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An evaluation of serological methods to diagnose tick-borne encephalitis from serum and cerebrospinal fluid

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ABSTRACT

Background: Tick-borne encephalitis (TBE) is an infectious disease endemic to large parts of Europe and Asia. Diagnosing TBE often relies on the detection of TBEV-specific antibodies in serum and cerebrospinal fluid (CSF) as viral genome is mostly not detectable once neurological symptoms occur.
Objectives: We evaluated the performance of TBEV IgM and IgG ELISAs in both serum and CSF of confirmed TBEV patients and discuss the role of (CSF) serology in TBEV diagnostics.
Study design: For the assay evaluation we collected specimen from confirmed TBEV patients. Assay specificity was assessed using sera from patients with a related flavivirus infection or other acute infection. A selected ELISA assay was used to analyze TBEV-specific antibodies in CSF and to evaluate the use in confirming TBE diagnosis.
Results: In this study the overall sensitivity of the IgM TBEV ELISAs was acceptable (94 -100 %). Four out of five IgM ELISA's demonstrated an excellent overall specificity from 94 -100% whereas a low overall specificity was observed for the IgG TBEV ELISAs (30-71%). Intrathecal antibody production against TBEV was demonstrated in a subset of TBE patients.
Conclusions: In four out of five ELISAs, IgM testing in serum and CSF of TBE patients is specific and confirmative. The lack of IgG specificity in all ELISAs emphasizes the need of confirmatory testing by virus neutralisation, depending on the patient's background and the geographic location of exposure to TBEV. A CSF-serum IgG antibody index can support the diagnosis specifically in chronic disease or once IgM has disappeared.

1. Background

Tick-borne encephalitis (TBE) is a zoonotic, infectious disease endemic to large parts of Europe and Asia [1,2]. It is caused by the tick-borne encephalitis virus (TBEV, family *flaviviridae*, genus *flavivirus*). Three subtypes can be distinguished, namely the European (TBEV-Eur), Siberian (TBEV-Sib) and Far Eastern (TBEV-FE) subtypes with a close antigenic relationship and inducing cross-protection. [3,4] TBEV is transmitted to humans through tick bites from *Ixodes spp*, but sporadic transmission by consumption of unpasteurised dairy products from infected livestock has also been reported. [5] In Europe, the prevalence of TBEV is increasing [2,6] and recently the first autochthonous cases were reported in the Netherlands [7,8].

The majority (75%) of TBEV infections are subclinical or asymptomatic. [9] Symptoms caused by TBEV vary from mild to severe, depending on a person's age and subtype, with severity increasing with age [10,11]. Typically, an infection with TBEV-Eur results in a biphasic course of illness, starting with a viraemic phase with non-specific, influenza-like symptoms, including fever, followed by an asymptomatic interval lasting several days [9–11]. In the second phase, which occurs in 72–87% of the symptomatic cases [9], patients may develop neurological symptoms, ranging from mild meningitis to severe encephalitis, with or without nerve paralysis and myelitis [10–12]. Neurological symptoms usually clear up completely, although headaches and

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cognitive impairment can last up to 6–12 months [9]. Exact data on case fatality rates of the eastern subtypes are lacking, but case fatality is thought to be much higher in TBEV-FE infections (20–40% [1,13]) than in TBEV-Sib (2–3% [1]) and TBEV-Eur (0.8–1.4% [10,11]). Infections of all subtypes can be prevented by vaccination, but vaccination breakthrough has been described [14–16].

Upon infection with TBEV, viral RNA is usually detected in blood or serum only during the first phase of infection, in which the patient is asymptomatic or symptoms are non-specific. Once neurological symptoms are manifest, TBEV RNA is rarely detected in blood and cerebrospinal fluid (CSF) [17], although persistent viraemia in an immunocompromised patient has been reported [18]. Usually, diagnosing TBEV infection relies on the detection of TBEV-specific antibodies in serum and CSF. TBEV-specific IgM antibodies are typically detected in serum when neurological symptoms are present. In CSF, the IgM response peaks later than in serum after the onset of neurological symptoms; usually it is detectable from the second week [19]. Specifically in TBEV-Sib infections, the neurological symptoms may develop more rapidly and be present before IgM seroconversion occurs [20].

