Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Comparison of three commercial IgG and IgM ELISA kits for the detection of tick-borne encephalitis virus antibodies

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ARTICLE INFO

Keywords:
Tick-borne encephalitis virus
ELISA
Neutralization test

ABSTRACT

Tick-borne encephalitis (TBE) is endemic in many parts of Europe and Asia. The diagnosis of this disease is essentially based on the demonstration of specific antibodies. For reasons of simplicity, automatization and quick availability of test results, enzyme-linked immunosorbent assays (ELISAs) are the method of choice for serological diagnosis of TBE. Here, we evaluated three commercially available anti-TBEV IgG and IgM ELISAs using 251 serum samples: the SERION ELISA *classic* FSME Virus/TBE Virus IgG and IgM kit (Virion\Serion), the RIDASCREEN® FSME/TBE IgG and IgM kit (R-Biopharm), and the anti-FSME/TBE virus ELISA "Vienna" IgG/anti-FSME/TBE virus ELISA IgM kit (Euroimmun). In total, discrepant test results for IgG and/or IgM were observed for 37/251 (14.7 %) of tested samples; differences were statistically significant. Reference values defined by serum neutralization test (SNT, n = 25) or results provided by EQA organizers (n = 2) were established for a subset of samples. In relation to these values, false-positive results were observed mainly for Euroimmun Vienna IgG and RIDASCREEN IgG, whereas false-negative results were primarily observed for Virion\Serion IgG and RIDASCREEN IgM kits. In routine diagnostics, specificity problems are of major relevance and may be addressed by analyzing the respective samples using SNT.

1. Introduction

Tick-borne encephalitis virus (TBEV) is the most important tick-borne arbovirus infecting humans in Europe and Asia. The TBEV species belongs to the mammalian tick-borne flavivirus group in the genus *Flavivirus*, family *Flaviviridae*. Based on antigenetic properties, it is subdivided into a Far Eastern, a European and a Siberian subtype. These subtypes correspond to the major TBEV genotypes 1, 2, and 3, respectively (Demina et al., 2010; Lindquist, 2014). TBEV is typically transmitted through bites of infected ticks, wherefore its distribution correlates with the presence of ixodid vectors. In Central Europe, TBEV is principally transmitted by *Ixodes ricinus* (Lindquist, 2014), although its presence in other tick species as well as the transmission via infected milk products has also been documented (Balogh et al., 2010; Holzmann et al., 2009; Mierzejewska et al., 2015).

Infections with TBEV are asymptomatic in 70–95% of cases. Symptomatic disease is typically biphasic when caused by European subtype viruses, including a viremic stage with flu-like symptoms starting about 8 days (4–28 days) after the tick bite, an asymptomatic interval of about one week (range 1–33 days), and a second stage with neurological manifestations ranging from mild meningitis to severe encephalitis with or without myelitis and spinal paralysis (Lindquist, 2014; Lindquist and Vapalahti, 2008). In contrast, Far Eastern and Siberian viruses most often induce monophasic diseases. Chronical forms may be observed in association with the Siberian subtype (Gritsun et al., 2003). Case fatality rates range between 0 and 1.4 % and increase with age for European subtype viruses. For infections with the Siberian and Far Eastern subtypes, mortality ranges between 2 and 3 % and about 35 %, respectively. This latter high rate, however, might be due to the lack of detection and reporting of mild cases (Charrel et al., 2004; Gritsun et al., 2003; Kaiser, 1999; Lindquist, 2014).

In biphasic tick-borne encephalitis (TBE), the virus can be detected by polymerase chain reaction (PCR) in blood during the viremic phase of illness. However, patients typically seek medical care only during the second phase of illness, when neurological symptoms occur. During this phase, direct detection of the virus is rarely successful, wherefore

Abbreviations: CMV, cytomegalovirus; CSF, cerebrospinal fluid; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assays; EQA, external quality assessments; Ig (G/M), immunoglobulin type G/M; PCR, polymerase chain reaction; SNT, serum neutralization test; TBE, tick-borne encephalitis; TBEV, tick-borne encephalitis virus

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https://doi.org/10.1016/j.ttbdis.2018.03.031
Received 22 November 2017; Received in revised form 21 March 2018; Accepted 27 March 2018

Table 1
Summary of the tested commercial anti-TBEV IgG and IgM ELISA kits[a].

