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

Martina Ceconi [1], Kevin K. Ariën [2,3], and Peter Delputte [1,*]

[1] Laboratory for Microbiology, Parasitology and Hygiene, and Infla-Med Centre of Excellence, University of Antwerp, Antwerp 2600, Belgium.

[2] Virology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine Antwerp, Antwerp 2000, Belgium.

[3] Department of Biomedical Sciences, University of Antwerp, Antwerp 2600, Belgium

*Correspondence: peter.delputte@uantwerpen.be

Abstract: In the last decades, the presence of flaviviruses of concern for human health in Europe has drastically increased, also due to climate change, which has allowed the vectors of these viruses to expand in new territories. Co-circulation of West Nile virus, Usutu virus, and tick-borne encephalitis virus 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 further complicates accurate diagnosis. Current flavivirus diagnosis is mainly based 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 both on 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.

26 **Keywords:** Flavivirus; Europe; Diagnosis; NS1.

27

28 **1. An introduction to flaviviruses**

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

38 Flaviviruses can be divided into three groups according to their dominant vector [6,7] (Figure
39 1a):

40 1. tick-borne viruses;
41 2. mosquito-borne viruses;
42 3. and viruses for which the vector is unknown

43 The mosquito-borne virus group can be subdivided into viruses transmitted predominantly by
44 *Culex* or *Aedes* mosquitoes which have different vertebrate hosts and pathogenesis. The *Culex*
45 species use birds as reservoirs and are the main ones responsible for spreading neurotropic
46 flaviviruses, which can cause severe meningoencephalitis. Flaviviruses mainly transmitted by
47 *Aedes* mosquitoes have primate reservoirs and do not show neurotropism (except for Zika
48 virus) and cause acute fever with arthralgias, myalgias, and, in extreme cases, hemorrhagic
49 fever (dengue and yellow fever). The tick-borne viruses also form two groups: one group

50 circulates among seabirds, while the other, the tick-borne encephalitis group, is primarily
51 associated with rodents [8–10].
52 Serological studies enable the definition of different serocomplexes based on antigenic cross-
53 reactivity [9,10]. The majority of flaviviruses relevant to human health can be clustered into 8
54 serocomplexes and 17 independent viruses that are not antigenically related enough to be
55 included in a serocomplex [11] (Figure 1c).
56 Because of the increasing global burden of flavivirus-associated diseases, the necessity of
57 diagnostic methods for accurate, specific, and straightforward discrimination between the
58 different flaviviruses, which can also be used during different stages of the disease, is crucial
59 [3]. Developing a diagnostic test that detects a specific flavivirus remains challenging. This is
60 especially the case in regions where antigenically related viruses co-circulate, as traditional
61 serological assays routinely performed in hospitals and laboratories may suffer from cross-
62 reactivity [12,13].
63 This review will focus on flaviviruses that co-circulate in Europe, highlighting the limits of the
64 current diagnostic methods and the potential of NS1-based tests to improve the diagnosis of
65 different flaviviruses that co-circulate in the same geographic areas.
66

67 **2. Flavivirus epidemiology in Europe**

68 Factors such as climate change, rapid urbanization, increased transportation, commerce, and
69 travel increasingly contribute to changes in the distribution, spread, and seasonality of
70 flaviviruses and their vectors in Europe [14,15]. Global warming further creates an
71 environment conducive to the spread and establishment of ticks and mosquitos in more
72 temperate countries [10,16]. Moreover, rising temperatures are extending the seasonal
73 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 Usutu virus (USUV), and to some extent also JEV, are of particular concern for public health.

2.1. West Nile virus

WNV has the widest geographical distribution in Europe, with the largest number of vectors and non-human 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 America 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 European Union (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 4 had an unknown importation status and unknown place of infection [23]. 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 less than 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 [20,24]. 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 [21,25].

2.2. Usutu virus

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 [22,26], and in 2009, the first cases of neurological symptoms associated with USUV infection were reported in two immunocompromised patients in Italy [26,27]. 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 United Kingdom) by virus isolation/detection or serologically [22].

As for WNV, USUV belongs to the Japanese encephalitis virus serocomplex of flaviviruses. These viruses mostly share the same vector and hosts and have a similar life cycle [10,20,22,24]. In contrast to WNV, USUV seems more virulent in birds, causing significant blackbird mortality, especially in central Europe, while human infection seems less common [20,24]. Between 2012 and 2021, 105 cases of human USUV infection were reported in Europe, 12 of which showed neurological symptoms [28]. Most of the cases were found in

