaviviruses [13]. Evidence has been provided that the use of recombinant NS1 proteins for the detection of IgM/IgG antibodies is less prone to cross-reactivity if compared to commercial kits based on E protein or whole-virus antibody detection [44,75]. However, contradictory results related to cross-reactivity still hinder the detection of IgM antibodies in patients from flavivirus-endemic regions, fueling debates over the usefulness of NS1 as an antigen for antibody detection [44].

NS1 in diagnosis of WNV

Today, an NS1 antigen capture ELISA for WNV is available only for avian and mosquito surveillance but not for human diagnosis [8].

The presence of NS1 in the serum can be detected between days 3 and 8 post-infection, and during this window no significant difference is observed between results obtained through the NS1 assay and RT-PCR. Additionally, the NS1 assay is superior to IgM or plaque assay techniques. The time period during which NS1 was present in the serum is found to coincide with the appearance of clinical symptoms [76,83].

Different attempts to develop an NS1 antigen capture ELISA have been made using a recombinant WNV NS1 protein to generate specific antibodies that recognize the protein present in the serum [84].

In vivo studies in WNV-infected hamsters have shown the secretion level of NS1 antigen ranges from 100 to 8000 ng/ml. These levels are significantly higher than the detection limit of the ELISA system developed by Saxena *et al*., which can detect up to 5 ng/ml of NS1 antigen. The sensitivity and specificity of the recombinant NS1 sandwich ELISA in this study suggest that the test can be used as a cost-effective and accurate tool for surveillance and early diagnosis of WNV infection in endemic areas [77]. The main limit of this assay is that it does not effectively distinguish between WNV and other flaviviruses because it is based on flavivirus NS1 protein cross-reactive mAbs. Even if the WNV NS1 is used as the antigen to generate mAbs, they can still show cross-reactivity with other flaviviruses, such as JEV and USUV, which are part of the same serocomplex. These mAbs should be tested against at least the flaviviruses that are more prevalent in a specific region. This could allow for the exclusion of a large part of the antibodies that show cross-reactivity, helping to increase the specificity of the assay for the WNV NS1 protein. Based on these considerations, Ding *et al*. developed an NS1 antigen capture ELISA using two mAbs that recognized distinct epitopes of the NS1 protein of WNV and showed no cross-reactivity with JEV and TBEV, while no data are available regarding potential cross-reactivity with USUV. The detection limit of the antigen-capture ELISA was as low as 15 pg/ml, which was much more sensitive than the WNV-NS1 ELISA reported by Saxena *et al*. and WNV-NS1 could be detected in the serum one day after infection. Ding's NS1 antigen-capture ELISA displayed greater sensitivity than real-time RT-PCR from 1 to 7 days in WNV-infected mouse serum samples, which might be attributed to the short duration of viremia and low viral RNA titers after WNV infection [78].

In both the assays of Saxena*et al.* and Ding*et al.,* the formation of NS1-immune complexes affected the sensitivity of the capture ELISA under standard conditions by preventing the detection of free, soluble NS1. Treating plasma with an alkaline solution and a non-ionic detergent partially dissociated NS1 immune complexes and improved the sensitivity of the capture ELISA. Disrupting immune complexes in plasma samples seemed to extend the time window for measuring the antigen beyond the detection of viral RNA by quantitative RT-PCR [77,78,83].

The mAbs developed by Ding *et al*. were also used to develop a portable surface-enhanced Raman scattering (SERS)–lateral flow immunoassay (LFIA) detector for the detection of recombinant NS1 that shows a visual detection limit of 10 ng/ml besides high sensitivity and specificity for WNV when it was compared to dengue, yellow fever, and Zika [79].

NS1 in USUV diagnosis

USUV has only recently been identified as a pathogen of concern, and hence diagnostic solutions for this infection are still limited. No attempts have been made so far to create an assay to measure the presence of NS1 in infected patients, although, in theory, all the considerations made for WNV should also apply to USUV, as the two viruses belong to the same serocomplex and are therefore antigenically very similar. This similarity could also have repercussions on WNV diagnostic tests, leading to false positives that cause a USUV infection to be misdiagnosed as a WNV infection. Until now, USUV was considered a pathogen of marginal importance for human health and was not frequently included among the flaviviruses to test for cross-reactivity with WNV.