Name	TBEV strain	Sample dilution	Processing	OD measurement	Standards/controls	Unit	Cut-offs	Test evaluation	Inter-assay variation (vc)	Intra-assay variation (vc)
IgG										
SERION ELISA classic FSME/TBE Virus IgG (Virion \Serion)	MoscowB4	1:101	sample incubation 60 min. 37 °C; wash 4x; conjugate incubation 30 min. 37 °C; wash 4x; substrate incubation 30 min. 37 °C; stop	405 nm/650 nm	2 standard sera: standard control, negative control	U/ml	negative < 100 U/ml; equivocal 100–150 U/ml; positive > 150 U/ml	quantitative, 4-parameter logistic equation function, lot-specific parameters A-D provided by the manufacturer	8.8–13.4 %	4.3–8.3%
RIDASCREEN® FSME/TBE IgG (R-Biopharm)	Neudörfl	1:100	sample incubation 30 min. 37 °C; wash 4x; conjugate incubation 30 min. 37 °C; wash 4x; substrate incubation 30 min. 37 °C; stop	450 nm/620 nm	2 standard sera: standard control, negative control	U/ml	negative < 100 U/ml; equivocal 100–126 U/ml; positive > 126 U/ml	quantitative, 4-parameter logistic equation function, lot-specific parameters A-D provided by the manufacturer	11.5–22.7 %	6.9–9.1%
Anti-FSME/TBE Virus ELISA Vienna (Euroimmun)	K 23	1:101	sample incubation 60 min. RT; wash 3x; conjugate incubation 60 min. RT; wash 3x; substrate incubation: 30 min. RT; stop	450 nm/620 nm	4 calibration sera: 1′000 VIEU/ml, 300 VIEU/ml, 150 VIEU/ml (cut-off), 15 VIEU/ml	VIEU/ml	negative < 120 VIEU/ml; equivocal 120 − < 165 U/ml; positive ≥165 U/ml	quantitative, 4-parameter logistic equation function, standard curve based on measured values of calibration sera calculated by user	3.9–10.5%	2.7–7.9%
IgM										
SERION ELISA classic FSME/TBE Virus IgM (Virion \Serion)	MoscowB4	1:101	IgG/RF absorption 15 min. RT; sample incubation: 60 min. 37 °C; wash 4x; conjugate incubation 30 min. 37 °C; wash 4x; substrate incubation 30 min. 37 °C; stop	405 nm/650 nm	2 standard sera: standard control, negative control	U/ml	negative < 10 U/ml; equivocal 10–15 U/ml; positive > 15 U/ml	quantitative, 4-parameter logistic equation function, lot-specific parameters A-D provided by the manufacturer	3.7–7.0 %	3.1–7.9%
RIDASCREEN® FSME/TBE IgM (R-Biopharm)	Neudörfl	1:100	IgG/RF absorption: 15 min. RT; sample incubation: 30 min. 37 °C; wash 4x; conjugate incubation 30 min. 37 °C; wash 4x; substrate incubation: 30 min. 37 °C; stop	450 nm/620 nm	2 standard sera: standard control, negative control	U/ml	negative < 100 U/ml; equivocal 100–126 U/ml; positive > 126 U/ml	quantitative, 4-parameter logistic equation function, lot-specific parameters A-D provided by the manufacturer	18.5–24.2 %	11.4–14.7 %
Anti-FSME/TBE Virus ELISA IgM (Euroimmun)	K 23	1:101	IgG/RF absorption: 10 min. RT; sample incubation: 30 min. RT; wash 3x; conjugate incubation 30 min. RT; wash 3x; substrate incubation: 15 min. RT; stop	450 nm/620 nm	2 standard sera: positive control, negative control	Ratio	negative ratio < 0.8; equivocal ratio ≥0.8 − < 1.1; positive ratio ≥1.1	semi-quantitative, extinction sample/extinction calibrator = ratio	3.9–5.7 %	3.8–5.5 %

[a] ELISA, Enzyme-linked immunosorbent assay; RF, rheuma factor; RT, room temperature; TBE, tick-borne encephalitis; TBEV, tick-borne encephalitis virus; U, Units; vc, variation coefficiant; VIEU, Vienna Units. All information is given as specified by the manufacturers.

molecular biological methods are of minor importance for laboratory diagnostics. The method of choice for the diagnosis of TBE is the detection of specific antibodies. Typically, IgM and IgG antibodies are present in serum samples at the beginning of the second phase and rise to maximum titers within 2–6 weeks. In cases where only IgM antibodies are detected, a follow-up sample is needed to demonstrate an IgG seroconversion and therewith establish a diagnosis. IgM typically remains detectable for 6–8 weeks but may persist for up to 10 months, whereas IgG antibodies persist for a whole life. In cerebrospinal fluid (CSF), intrathecal IgM and IgG antibodies may be detected 10 days after the onset of neurological symptoms (Bogovic and Strle, 2015; Charrel et al., 2004; Holzmann, 2003).