121 Italy, but USUV infections were also detected in Croatia, Germany, Czechia, Austria, Hungary,
122 and France [20,28,29].
123
124 *2.3. Tick-borne Encephalitis virus*
125 TBEV is considered the most medically significant arbovirus in Europe, infecting over 10,000
126 humans every year [8,30]. Its presence in Europe was reported for the first time in 1931 in
127 south-eastern Austria, and today it is considered endemic in 27 European countries, mostly
128 East-European countries with Slovenia showing the highest reported incidence [31,32]. In
129 Europe, all three different subtypes of TBEV have been identified: the European subtype
130 (TBEV-Eu), transmitted by *Ixodes ricinus* ticks and endemic in rural and forested areas of
131 central, eastern, and northern Europe; the Siberian subtype (TBEV-Sib), transmitted by *Ixodes*
132 *persulcatus* and endemic in the Urals region, Siberia, Russia and in some areas in north-
133 eastern Europe; and the Far Eastern subtype (TBEV-FE), transmitted by *Ixodes persulcatus* and
134 mainly typical of Asia but has been found in several Eastern European countries [32,33].
135 Approximately two-thirds of human TBEV infections are asymptomatic, but 10-30% of patients
136 can develop non-specific symptoms such as fever, fatigue, headache, and myalgia. In rare
137 cases, the nervous system could be involved in patients showing meningitis (50%),
138 meningoencephalitis (40%), meningoencephalomyelitis (10%), paralysis, and radiculitis
139 [25,34]. TBEV-FE is associated with the most severe neurological manifestations and has a
140 fatality rate of around 20%. In comparison, the European subtype shows milder disease and
141 mortality rates below 1%, with severe neurological sequelae in up to 10% of patients
142 [33,35,36].
143 Tick vectors are responsible for transmitting the virus to animals (mostly rodents and deer,
144 which act as amplifying hosts) and humans, who act as dead-end hosts [25]. According to data

145 published in February 2023 in the context of the VectorNet project, the presence of *Ixodes*
146 *ricinus*, the main vector of TBEV, was detected in all European countries. This makes it
147 possible, at least theoretically, for TBEV to spread even in areas where it is not considered
148 endemic yet [37]. In this regard, the first three autochthonous TBEV cases were reported in
149 Belgium during the summer of 2020, but the common vector of TBEV was already widespread
150 in the country, and TBEV antibodies were detected in animals such as dogs, cattle, roe deer,
151 and wild boar before evidence of human infection [38].
152
153 *2.4. Japanese encephalitis virus*
154 JEV is the prototype of the JEV serogroup that also includes WNV and USUV [39]. It is one of
155 the leading causes of viral encephalitis, with an annual number of cases between 30,000 and
156 50,000 [40]. It is considered endemic in at least 24 countries in Asia and Oceania, and it is
157 estimated that around 3 billion people live in JEV epidemic areas [39,41].
158 JEV infection generally causes mild febrile symptoms, while approximately 1% of patients can
159 develop a severe neuroinvasive illness characterized by high fever, headache, neck stiffness,
160 disorientation, coma, seizures, and spastic paralysis, which a mortality rate of around 30%
161 [40,42]. The neuroinvasive disease may also be responsible for lifelong disabilities or cognitive
162 impairments in approximately 30% of patients who recover after JEV infection [42,43].
163 JEV is mainly spread by *Culex* mosquitos and circulates in various species of birds that are the
164 natural reservoir, while pigs are considered the main maintenance or amplifying host [19]. As
165 for WNV and USUV, humans are dead-end hosts because viremia is insufficient to transmit the
166 infection to another vector [44].
167 The introduction of the Japanese encephalitis virus in Europe is a potential risk due to
168 international travel and commerce with Asia and Oceania. This could lead to the introduction

169 of infected mosquitoes. If the virus is introduced, it could become established in Europe due
170 to the presence of susceptible mosquito vectors and vertebrate hosts [40]. In 1996-97, the
171 first evidence of JEV antibodies and RNA in Italian birds was reported [45,46]. Afterward, JEV-
172 like sequences were detected in *C. pipiens* specimens in northern Italy in the summer of 2010
173 [46]. JEV gene amplification was detected in bird specimens collected in Tuscany, where JEV-
174 positive mosquitoes were also found. According to the epidemiological analysis, no JE patients
175 were found where the bird specimens were collected [39]. This suggests that there was a
176 limited epidemic cycle of JEV between birds and mosquitoes in southern Italy. The lack of pigs,
177 the primary hosts of JEV, may have prevented the virus from spreading on a larger scale. The
178 detection of JEV in both birds and mosquitoes indicates that the virus has spread to Europe,
179 specifically to Italy, from traditional JEV epidemic areas in Asia [39,45].
180
181 *2.5. Flavivirus co-circulation in Europe*
182 The era of global change has brought significant modifications to the distribution of
183 flaviviruses in the European continent, and in the coming years, the increasingly tangible
184 global warming is expected to cause further alterations. In this context, the tick species *Ixodes*
185 *ricinus*, which is the primary vector of the European variant of TBEV, has been discovered at
186 higher altitudes where it was previously absent, and in greater numbers in areas where it was
187 originally present. Similar behavior has been observed for vectors of WNV and USUV in
188 Europe, such as mosquito species *Culex pipiens s.l.* and *Culex modestus,* which are considered
189 the main bridge vectors of WNV from avian reservoirs to dead-end hosts, including humans.
190 In the last decade, this thermophilic species has expanded its territory northward and has
191 been reported in several European countries for the first time [14,20].