Only the EuroImmun USUV IgG ELISA is available on the market, while for IgM detection, there are no commercially available assays. The EuroImmun USUV IgG ELISA is based on the viral structural E protein, and it suffers from broad antigenic cross-reactivity between anti-flavivirus antibodies [26]. Detection of IgG antibodies using purified NS1 instead of E protein has been reported to show a low degree of cross-reactivity between related viruses. Thus, IgM/G ELISA based on recombinant NS1 for USUV is being established and tested in comparison with the highly homologous and geographically overlapping WNV. Careful analysis in immunized mice allowed a better characterization of sensitivity and specificity, showing that immune IgM sera targeting TBEV and WNV did not cross-react with USUV, while some cross-reactivity for WNV IgG was detected [80].

NS1 in TBEV diagnosis

NS1 is studied mainly in mosquito-borne flaviviruses, while the information available for TBEV is limited, and no studies have been conducted on NS1 as a possible marker for early detection of the infection during the acute phase.

Regarding the antibody response against NS1, a study evaluated the sensitivity and specificity of the recombinant NS1-based ELISA test for the detection of IgM/IgG antibodies from a cohort of patients infected by TBEV. The results showed that the recombinant NS1-based ELISA test had high sensitivity and specificity for the detection of TBEV-specific IgG antibodies [85].

Another study reported the development and validation of a TBEV NS1 IgG ELISA that facilitates precise identification of TBEV infections and the differentiation of TBEV infections from vaccination antibody responses [44,81]. The study used an IgG ELISA to detect the presence of TBEV NS1 protein in patients' sera. Since the available vaccines (FSME Immun® by Pfizer and Encepur® previously by GSK, now divestment to Bavarian Nordic) are highly purified and inactive, there is no replication of TBEV and thus no formation of NS1 protein or NS1-specific antibodies [30,81]. The absence of NS1 IgG in vaccinated patients makes it possible to discriminate between infection and vaccination leading to a better understanding of TBEV infection rate and epidemiology. The cross-reactivity of antibodies specific to TBEV NS1 with other flaviviruses appears to be lower than that of commercial whole-virus ELISAs. This may be due to the low degree of similarity between the NS1 proteins of different flaviviruses compared to the E protein [81]. Interestingly, TBEV NS1 IgG ELISA showed cross-reactivity with serum from patients vaccinated against YFV, but this was limited, probably because TBEV and YFV NS1s share the

greatest homology. As YFV vaccine is a live-attenuated vaccine, NS1 can be produced in the same way that occurs during a natural infection, and antibodies against NS1 can be detected both in vaccinated and infected patients [44,81].

NS1 in JEV diagnosis

There is an urgent need to develop a rapid diagnostic method for the detection of JEV infection to detect possible outbreaks, not only in humans but also in swine, which are the principal amplification hosts. Swine serum can be used to screen for the JEV NS1 protein for early detection, surveillance, and prevention of human outbreaks, as the virus is known to seroconvert in swine 2–4 weeks before human infection [82,86].

NS1 antigen capture ELISA can be used to diagnose JEV infection as early as on the first day of illness, and NS1 can be detected even when viral RNA is not found by RT-PCR or in the presence of IgM antibodies. In addition, the assay is quantitative, considerably stable, and less affected by variations in physical conditions than the quantification of viral RNA [71].

Li *et al*. developed a JEV-specific and quantitative NS1 antigen-capture ELISA using a flavivirus-specific and a JEV-specific mAb, respectively, and they analyzed NS1 secretion in JEV-infected cell culture and NS1 in sera of JEV-infected mice and patients. The capture assay could detect protein levels as low as 0.2 ng/ml. However, only 29.3% of human sera and 10.5% of CSF tested positive for NS1 protein, likely due to late sample collection after the mosquito bite, as indicated by the presence of anti-JEV IgM antibodies. Further research on early virus detection during febrile illness may increase the chances of virus recovery and NS1 capture [87]. A recent study using NS1-capture sandwich ELISA on sera and CSF during the acute phase of JEV infection showed a 97% agreement with RT-PCR [71].