Interpretation of serologic test results is hampered by the high cross-reactivity of the antigenic structure among flaviviruses, especially in areas where other flaviviruses co-circulate, or where vaccination against other flaviviruses are regularly used (Lindquist, 2014). Among the different serological assays available, the serum neutralization test (SNT) is the most specific method, which is however only performed in specialized laboratories. Most laboratories use enzyme-linked immunosorbent assays (ELISAs), some may also use immunofluorescence assays or haemagglutination inhibition assays (Litzba et al., 2014). These latter three types of tests are known to produce false-positive results with antibodies directed against other flaviviruses. Nevertheless, if contact to or vaccination against other flaviviruses can be ruled out, ELISAs remain the method of choice due to their simplicity and quick availability of test results.

In this study, we compared the results of both IgG and IgM detection for three commercially available ELISAs using a total of 251 clinical samples. In addition, serum neutralization testing was done with a subset of 35 samples of special interest.

2. Materials and methods

2.1. Study population

the study included anonymized serum samples sent to a routine diagnostic laboratory for serological testing (accredited according to ISO/IEC 17025:2005 and 15189:2007) as well as samples originating from external quality assessments (EQA). Samples had been collected during the years 2005–2015 and stored at −35 °C. They were thawed/frozen for a maximum of 5 cycles before the study. During the testing period, sera were thawed and temporarily stored at 4 °C.

2.2. Sample selection

Based on the routine testing results, a total of 251 samples (241 clinical samples, 10 samples from EQA) were selected, which are divided into 5 categories: a) samples positive for anti-TBEV IgG (n = 53), b) samples positive for anti-TBEV IgM (n = 16), c) samples positive for anti-TBEV IgG and IgM (n = 33), d) samples negative for anti-TBEV IgG and IgM (n = 96), and e) samples positive for IgM against other pathogens (*Borrelia burgdorferi* (n = 15), rubella virus (n = 3), human cytomegalovirus [CMV] (n = 12), Epstein-Barr virus [EBV] (n = 9), and *Toxoplasma gondii* (n = 14). Information on the clinical status of the respective patients was not available. The tests used for routine testing during the sample collection period were the Progen IMMUNOZYM® FSME (TBE) IgG/IgM (January 2005 to September 2009) or the SERION ELISA classic FSME Virus/TBE Virus IgG/IgM (October 2009 to December 2015) for TBEV, the IgM Capture Borrelia (Dako, Oxoid) for *B. burgdorferi*, Rubella IgM Vidas (BioMérieux) for rubella virus, CMV IgM Vidas (BioMérieux) for CMV, EBV VCA IgM Vidas (BioMérieux) for EBV, and Toxo IgM Vidas (BioMérieux) for *T. gondii*, respectively.

2.3. ELISA-testing using commercial anti-TBEV IgG and IgM ELISA-Kits

All samples were subjected to testing with anti-TBEV IgG and IgM ELISA kits from three different companies: the SERION ELISA *classic* FSME Virus/TBE Virus IgG and IgM kit (Virion\Serion GmbH, Würzburg, Germany), the RIDASCREEN® FSME/TBE IgG and IgM kit (R-Biopharm AG, Darmstadt, Germany), and the anti-FSME/TBE virus ELISA "Vienna" IgG/anti-FSME/TBE virus ELISA IgM kit (Euroimmun, Lübeck, Germany). Analyses were performed manually and according to the manufacturer's instructions (Table 1). For OD measurements and test evaluation, the DYNEX DSX® automated ELISA processing system was used. Results of type , > 1000 U/ml' in the Euroimmun "Vienna" IgG kit were replaced by an arbitrary constant value larger than 1000 as no larger values had actually been measured. Since all statistical analyses of the quantitative results are based on the ranks of the observed values only, the arbitrary value selected will not have any influence on these analyses. In one sample, however, the standardized value for IgM according to Virion\Serion was " > 120 U/ml", which was treated as missing since other values greater than 120 were actually obtained for other samples. This sample was then omitted from the analyses involving this variable.