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

201 The presence of these viruses on European territory, as well as their possible co-circulation,
202 could be underestimated by the fact that most countries have no active surveillance programs
203 to detect flavivirus circulation both in humans and animals [22]. Furthermore, specific
204 serology tests are lacking to study seroprevalence and do systematic serosurveillance [47].
205 Increased travel and transportation from endemic areas raise the risk of introducing other
206 neurotropic flaviviruses in Europe, such as JEV [48], causing further problems in the diagnosis
207 and surveillance of this family of viruses [25,49].

208

209 3. Diagnostic methods to detect flavivirus infection

210 Given the increasing public health risk posed by the spread of various flaviviruses in Europe,
211 it is now more crucial than ever to be able to accurately diagnose the virus responsible for the
212 infection. The early and precise diagnosis of the infectious agent is necessary for appropriate
213 clinical care before symptoms exacerbate (i.e., patients can rapidly progress to life-threatening
214 neurological complications), but also for surveillance and epidemiology [3,12,50]. The ability
215 to discriminate between different members of this family, especially when they are part of the

216 same serocomplex, is essential to understanding which viruses circulate in a given region and
217 time to take appropriate precautions, such as vector control and One Health surveillance.
218 Furthermore, the implementation of a surveillance system and the possibility to detect timely
219 autochthonous and imported infections is becoming a priority in non-endemic areas to avoid
220 new outbreaks caused by the spread of these viruses in new areas [51].
221 The standard method for diagnosing flavivirus infections involves detecting the pathogen, its
222 nucleic acids, or specific viral antigens during the acute phase of the disease, followed by
223 measuring specific antibodies present in the patient during the convalescence phase [51,52].
224

225 *3.1. Molecular diagnostics*

226 Flavivirus infection can be confirmed by the detection of the viruses in body fluids (usually
227 blood, serum, or plasma, but they can also be detected in urine and cerebrospinal fluids). Viral
228 nucleic acids can be detected by performing an RT-PCR or an RT-qPCR [51]. This is the most
229 specific and sensitive technique available because it can distinguish between two different
230 flaviviruses at the RNA level. RT-PCR allows the detection of the presence of infection from
231 the onset of symptoms until 7-10 days post-infection, allowing for rapid diagnosis of suspected
232 cases. It is also highly standardized and allows for a high degree of repeatability and
233 reproducibility [15,21].
234 The acute viremic phase of flavivirus infection lasts 5-7 days on average (Figure 3) and is often
235 missed due to the generic flu-like symptoms that the patients develop during this phase,
236 which can be confused with those of more common infections [1]. Usually, patients only visit
237 a doctor when their symptoms persist or worsen, and, at that point, viral nucleic acids are
238 often no longer detectable in the blood, excluding RT-PCR for diagnosis [8,15]. WNV, USUV,
239 and TBEV can persist in the kidneys for extended periods and be excreted in the urine. In cases

240 of neuroinvasive WNV infection, the diagnosis from urine samples can be more reliable and
241 effective than from cerebrospinal fluid. However, urine samples do not yet seem to be
242 routinely collected as standard sample material [14]. Furthermore, RT-PCR is complex,
243 expensive, and requires specialized equipment and trained personnel to be performed [12].
244
245 *3.2. Serological diagnosis*
246 Serological assays to detect antibodies are the preferred diagnostic method in most
247 laboratories or hospitals. IgM is detectable from the first week post-infection, and titers start
248 to decline in the following 2 to 3 months (Figure 3). However, in the case of WNV infection,
249 IgM has been reported to persist even for a year [15]. IgG levels, instead, can be measured
250 with a few days delay relative to IgM but usually remain detectable for several months or years
251 after exposure to the antigen [51] (Figure 3). During secondary infection, instead, the rise of
252 IgM levels is often delayed compared to IgG, which can be rapidly detected within 2 days after
253 the onset of the disease [15].
254 Detection of antibody levels against flaviviruses is the most widely used diagnostic method
255 due to its rapidity, sensitivity, reproducibility, and affordability. It is cheaper than an RT-PCR
256 and does not require complex equipment [8,21]. IgM Ab-capture immunoassay (MAC-ELISA)
257 can be performed to detect an acute infection, while IgG indirect ELISA is more useful to
258 diagnose a secondary infection [8]. The main problems with antibody detection as a diagnostic
259 method are that the very early phase of infection might not be accurately detected as
260 antibodies might not be produced yet, and the presence of high cross-reactivity found
261 between different flaviviruses, particularly those belonging to the same serocomplex, such as
262 for example WNV and USUV [53,54]. These viruses share a high degree of structural and
263 sequence homology, which results in a similar antibody response that causes extensive cross-