Recently, a colorimetric sandwich-based lateral flow assay (LFA) has been developed using gold nanoparticles (AuNPs) labeled with the JEV NS1-specific polyclonal antibody for rapid, sensitive, and specific NS1 detection in serum samples. This portable, cost-effective, and disposable immunochromatographic strip has a visual detection limit of 10 pg/ml for JEV NS1 Ag in JEV-negative serum and can detect JEV NS1 Ag in clinical swine serum samples within 10 min. It shows negligible nonspecific binding with other flavivirus NS1 and may be developed into a point of care (PoC) diagnostic kit for rapid mass screening, especially in rural areas with limited laboratory resources. At the moment, this assay is limited to swine NS1 detection, but it could represent an interesting solution even for human diagnosis [82].

NS1 stimulates high levels of antibody production *in vivo*. When Zhou *et al*. tested for the JEV NS1 antibody in five immunized swine, four had detectable NS1 antibodies 7 days post-immunization, while only one was found to be positive for the E protein antibody. Based on this evidence, they developed a blocking ELISA using a high-affinity anti-JEV NS1 mAb for serological monitoring of JEV infection and evaluation of the immune status of swine following JEV vaccination. Of newly JEV-vaccinated swine, 80% showed seroconversion within 7 days post-immunization, while the commercial E-protein-based indirect ELISA detected seroconversion in only 20% of the newly vaccinated swine [75].

Concluding remarks

WNV, USUV, and TBEV co-circulation in Europe is rapidly becoming a matter of concern, and the limited availability of precise surveillance and diagnostic systems might exacerbate the problem in the future. This might especially hold true if other flaviviruses, such as JEV, are introduced into Europe.

Outstanding questions

How important is it to discriminate between different flaviviruses regarding clinical care, considering that no specific treatment is available for these viruses? What are the main consequences if a flavivirus is misdiagnosed for another?

How can antibodies highly specific for the NS1 of a single flavivirus be developed?

How can the absence of cross-reactivity be assured?

Why has no NS1-based diagnostic test been commercialized yet for WNV, USUV, TBEV, and JEV, as it seems to be the best alternative for PCR for accurate, early, and rapid diagnosis?

In this context, the need to introduce effective diagnostic methods that can clearly discriminate between flaviviruses is relevant and urgent. With currently available diagnostic methods either on the market or used in laboratories, it is difficult to diagnose flavivirus infections at their onset, and above all, it is complex to unequivocally identify the responsible pathogen due to high cross-reactivity between members of this family. Cross-reactivity represents a major obstacle because, in addition to the uncertainty in diagnosis, it can also lead to an incorrect definition of the epidemiology of these viruses in areas where co-circulation is observed (see Outstanding questions).

NS1 currently represents the most promising diagnostic marker that would allow an early diagnosis, as it can be detected in the blood during the acute phase of flavivirus infection. Furthermore, NS1 diagnosis has the potential to reduce the problem of cross-reactivity, as antibodies against this protein seem more specific than those directed against the E protein on which commercially available assays are based. NS1 antigen capture ELISAs have already been commercialized for dengue diagnosis, and several studies show that WNV can be diagnosed in a highly specific manner using the same type of assay. Further investigations need to be conducted on USUV and TBEV, for which currently little or no information is available regarding the use of NS1 as a marker for early diagnosis. However, developing mAbs that are highly specific for the NS1 of a given flavivirus and do not show cross-reactivity with members of the same serocomplex remains a major challenge. Moreover, in addition to being used in rapid antigen tests (or capture ELISA) to detect the NS1 antigen, these specific antibodies can be used for the detection of flavivirus antibodies by competitive ELISA, expanding the time frame in which a specific diagnosis can be made. The development of such mAbs and further research in this area could provide a concrete solution to the problem of early, specific, rapid, and low-cost diagnosis of WNV, USUV, and TBEV in Europe.

Declaration of interests

The authors declare no competing interests.

Resources

[i] www.ecdc.europa.eu/en/news-events/epidemiological-update-west-nile-virus-transmission-season-europe-2022

[ii] www.cdc.gov/tick-borne-encephalitis/geographic-distribution/index.html

[iii] www.ecdc.europa.eu/en/tick-borne-encephalitis/facts/factsheet

[iv] www.ecdc.europa.eu/en/publications-data/ixodes-ricinus-current-known-distribution-february-2023

[v] www.ecdc.europa.eu/en/japanese-encephalitis/facts

[vi] www.cdc.gov/japaneseencephalitis/maps/index.html

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