The current laboratory-case definitions state that brain-derived IgM detection in CSF confirms the diagnosis of TBEV encephalitis. [21,22] A CSF-serum antibody index can be used to differentiate between blood-derived and brain-derived specific antibody fraction [23]. After TBEV infection, specific, neutralising IgG have lifelong persistence in protecting against reinfection, whereas IgM is typically detected up to 3 months, with persistence occasionally lasting up to 9 months [24,25]. Unfortunately, the interpretation of TBEV serology is severely hampered by extensive cross-reactivity among flaviviruses and the phenomenon of original antigenic sin, where an acute flavivirus infection might boost cross-reactive antibodies due to previous infection with, or vaccination against, another flavivirus [26,27]. Therefore, the choice of serological test in a diagnostic setting needs careful consideration and an evaluation of the available tests using sample cohorts relevant for the local setting.

2. Objectives

We have evaluated the analytical and diagnostic performance of five commercially available TBEV IgM and IgG ELISAs, using a panel of virus-neutralisation and/or RT-PCR-confirmed TBEV patients. TBEV-specific antibodies in CSF were determined by an ELISA with a specific CSF protocol, applicable within routine diagnostics. The results were evaluated and the use of CSF serology in confirming TBE diagnosis is discussed.

3. Study design

3.1. Evaluation cohorts

For the assay evaluation we collected 18 serum and plasma samples from TBEV-infected patients. TBEV infection was confirmed by the presence of TBEV neutralising antibodies, and in three patients TBEV genomic RNA was detected by a TBEV-specific RT-PCR (Table 1). Between 2010 and 2017 material was collected from three reference laboratories: 1) Erasmus MC Rotterdam, the Netherlands; 2) the Faculty of Medicine in Ljubljana, Slovenia; and 3) the Helsinki University Hospital, Helsinki, Finland. Ethical approval to anonymously analyse the used samples of all patients was obtained from the Erasmus MC Medical Ethical Committee (MEC-2015-306).

Assay specificity was assessed using a total of 42 sera from 42 patients with a related flavivirus infection, *i.e.* dengue virus (DENV), Japanese encephalitis virus (JEV) and zika virus (ZIKV), or vaccinated against a related flavivirus, *i.e.* JEV and yellow fever virus (YFV). In addition, assay specificity was assessed using 17 sera or plasma samples from patients with an acute human cytomegalovirus infection or Epstein-Barr virus infection and 10 sera from patients with a confirmed chikungunya virus infection. Given the lack of detailed clinical information on the control groups, *e.g.* flavivirus vaccination history and previous flavivirus exposure, all control-group samples were tested in the TBEV VNT so that the presence of antibodies reactive against TBEV could be ruled out. To evaluate the role of CSF serology, we collected eight paired/serum CSF samples from clinical TBE patients, which were laboratory confirmed by the TBEV virus neutralisation test (VNT).

Table 1
Evaluation cohorts.

Sensitivity panel		
TBEV confirmed samples	*Confirmation level*	
	VNT positive	15
	VNT and PCR positive	3
	Total	18
Background information	*Gender*[a]	*#patients(%)*
	Male	8(73%)
	Female	3(27%)
	Reported symptoms	*#patients(%)*
	Fever	6(33%)
	Headache	9(50%)
	Meningitis and/or encephalitis	7(39%)
	Reported tick bite	6(33%)

Specificity panel			
Flavivirus	*Confirmation level*	*#*	*VNT TBEV*
DENV patients	DENV IgM + IgG +	8	neg
	DENV NS1 + IgM +	9	neg
JEV patients	JEV IgG + JEV VNT +	2	neg
ZIKV patients	ZIKV IgM + IgG + ZIKVVNT +	9	neg
	ZIKV IgM + IgG- ZIKV VNT +	1	neg
YFV post vaccination	YFV IgG +	10	neg
JEV post vaccination	JEV IgG + JEV VNT +	2	neg
Acute infection with non-flavivirus			
CMV	CMV IgM + IgG + CMV avidity low	8	neg
EBV	EBV NA IgG- EBV VCA IgM + PCR EBV +	9	neg
CHIKV	CHIKV IgM + IgG +, CHIKV VNT +	10	neg

CMV: cytomegalovirus, CHIKV: Chikungunya virus, DENV: dengue virus, EBV: Epstein Barr virus, JEV: Japanese encephalitis virus, TBEV: tick-borne encephalitis virus, VNT: virus neutralisation test, YFV: yellow fever virus, ZIKV: Zika virus, neg: negative, #:number

[a] All other patients unknown.