2.4. Serum neutralization test (SNT)

Selected samples, including 25 samples with discordant and five samples with concordant results for the different ELISAs were subjected to SNT. Sera were heat-inactivated for 30 min. at 56 °C, then serially diluted in Leibovitz L-15 medium in microtiter plates in 2-fold steps, starting at a dilution of 1:8. Virus (100 TCID50/ml, TBEV strain Hypr) was added and plates were incubated over night at 4 °C and subsequently for 1 h at 37 °C without CO_2. Porcine kidney stable cells were then seeded to the plates (15,000 cells/well), and plates were further incubated at 37 °C without CO_2. On day 4, neutral red dye in Dulbecco's phosphate-buffered saline (Sigma Aldrich) was added to each well at a final concentration of 0.000165 %. On day 5 the liquid was removed and the presence or absence of neutral-red-stained cells was used to assess the virus-induced cytopathic effect. The neutralization titer was defined as the reciprocal dilution resulting in 50 % virus neutralization Each serum was tested at least in duplicate and geometric mean titers (total Ig) were calculated.

2.5. Statistical analyses

The stats, coin, and vcd packages of the R software were used for all statistical analyses. To assess whether there was an overall difference (n = 251) between qualitative (negative, equivocal, positive) results of the different IgG or IgM kits, the asymptotic generalized Cochran-Mantel-Haenszel (CMH) test, stratified for samples, was used; a p-value < 0.05 was regarded as significant. Qualitative test results were then pairwise compared in contingency tables, and the degree of agreement between kits was measured by weighted Kappa (κ), including approximate 95 % confidence intervals. In addition, an asymptotic linear-by-linear association test was applied to detect whether the kit test results significantly differ from each other, and a p-value < 0.05 was regarded as significant. To investigate whether one of the tested kits significantly more often yielded equivocal test results, qualitative data were arranged in two groups (unequivocal, i.e. positive or negative, and equivocal). Again, the CMH test was applied and a p-value < 0.05 was regarded as significant; pairwise comparison was done using McNemar's test with a continuity correction, with a p-value < 0.05 being regarded as significant. Correlation of quantitative results (U/ml or ratio) was assessed using Spearman's rank correlation coefficient. Approximate 95 % confidence intervals for Spearman's rank correlation coefficient were obtained via confidence intervals for the Pearson product-moment correlation applied to the ranks. Statistical analyses were done on the overall study population (n = 251) but not separately for the different sample categories a) to e) due to the limited sample size for these categories.

Table 2
Pairwise comparison of anti-TBEV IgG and IgM qualitative test results[a].

		RIDASCREEN IgG			Serion IgG		
		neg	equ	pos	neg	equ	pos
Euroimmun Vienna IgG	neg	145	3	5	151	1	1
	equ	4	0	2	6	0	0
	pos	6	0	86	10	4	78

		RIDASCREEN IgM			Serion IgM		
		neg	equ	pos	neg	equ	pos
Euroimmun IgM	neg	208	0	0	207	0	1
	equ	5	0	0	3	1	1
	pos	3	0	35	2	1	35

		RIDASCREEN IgG			Serion IgG		
		neg	equ	pos	neg	equ	pos
Euroimmun Vienna IgG	neg	155	0	0	155	0	0
	equ	3	0	0	3	0	0
	pos	9	5	79	9	5	79

		RIDASCREEN IgM			Serion IgM		
		neg	equ	pos	neg	equ	pos
Euroimmun IgM	neg	211	1	4	211	1	4
	equ	0	0	0	0	0	0
	pos	1	1	33	1	1	33

[a] The number of samples with the respective results are given; total number of samples tested = 251. Pos, positive; equ, equivocal; neg, negative; Euroimmun Vienna IgG, anti-FSME/TBE virus ELISA "Vienna" IgG, Euroimmun; Euroimmun IgM, anti-FSME/TBE virus ELISA IgM kit, Euroimmun; RIDASCREEN IgG, RIDASCREEN® FSME/TBE IgG kit, R-Biopharm; RIDASCREEN IgM, RIDASCREEN® FSME/TBE IgM kit, R-Biopharm; Serion IgG, SERION ELISA *classic* FSME Virus/TBE Virus IgG kit, Virion\Serion; Serion IgM, SERION ELISA *classic* FSME Virus/TBE Virus IgM kit, Virion\Serion.