264 reactivity [1,55]. This can lead to incorrect interpretation of diagnostic results and can also
265 result in underestimating the presence of a flavivirus in a given geographic area, as may be
266 happening in Europe for USUV, which, being less known and studied than WNV, could be
267 confused with the latter [20]. TBEV and WNV show lower cross-reactivity compared to WNV
268 and USUV, probably because they are not part of the same serocomplex, and they also have
269 different vectors and amplification hosts. Despite this, cross-reactivity between WNV and
270 TBEV was observed in Greece during the WNV outbreak in 2010, confirming that the
271 possibility of cross-reaction also exists with more distantly related viruses [56]. Since both IgM
272 and IgG antibodies circulate for multiple months following the onset of the infection, it can be
273 challenging to determine if the positive antibody titer is the result of an acute infection or if it
274 is the remnant of a previous infection or vaccination [8].
275 The diagnosis made by antibody detection can be further complicated by the fact that patients
276 could have previously been vaccinated against one or more flaviviruses. In Europe, human
277 vaccines are available only for TBEV, JEV, and YFV [57,58]. JEV and YFV vaccination is not
278 routinely performed, and they are recommended only in case of travel to endemic areas, such
279 as tropical and subtropical regions of Africa, South America, or Southeast Asia [8]. In contrast,
280 TBEV vaccination is recommended and implemented in the countries in which TBEV is
281 considered endemic. A cross-sectional study conducted in 2015 found that the average TBE
282 vaccination rate of all the European countries evaluated was 25% of the total sample. Finland
283 and Slovakia had the lowest vaccination rates (~10%) [59], while Austria is the European
284 country with the highest vaccination coverage, corresponding to 88% [8]. In general, the
285 vaccination rates in Europe are highly variable from country to country, with an overall low
286 vaccination coverage [59]. Since the serological tools are not able to distinguish between

287 naturally infected and vaccinated people, vaccination history and rates in a country should be
288 investigated when interpreting diagnostic results [60].
289 When the samples are not distinguishable by antibody detection-based methods, it is
290 recommended to perform a plaque-reduction neutralization assay (PRNT), which is
291 considered the gold standard in flavivirus serological diagnosis [12]. This method requires the
292 manipulation of live flaviviruses at a biosafety laboratory level (BSL) 2 for USUV or 3 for WNV,
293 TBEV, and JEV, which can be performed routinely only in a few laboratories in Europe [8,20].
294 Moreover, the use of live viruses leads to high variability between assays and between
295 laboratories due to the differences in cell lines used, the virus strain, other inter-laboratory
296 variations, and overall lack of international standardization [12].
297
298 *3.3 Viral antigen capture*
299 Viral antigens can be used to diagnose viral infections in the early stages of the disease by
300 detecting viral antigens directly in the clinical specimen [61]. Viral antigen detection by ELISA
301 is a cost-effective, rapid, and accurate diagnostic assay that could facilitate early viral detection
302 [62]. However, viral antigen detection kits are commercially available only for dengue
303 diagnosis [63], while for WNV, USUV, TBEV, or JEV, the kits are limited to research purposes
304 and not suitable for diagnosis in clinical settings. The potential and limitations of this
305 methodology will be discussed in detail in the next section.
306
307 Different flavivirus diagnostic methods have some advantages but also several limitations
308 (listed in Table 1). It is evident that there is an urgent need to introduce on the market new
309 diagnostic tests for flavivirus infection that can be easily implemented without expensive
310 equipment, show high specificity and sensitivity, allow the diagnosis during the acute phase

311 of the disease, and are not affected by the cross-reactivity of co-circulating flavivirus. In
312 Europe, there is a clear necessity to have a diagnostic tool that can discriminate principally
313 between WNV, TBEV, and USUV that co-circulate. Furthermore, other viruses that are
314 antigenically related, such as JEV, could potentially be introduced into Europe. These viruses
315 show cross-reactivity with WNV and USUV, and it is crucial to accurately differentiate between
316 them.

317

318 **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
Plaque-reduction neutralization assay (PRNT)	• Less cross-reactivity than antibody detection • Golden standard for serological diagnosis of flaviviruses	• Requires Biosafety Laboratories (levels 2 to 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

319

320 **4. NS1 antigen capture for West Nile, Usutu, tick-borne encephalitis, and Japanese**
321 **encephalitis diagnosis**

322 In the search for an ideal diagnostic assay that can discriminate between different flavivirus
323 infections, non-structural protein 1 (NS1) is a key viral protein that can be used to develop
324 new diagnostic assays for flavivirus infections [3].

325 The flavivirus genome is composed of a single-strand RNA of positive polarity, which is non-
326 segmented and around 10-11 kbp in length. The genome encodes a large polyprotein
327 precursor, which is co- and post-translationally processed by viral and host-derived proteases
328 into three structural proteins (Capsid, prM, and Envelope) and seven non-structural proteins
329 (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [5,64]. The structural proteins are responsible
330 for the assembly of the virion, while the non-structural proteins contribute to viral replication
331 [1,57].

332 NS1, a conserved glycoprotein with a molecular weight ranging from 46-55 kDa depending on
333 the extent of glycosylation among the non-structural proteins of flavivirus, has been found in
334 various intracellular compartments in infected cells as membrane-bound protein (mNS1) and
335 secreted protein (sNS1) [3,13]. After the NS1 protein is synthesized in cells, it forms a dimer
336 that is transported to the plasma membrane with its C-terminals in a head-to-head
337 configuration. In the Golgi apparatus of infected cells, the NS1 dimer is processed by
338 glucosidase and glycosyltransferase to remove complex sugars. As a result, NS1 becomes
339 soluble and is secreted [65].