3.2. Immunoassays for TBEV antibody detection

Based on the presence of European Union CE certification marking, TBEV ELISAs of five different manufacturers were selected. These were: 1. Serion ELISA TBE Virus (Virion\Serion, Würzburg, Germany; TBEV strain Moscow B-4); 2. Immunozym FSME (Progen, Heidelberg, Germany; TBEV strain Neudörfl); 3. Reagena TBE (Reagena, Toivala, Finland; TBEV-Eu strain Kumlinge A52); 4. Euroimmun Anti-TBE Virus ELISA (Euroimmun, Lübeck, Germany; TBEV strain K23); and 5. Enzygnost Anti-TBE (Siemens, Marburg, Germany; strain K23). All ELISAs indirectly detect TBEV-directed antibodies using inactivated TBEV virus antigen coated wells, except for Reagena IgM and IgG, which are capture ELISAs that use recombinant TBEV antigen. The ELISAs were carried out and the results were interpreted according to the manufacturers' instructions.

3.3. Virus neutralisation test

Virus neutralisation is the gold standard in discriminating flavivirus serology. The presence of TBEV-specific neutralising antibodies was determined using an in-house micro-neutralisation assay. Twofold serum dilution series were incubated with 100 $TCID_{50}$ of TBEV strain (Neudörfl H2J (Isolate Arb 131)) at 36.5 °C, and transferred to Vero

cells (Vero ATCC CCL-81) for 1 h. Then the Vero cells were refreshed with Dulbecco MEM Eagle Medium (DMEM) supplemented with 1% Penicillin/streptomycin (LO DE17-602E), 1% L-Glutamine (LO BE17-605E), 1 M HEPES (BE17-737E), 7,5% NaHCO3 (BE17-613E), 3% Foetal Bovine Serum (FBS, F7524) and incubated at 36.5 °C and 5% CO_2. TBEV infection was determined by cytopathic effect at 7 days post infection. A VNT TBEV reciprocal titre of ≥1/32 was considered as positive.

3.4. Analysis

Sensitivity values for IgM and IgG were calculated for each of the five test systems by using the TBEV VNT as a gold standard for IgG, while an alternative strategy was used for IgM. In the absence of a gold standard, an IgM result (positive, negative or equivocal) obtained by ≥ three of the five test systems was interpreted as the correct result. Equivocal results were consistently interpreted as a positive result in all calculations for IgM and IgG sensitivity and specificity.

Specificity values were calculated by analysing the amount of reactivity in the TBEV ELISAs, of sera from patients with confirmed other viral infections, not responsive in TBEV VNT.

3.5. TBEV RNA detection

TBEV RNA detection in CSF was performed using an in-house real-time reverse transcription-polymerase chain reaction. [28]

3.6. Analysis of CSF

The first step when calculating a CSF-serum Antibody Index (AI) is the quantification of the blood-brain barrier (BBB) functioning, which is done by calculating the age-dependent albumin CSF-serum quotient. Next, a CSF-serum immunoglobulin quotient has to be calculated to correct for high levels of intrathecal IgG. The AI is the ratio between the CSF-serum quotient of the specific TBEV IgG antibodies and the total IgG antibodies, following Reiber. [23,29] Theoretically, the normal AI value must be AI = 1.0, whereas clinically relevant, pathological AI values are > 1.5. [23].

Albumin and total IgG in both serum and CSF were measured by nephelometry at the laboratory of clinical chemistry at Erasmus MC. Quantitative detection, of both anti-TBEV IgM and IgG in paired serum and CSF, was carried out with the Euroimmun TBEV ELISA (Lübeck, Germany), which is the testing benchmark in our laboratory, using the included CSF protocol. Measured immunoglobulin titres were quantified by transforming the optical density (at 450 nm) into mg/L, using four different CSF calibrators, in accordance with the manufacturer's instructions.

4. Results

4.1. Sensitivity comparison

The sensitivity of the five commercial ELISAs was assessed using 18 serum samples of confirmed European TBEV patients (Table 1, Fig. 1, Suppl. Table 1). Serion, Progen and Siemens ELISAs had a sensitivity of 94% for IgG, while Reagena and Euroimmun ELISAs had a sensitivity of 83%. For IgM detection, the ELISAs demonstrated a comparable sensitivity (94–100%).