340 The intracellular form of NS1 is central to viral replication, whereas the secreted one can be
341 detected in serum and other body fluids and plays a role in immune evasion. A study identified
342 a unique mutation in NS1 that causes the protein to lose its secretory capacity while retaining
343 its role in viral genome replication, suggesting that sNS1 plays a role in the particle formation

344 of flaviviruses through its interaction with the lipid membrane [68]. NS1 is also crucially
345 involved in the pathogenesis of flaviviruses, directly causing endothelial dysfunction and
346 stimulating immune cells to produce proinflammatory cytokines [69]. Soluble NS1 could re-
347 attach itself to the surface of both infected and uninfected cells, which can affect the integrity
348 of the endothelium and, hence, the permeability of blood-tissue barriers [70,71]. Moreover,
349 the immune response to NS1 may harm endothelial cells due to the cross-reaction of
350 antibodies and the formation of immune complexes [72]. This can trigger the production of
351 autoantibodies that react with platelets and extracellular matrix proteins [65]. The presence
352 of specific antibodies to mNS1 and sNS1 can further intensify the activation of the
353 complement system [72]. While it is thought that extracellular NS1 plays a role in disease
354 progression during infection, it also stimulates an immune response and triggers the
355 production of antibodies. Monoclonal antibodies specifically targeting NS1 have been
356 identified and have been shown to protect against lethal challenge models for viruses such as
357 YFV, DENV, ZIKV, JEV, and WNV in mice. Moreover, mice vaccinated with NS1 were protected
358 from lethal infection in multiple flavivirus models. NS1 represents thus a captivating target to
359 develop new vaccinal or therapeutic strategies against flavivirus infection antagonizing NS1
360 pathogenic effects [69,71,73–75].
361 An extended version called NS1' has a molecular weight of 52-53 kDa and was discovered
362 during JEV, WNV, and DENV infection. Its presence seems to correlate with flavivirus neuro-
363 invasiveness [3].
364
365 *4.1. Detection of NS1 antigen*
366 NS1 can be detected in the blood even before the onset of symptoms, generally as early as
367 the detection of viral RNA (Figure 3). Thus, an NS1 antigen capture assay that evaluates the

368 presence of NS1 in serum samples has the potential to be a valuable tool for early diagnosis
369 of flavivirus infections [76] (Table 2).
370 While the detection of NS1 using an antigen capture assay seems to be less sensitive than
371 detecting viral RNA using RT-PCR, the assay can be performed with a simple laboratory setup
372 that can process hundreds of samples relatively quickly and with minimal effort [77,78].
373 Additionally, the NS1 antigen capture assay is a semi-quantitative test that is easier to perform
374 and requires a lower level of complexity in laboratory infrastructure and staff training than RT-
375 PCR quantifying viral RNA [77,79]. NS1 can be used as a biomarker to develop rapid tests
376 according to the World Health Organization "ASSURED" criteria. These criteria describe the
377 ideal characteristics of a diagnostic test that can be used at all levels of the healthcare system:
378 affordable, sensitive, specific, user-friendly, rapid, equipment-free, and delivered to those
379 who need it. Rapid tests can serve as a cost-effective method for patient screening during
380 regular medical check-ups, helping to detect asymptomatic infections in individuals residing
381 in endemic regions. This plays a vital role in epidemiological monitoring, which is crucial for
382 both patient treatment and epidemic preparedness [80].
383 However, previous studies have reported difficulty in detecting NS1 in secondary infections,
384 which could represent a disadvantage compared to RT-PCR. In primary infections, NS1 can be
385 found in infected serum or plasma samples until 9-12 days after disease onset, while during a
386 secondary infection with the same virus, the IgG present in the blood could react with the
387 protein forming immune complexes and making it impossible to detect NS1 in secondary-
388 infected patients beyond 5-7 days after onset of symptoms [13]. To avoid the issue, NS1
389 detection can be supported by the measurement of specific IgM antibodies [13,77].
390 Furthermore, a study shows that acid treatment to dissociate immune complexes has been
391 found to increase NS1 detection from 27% to 78% in secondary infections [81], while another

392 study indicates no statistically significant difference in NS1 detection rates between primary
393 and secondary infections [82]. NS1 can also be detected in urine at a later stage than in serum
394 samples, remaining consistent until day 14. This extends the time window during which a
395 flavivirus infection can be diagnosed using an NS1 antigen-capture assay [72]. In addition,
396 using a urine sample could be a less invasive way to diagnose an infection without having to
397 resort to a blood draw [14].
398 NS1 has been suggested as a marker for viremia because its concentration in the blood seems
399 directly correlated with the viral titer [13,78]. Therefore, it may be used to predict the risk of
400 developing severe symptoms and allow the clinicians to determine the most appropriate
401 treatment for the patients, acting promptly towards patients with increased risk for severe
402 disease and avoiding hospitalizations and unnecessary treatment for those at low risk.
403 However, the correlation between NS1 levels in serum and disease severity has not been
404 clearly demonstrated yet [72].
405 NS1 antigen-capture ELISA can limit the issue of cross-reactivity between antibodies of
406 homologous and heterologous flavivirus antigens [78]. However, the effectiveness of
407 immunoassays is largely determined by the quality of the antibodies employed and the
408 distinctiveness of the epitopes that those antibodies target. The specificity of these assays can
409 be weakened by the presence of fewer unique epitopes, particularly when antibodies
410 produced against antigenic domains of related pathogens show varying affinities towards the
411 targeted epitope regions [76,77].
412

413 *4.2. Detection of anti-NS1 antibodies*

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

416 responses has led to the creation of ELISA-based tests that can determine the infecting
417 serotype, whether the infection is primary or secondary, and differentiate between different
418 flaviviruses [13]. Evidence has been provided that the use of recombinant NS1 proteins for the
419 detection of IgM/ IgG antibodies is less prone to cross-reactivity if compared to commercial
420 kits based on Envelope (E) protein or whole virus antibody detection [50,87]. However,
421 contradictory results related to cross-reactivity still hinder the detection of IgM antibodies in
422 patients from flavivirus-endemic regions, fueling debates over the usefulness of NS1 as an
423 antigen for antibody detection [50].
424

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

Virus	Method	Source
WNV	NS1 antigen capture ELISA	Macdonald et al., (2005) [88]
	NS1 antigen capture ELISA	Saxena et al., (2013) [84]
	NS1 antigen capture ELISA	Ding et al., (2014) [89]
	Lateral flow immunoassay for NS1 detection	Jia et al., (2021) [90]
	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®
	NS1 IgG Antibody ELISA Kit (Cat. Number: VACY-1022-CY633) NS1 IgM Antibody ELISA Kit, Human (Cat. Number: VACY-1022-CY632)	Creative biolabs®
USUV	rNS1-based ELISA for IgM/G	Caracciolo et al., (2020) [91]
TBEV	Anti NS1 IgG ELISA	Girl et al., (2020) [92]

JEV	NS1 antigen capture ELISA	Kumar et al., (2011) [79]
	NS1 antigen capture ELISA	Li et al., (2012) [43]
	Lateral flow immunoassay for NS1 detection	Roberts et al., (2022) [93]
	NS1 mAb-based blocking ELISA	Zhou et al., (2019) [87]
	NS1 Protein IgM ELISA kit (Cat. Number: 910-175-JEM) NS1 Protein IgG ELISA kit Cat. Number: 910-170-JEG)	Alpha diagnostics

426 * All the kits and lab-based diagnostic tests listed in the table are intended for research use

427 only, not for use in diagnostic procedures.

428

429 *4.3. NS1 in West Nile virus diagnosis*

430 Today, an NS1 antigen capture ELISA for WNV is available only for avian and mosquito

431 surveillance but not for human diagnosis [8].

432 NS1 presence in the serum can be detected between days 3 and 8 post-infection, and during

433 this window, no significant difference is observed between results obtained through the NS1

434 assay and RT-PCR. Additionally, the NS1 assay is superior to IgM or plaque assay techniques.

435 The time period during which NS1 was present in the serum is found to coincide with the

436 appearance of clinical symptoms [88,94].

437 Different attempts to develop an NS1 antigen capture ELISA have been made using a

438 recombinant WNV NS1 protein to generate specific antibodies that recognize the protein

439 present in the serum [95].

440 In vivo studies in WNV-infected hamsters have shown the secretion level of NS1 antigen ranges

441 from 100 to 8,000 ng/ml. These levels are significantly higher than the detection limit of the

442 ELISA system developed by Saxena et al., which can detect up to 5 ng/ml of NS1 antigen. The
443 sensitivity and specificity of the recombinant NS1 sandwich ELISA in this study suggest that
444 the test can be used as a cost-effective and accurate tool for surveillance and early diagnosis
445 of WNV infection in endemic areas [84]. The main limit of this assay is that it does not
446 effectively distinguish between WNV and other flaviviruses because it is based on flavivirus
447 NS1 protein cross-reactive monoclonal antibodies (mAbs). Even if the WNV NS1 is used as the
448 antigen to generate monoclonal antibodies, they can still show cross-reactivity with other
449 flaviviruses, such as JEV and USUV, which are part of the same serocomplex. These mAbs
450 should be tested against at least the flaviviruses that are more prevalent in a specific region.
451 This could allow for the exclusion of a large part of the antibodies that show cross-reactivity,
452 helping to increase the specificity of the assay for the WNV NS1 protein. Based on these
453 considerations, Ding et al. developed an NS1 antigen capture ELISA using two monoclonal
454 antibodies that recognized distinct epitopes of the NS1 protein of WNV and showed no cross-
455 reactivity with JEV and TBEV, while no data are available regarding potential cross-reactivity
456 with USUV. The detection limit of the antigen-capture ELISA was as low as 15 pg/ml, which
457 was much more sensitive than the WNV-NS1 ELISA reported by Saxena et al. and WNV-NS1
458 could be detected in the serum one day after infection. Ding's NS1 antigen-capture ELISA
459 displayed greater sensitivity than real-time RT-PCR from 1 to 7 days in WNV-infected mouse
460 serum samples, which might be attributed to the short duration of viremia and low viral RNA
461 titers after WNV infection [89].
462 In both Saxena and Ding's assays, the formation of NS1-immune complexes affected the
463 sensitivity of the capture ELISA under standard conditions by preventing the detection of free,
464 soluble NS1. Treating plasma with an alkaline solution and a non-ionic detergent partially
465 dissociated NS1 immune complexes and improved the sensitivity of the capture ELISA.