4.2. Specificity comparison

To address the amount of cross-reactivity, we assessed the specificity of the five commercial assays by using a panel of 42 sera from patients infected or vaccinated with related flaviviruses and a panel of 27 sera from patients with non-related viral infections (Tables 1 and Suppl. Table 1). All commercial TBEV IgG tests scored low on specificity when tested with a related flavivirus panel, with specificities ranging from 12 to 67%. Of the TBEV IgM assays, only the Reagena ELISA demonstrated 100% specificity in the flavivirus panel. For the panel of sera from non-flavivirus infections, all ELISAs demonstrated 100% specificity for IgM, while the specificity for IgG ranged between 59 and 89%.

4.3. Serum-CSF serology

To assess the added value of comparative serum-CSF serology in confirming the diagnosis of TBE in clinically suspect patients, we retrospectively analysed eight serum-CSF pairs obtained from patients infected in different areas in Europe and presenting with neurological signs of TBE. All patients had an intact BBB and no evidence of polyclonal intrathecal antibody production. The serological diagnosis was confirmed by performing a TBEV VNT in all patients (Table 2). Paired serum-CSF samples were tested by using the CSF protocol of the Euroimmun assay, which is routinely performed in our laboratory and had acceptable performance characteristics. All patients had detectable levels of IgM in both serum and CSF, which is considered to be confirmative for the diagnosis of TBE. [21,22] IgG levels were detected in both serum and CSF in seven of the eight patients. The IgG TBEV AI was calculated in these seven patients and in five of them it was found to be above the manufacturer's cut-off of 1.5, which is indicative of the presence of intrathecal IgG immunoglobulins directed against TBEV. In two patients the index was < 1, which is interpreted as non-pathological.

5. Discussion

TBE has been a serious and notifiable disease in the EU since 2012. Between 2012 and 2016 some 12,500 TBE cases were reported to the ECDC, by 23 EU countries. While the overall TBE notification rate has remained stable, increased geographic prevalence has been observed. [30] Accurate diagnostics are the cornerstone of an adequate clinical and public-health response, including the implementation of surveillance programmes [31]. The routine detection of TBEV-specific antibodies in serum and CSF is a key tool for diagnosing TBEV infection [22]. However, flavivirus serology is known to be complex and the market supply of commercial tests is rather diverse. A serological assay that can thoroughly analyse both clinical sample types is thus a key requirement for obtaining reliable test results.

Although a few studies have evaluated commercially available TBEV ELISAs [32–35], the putative cross-reactive panels in these studies were limited. This is an important study limitation because TBEV testing in diagnostic laboratories is often part of a much broader differential diagnostic panel of viruses, while (previous) exposure to related flaviviruses might interfere with test interpretation [36]. For example, the increased seroprevalence of antibodies against ZIKV in the population makes the inclusion of ZIKV patients in flavivirus test evaluations part of accurate test evaluation [36], while acute EBV and CMV patients are notoriously cross-reactive in a broad range of serological tests [36–38]. Indeed, the importance of such evaluations was underlined by the extremely low specificity we observed for the IgG TBEV ELISAs. All IgG assays had specificity problems (observed range 12–67%) with regard to the flavivirus panel, while Progen and Reagena ELISAs had specificity below 80% within the non-flavivirus panel as well. These observations were in line with the results of a 2013 external quality assessment (EQA) [33], for which results were submitted for 15 commercially available TBEV IgG ELISAs, including Serion, Progen, Euroimmun and Siemens. Our observations once again stress the complexity of interpreting flavivirus serology and the necessity to run gold standard neutralisation tests, in case infection is presumed on the basis of IgG responses. This should be emphasised in patients with presumed exposure to multiple flaviviruses (in the past) or if positive predictive value is low in the area of exposure.