466 Disrupting immune complexes in plasma samples seemed to extend the time window for
467 measuring the antigen beyond the detection of viral RNA by quantitative RT-PCR [84,89,94].
468 The monoclonal antibodies developed by Ding et al. were also used to develop a portable
469 surface-enhanced Raman scattering (SERS)–lateral flow immunoassay (LFIA) detector for the
470 detection of recombinant NS1 that shows a visual detection limit of 10 ng/ml besides high
471 sensitivity and specificity for WNV when it was compared to Dengue, Yellow fever, and Zika
472 [90].
473
474 *4.4. NS1 in Usutu virus diagnosis*
475 USUV has only recently been identified as a pathogen of concern and hence diagnostic
476 solutions for this infection are still limited. No attempts have been made so far to create an
477 assay to measure the presence of NS1 in infected patients, although, in theory, all the
478 considerations made for WNV should also apply to USUV, as the two viruses belong to the
479 same serocomplex and are therefore antigenically very similar. This similarity could also have
480 repercussions on WNV diagnostic tests, leading to false positives that cause a USUV infection
481 to be misdiagnosed as a WNV infection. Until now, USUV was considered a pathogen of
482 marginal importance for human health and was not frequently included among the
483 flaviviruses to test for cross-reactivity with WNV.
484 Only the EuroImmun USUV IgG ELISA is available on the market, while for IgM detection, there
485 are no commercially available assays. The EuroImmun USUV IgG ELISA is based on the viral
486 structural E protein, and it suffers from broad antigenic cross-reactivity between anti-flavivirus
487 antibodies [27]. Detection of IgG antibodies using purified NS1 instead of E protein has been
488 reported to show a low degree of cross-reactivity between related viruses. Thus, IgM/G ELISA
489 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 [91].

4.5. NS1 in tick-borne encephalitis virus diagnosis

NS1 is mainly studied 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 [96].

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 [50,92]. 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 [32,92]. 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

514 be due to the low degree of similarity between the NS1 proteins of different flaviviruses
515 compared to the envelope (E) protein [92]. Interestingly, TBEV NS1 IgG ELISA showed cross-
516 reactivity with serum from patients vaccinated against YFV, but this was limited, probably
517 because TBEV and YFV NS1s share the greatest homology. As YFV vaccine is a live-attenuated
518 vaccine, NS1 can be produced in the same way that occurs during a natural infection, and
519 antibodies against NS1 can be detected both in vaccinated and infected patients [50,92].
520
521 *4.6. NS1 in Japanese encephalitis virus diagnosis*
522 There is an urgent need to develop a rapid diagnostic method for the detection of JEV infection
523 to detect possible outbreaks, not only in humans but also in swine, which are the principal
524 amplification hosts. Swine serum can be used to screen for the JEV NS1 protein for early
525 detection, surveillance, and prevention of human outbreaks, as the virus is known to
526 seroconvert in swine 2-4 weeks before human infection [93,97].
527 NS1 antigen capture ELISA can be used to diagnose JEV infection as early as on the first day of
528 illness, and NS1 can be detected even when viral RNA is not found by RT-PCR or in the presence
529 of IgM antibodies**.** In addition, the assay is quantitative, considerably stable, and less affected
530 by variations in physical conditions than the quantification of viral RNA [79].
531 Li et al. developed a JEV-specific and quantitative NS1 antigen-capture ELISA using a flavivirus-
532 specific and a JEV-specific mAb, respectively, and they analyzed NS1 secretion in JEV-infected
533 cell culture and NS1 in sera of JEV-infected mice and patients. The capture assay could detect
534 protein levels as low as 0.2 ng/ml. However, only 29.3% of human sera and 10.5% of CSF tested
535 positive for NS1 protein, likely due to late sample collection after the mosquito bite, as
536 indicated by the presence of anti-JEV IgM antibodies. Further research on early virus detection
537 during febrile illness may increase the chances of virus recovery and NS1 capture [98]. A recent

538 study using NS1-capture sandwich ELISA on sera and CSF during the acute phase of JEV
539 infection showed a 97% agreement with RT-PCR [79].
540 Recently, a colorimetric sandwich-based lateral flow assay (LFA) has been developed using
541 gold nanoparticles (AuNPs) labeled with the JEV NS1 specific polyclonal antibody for rapid,
542 sensitive, and specific NS1 detection in serum samples. This portable, cost-effective, and
543 disposable immunochromatographic strip has a visual detection limit of 10 pg/ml for JEV NS1
544 Ag in JEV negative serum and can detect JEV NS1 Ag in clinical swine serum samples within 10
545 minutes. It shows negligible non-specific binding with other flavivirus NS1 and may be
546 developed into a point of care (PoC) diagnostic kit for rapid mass screening, especially in rural
547 areas with limited laboratory resources. At the moment, this assay is limited to swine NS1
548 detection, but it could represent an interesting solution even for human diagnosis [93].
549 NS1 stimulates high levels of antibody production *in vivo*. When Zhou et al. tested for the JEV
550 NS1 antibody in five immunized swine, four had detectable NS1 antibodies seven days post-
551 immunization, while only one was found to be positive for the Envelope (E) protein antibody.
552 Based on this evidence, they developed a blocking ELISA using a high-affinity anti-JEV NS1
553 mAb for serological monitoring of JEV infection and evaluation of the immune status of swine
554 following JEV vaccination. 80% of newly JEV-vaccinated swine showed seroconversion within
555 7 days post-immunization, while the commercial envelope-protein-based indirect ELISA
556 detected seroconversion in only 20% of the newly vaccinated swine [87].
557

558 **5. Concluding remarks**

559 WNV, USUV, and TBEV co-circulation in Europe is rapidly becoming a matter of concern, and
560 the limited availability of precise surveillance and diagnostic systems might exacerbate the

561 problem in the future. This might especially hold true if other flaviviruses, such as JEV, are
562 introduced into Europe.
563 In this context, the need to introduce effective diagnostic methods that can clearly
564 discriminate between flaviviruses is relevant and urgent. With currently available diagnostic
565 methods either on the market or used in laboratories, it is difficult to diagnose flavivirus
566 infections at their onset, and above all, it is complex to unequivocally identify the responsible
567 pathogen due to high cross-reactivity between members of this family. Cross-reactivity
568 represents a major obstacle because, in addition to the uncertainty in diagnosis, it can also
569 lead to an incorrect definition of the epidemiology of these viruses in areas where co-
570 circulation is observed.
571 NS1 currently represents the most promising diagnostic marker that would allow an early
572 diagnosis, as it can be detected in the blood during the acute phase of flavivirus infection.
573 Furthermore, NS1 diagnosis has the potential to reduce the problem of cross-reactivity, as
574 antibodies against this protein seem more specific than those directed against the E protein
575 on which commercially available assays are based. NS1 antigen capture ELISAs have already
576 been commercialized for Dengue diagnosis, and several studies show that WNV can be
577 diagnosed in a highly specific manner using the same type of assay. Further investigations
578 need to be conducted on USUV and TBEV, for which currently little or no information is
579 available regarding the use of NS1 as a marker for early diagnosis. However, developing
580 monoclonal antibodies that are highly specific for the NS1 of a given flavivirus and do not
581 show cross-reactivity with members of the same serocomplex remains a major challenge.
582 Moreover, in addition to being used in rapid antigen tests (or capture ELISA) to detect the NS1
583 antigen, these specific antibodies can be used for the detection of flavivirus antibodies by
584 competitive ELISA, expanding the time frame in which a specific diagnosis can be made. The

585 development of such monoclonal antibodies and further research in this area could provide a
586 concrete solution to the problem of early, specific, rapid, and low-cost diagnosis of WNV,
587 USUV, and TBEV in Europe.

588

589 **Declaration of interest**

590 The authors declare no competing interests.

591

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822
823 **Figures**

824 **Figure 1**. Geographic distribution of most relevant flaviviruses and their antigenic
825 relationships. (a) The map shows the global distribution of the most prevalent neurotropic
826 flaviviruses. The data are lacking for the grey areas. (b) The map shows the global distribution
827 of the most prevalent hemorrhagic flaviviruses. The data are lacking for the grey areas. The
828 figures were generated using an online tool, URL: https://mapchart.net. (c) The phylogenetic
829 tree shows the antigenic relationships among flaviviruses. The flavivirus names are colored
830 based on their respective serocomplex (legend on the right), while the arced lines cover the
831 viruses that share the same vector. The full-length polyprotein amino acid sequences from
832 various flaviviruses were obtained from the NCBI database and pairwise aligned using Muscle.
833 Phylogenetic analysis was inferred using the Neighbor-Joining method [99]. The evolutionary
834 distances were computed using the p-distance method [100] and are in the units of the
835 number of amino acid differences per site. The analyses were conducted in MEGA11
836 [101,102];
837
838 **Figure 2.** Geographic distribution of flaviviruses in Europe. The map shows the countries in
839 which WNV, USUV, and TBEV have been detected and their pattern of co-circulation. The
840 figure was generated using an online tool, URL: https://mapchart.net.
841

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

851

852 **Figure 4.** Structures of NS1 in its different oligomerization states. (a) structure of dimeric NS1
853 (PDB: 4O6B). One monomer is represented in grey, while in the other one, the β-roll domain
854 (residues 1–29) is colored in orange, the wing domain (residues 30–180) is colored in blue,
855 and the β-platform domain (residues 181–352) is colored in magenta. (b) Surface
856 representation of NS1 stable tetramer (PDB7: WUT). (c) Surface representation of NS1 loose
857 tetramer (PDB: 7WUU). (d) Surface representation of NS1 loose hexamer (PDB: 7WUV). In the
858 hexameric structure, the density of the central core is weak. Therefore, it is not possible to
859 distinguish the β-roll conformation [66]. In NS1 oligomeric structures, the domains of the
860 protein are colored following the same color code used for the dimer.