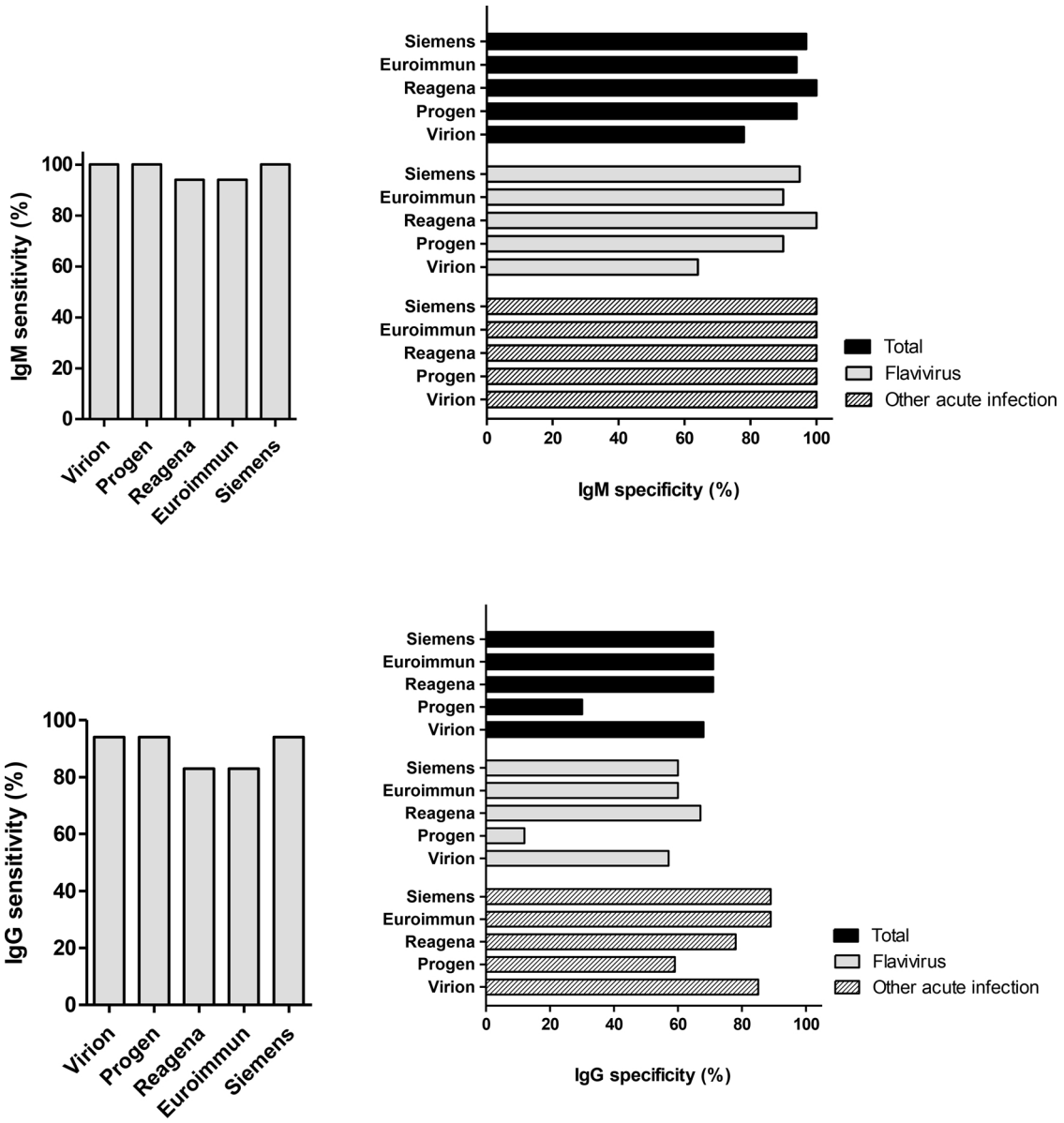

Fig. 1. Sensitivities and specificities of five ELISAs for the detection of TBEV-specific IgM and IgG antibodies.

In the EU clinical case definition of TBE, "any person with symptoms of inflammation of the CNS" should be considered as a clinical case. [22] Given that the clinical manifestations of TBE can be atypical, varying from meningitis to meningoencephalomyelitis [39], TBEV is not always in the differential diagnosis of the treating physician, or diagnostics are often performed with a delay, particularly in non-endemic regions. The official European laboratory definition of TBE [22] considers TBE to be confirmed when TBEV-specific IgM is detected in CSF. The presence of IgM in CSF is generally considered as locally synthesised but ideally, the proper functioning of the BBB should be assessed in parallel, because the BBB can be impaired by infectious, inflammatory or metabolic disorders [23].

We aimed to assess the value of a commercially available ELISA in detecting antibody responses in CSF of eight patients with a clinical presentation of TBE, in whom paired serum and CSF sampling was done within several days of the onset of the symptoms. In these patients the diagnosis TBE could be confirmed, based on the detection of TBE-specific IgM production in CSF. As IgM was also detected in the serum of all patients, and taking into account the good performance characteristics of the used assay, TBEV IgM testing can be regarded as a reliable diagnostic tool in patients with a neurological disease attributable to TBEV. Next, we calculated the TBEV IgG AI in seven of the patients with detectable IgG, and demonstrated that the AI was indicative for local IgG production in five out of seven cases. It should be emphasised, that the non-pathological result in two patients does not rule out TBEV as a cause for the neurological disease, because: 1) IgM was already detected; and 2) the antibody kinetics in CSF are known to be slower than in serum. [20] Seen in a broader context, calculating an IgG AI will be of added value, particularly in situations in which IgM might not, or no longer, be present. IgM responses can for example be altered when infections occur with a background of another flavivirus infection. Alternatively, TBEV might be part of the diagnostic panel performed late in the course of the disease, such as in patients with chronic neurological disease of unknown aetiology. In these patients, autoimmune diseases are often part of the work-up, and adequate assessments of both the functioning of the BBB and the total intrathecal IgG responses are crucial [23].

Our study has some limitations. As mentioned earlier, there is no accepted gold standard test for ascertaining the presence of specific TBEV IgM antibodies. This makes it a challenge to correctly interpret

Table 2
TBEV-specific antibody detection in CSF.

No	material	Country of infection	TBEV VNT	TBEV IgG	TBEV IgM	AI TBEV IgG	PCR
1	CSF	Sweden		neg	**pos**	n.d.	n.d.
	serum		**200**	neg	**pos**		
2	CSF	Sweden		**pos**	**pos**	0,79	neg
	Serum		**406**	**pos**	**pos**		
3	CSF	Sweden		**pos**	**pos**	0,96	neg
	Serum		**40**	**pos**	**pos**		
4	CSF	Germany		**pos**	**pos**	2,25	neg
	serum		**40**	**pos**	**pos**		
5	CSF	Austria		**pos**	**pos**	2,4	neg
	Serum		**101**	**pos**	**pos**		
6	CSF	Netherlands		**pos**	**pos**	3,69	n.d.
	serum		**32**	**pos**	**pos**		
7	CSF	Lithuania		**pos**	**pos**	7	n.d.
	serum		**256**	**pos**	**pos**		
8	CSF	Netherlands		**pos**	**pos**	9	n.d.
	serum		**161**	**pos**	**pos**		

AI: Antibody Index, CSF: cerebrospinal fluid, n.d.: not determined, PCR : polymerase chain reaction, TBEV : tick-borne encephalitis virus, TBEV IgG and IgM: measured by ELISA D, VNT: virus neutralisation test.

the performance of the IgM ELISAs, because it cannot be definitively established that TBEV IgM should be present in certain samples. For this reason, we have based the IgM interpretation on the agreement of the assays, which is only a proxy for the correct interpretation of the results. Furthermore, our study does not include the two other flaviviruses that are endemic in parts of Europe, *i.e.* the Usutu virus and West Nile virus. Nor did it include samples from TBE patients for which TBE was a secondary flavivirus infection. This was due to the lack of availability of these sera to the study consortium at the time of the study. Finally, background information on the surveyed patients was limited, whereas it should always be taken into account in clinical decision-making.

In summary, our study shows a crucial role of IgM testing in serum and CSF to confirm TBEV infection. We demonstrate the importance of a thorough evaluation of commercially available ELISAs, prior to implementation. Although the compared ELISAs are acceptably sensitive, the lack of IgG specificity underscores the need for confirmatory testing by virus neutralisation, particularly if (previous) exposure to multiple flaviviruses cannot be ruled out. In case of chronic disease, or once IgM has disappeared, an IgG AI can support the confirmed diagnosis.

Ethical approval

Ethical approval was obtained from the Erasmus MC Medical Ethical Committee (MEC-2015-306) to anonymously analyse the used samples of all the surveyed patients.

Author contributions

CR, MB and CGvK designed the study, analysed the data and drafted the manuscript. SR, SS and FC performed the serology and virus neutralisation experiments and acquired the corresponding data.

TA and OV provided the reference specimens. TA, OV and MK critically revised the manuscript. All authors have approved the final version of the manuscript.

Declaration of Competing Interest

None.

Acknowledgement

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.09.009.

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