3. Results

3.1. Qualitative test results

Although weighted κ was generally high (0.85 and more), the test results significantly differed between the different kits for both IgG ($p < 0.0001$, CMH test) and IgM ($p = 0.0388$, CMH test). In total, discrepant test results for IgG and/or IgM were observed for 37/251 samples (14.7 %). In pairwise comparison, the differences were significant for Euroimmun Vienna vs. Virion\Serion IgG, RIDASCREEN vs. Virion\Serion IgG, and Euroimmun vs. RIDASCREEN IgM (Tables 2 and 3). A significantly elevated proportion of equivocal test results could not be established for any of the kits (IgG: $p > 0.05$, CMH test; IgM: $p = 0.04$, CMH test, but $p > 0.05$ for all pairwise comparisons using McNemar's test).

Using SNT, the neutralizing titer (total Ig) for 25/37 discrepant samples was assessed; for six samples, sample volume was not sufficient to perform SNT; four samples had yielded discrepant results in both IgG and IgM ELISA testing within the same sample, wherefore they were not included in the evaluation described in this paragraph (a positive SNT result could not have been attributed to one immunoglobulin type). In addition to serum neutralization testing, results specifications provided by the EQA organizer were taken as reference results for two samples. With these data, a reference result (SNT titer or EQA result) was obtained for 27/37 discrepant samples (21 samples discrepant in IgG-testing, six in IgM-testing). These samples belonged to categories a) (n = 5), b (n = 5), d) (n = 10), and e) (n = 7). Table 4 summarizes the performance of the evaluated Anti-TBEV IgG or IgM ELISA tests for these samples. Detailed testing results are given in Table 5.

In addition to discrepant samples, five randomly selected samples with concordant positive anti-TBEV IgG ELISA test results were analysed using SNT. From these, four tested positive with titers ranging between 64 and > 1024, whereas one tested negative (Table 5).

3.2. Quantitative test results

The following Spearman rank correlation coefficients (95 % confidence intervals) were estimated for quantitative test results (U/ml or ratio): Euroimmun Vienna vs. RIDASCREEN IgG; 0.81 (0.77–0.85); Euroimmun Vienna vs. Virion\Serion IgG: 0.80 (0.75–0.84); RIDASCREEN vs. Virion\Serion IgG: 0.78 (0.73–0.83); Euroimmun vs. RIDASCREEN IgM: 0.63 (0.55–0.70); Euroimmun vs. Virion\Serion IgM: 0.63 (0.55–0.70); RIDASCREEN vs. Virion\Serion IgG: 0.72 (0.65–0.77); (Supplementary Fig. 1).

4. Discussion

TBE is an endemic disease in many parts of Europe and Asia (Lindquist, 2014). The diagnosis of TBE is based on the demonstration of specific antibodies (Holzmann, 2003).

Although ELISAs are known to produce false-positive results with antibodies directed against other flaviviruses, for reasons of simplicity and quick availability of test results, they remain the method of choice for serological diagnosis of TBE (Litzba et al., 2014). In this work, we compared three commercially available anti-TBEV IgG and IgM ELISAs. For a subset of 35 samples, SNT was performed as a reference method to evaluate the performance of the different ELISAs.

Qualitative test results overall (n = 251) showed a high level of agreement for IgG as well as IgM test when using weighted κ as criterion (0.86–0.89 for IgG, 0.90–0.92 for IgM). Nevertheless, in the linear-by-linear association test, ELISA test results significantly differed for Euroimmun Vienna vs. Virion\Serion IgG, RIDASCREEN vs. Virion \Serion IgG, and Euroimmun vs. RIDASCREEN IgM (Tables 2 and 3). Thus, the qualitative test results and therewith potentially the diagnosis of disease significantly depend on the test system used in the laboratory carrying out the analysis. This emphasizes the need of harmonization

Table 3
Kappa coefficients and asymptotic linear-by-linear association test *p*-values for pairwise comparison of anti-TBEV IgG and IgM qualitative test results[a].

Compared kits	weighted κ	κ lower CI	κ upper CI	p-value association test
IgG				
Euroimumn Vienna IgG vs. RIDASCREEN IgG	0.8686	0.8101	0.9270	n.s.
Euroimmun Vienna IgG vs. Serion IgG	0.8562	0.7957	0.9167	0.00027
RIDASCREEN IgG vs. Serion IgG	0.8866	0.8320	0.9411	< 0.0001
IgM				
Euroimmun IgM vs. RIDASCREEN IgM	0.9143	0.8515	0.9772	0.00763
Euroimmun IgM vs. Serion IgM	0.9169	0.8568	0.9771	n.s.
RIDASCREEN IgM vs. Serion IgM	0.9038	0.8314	0.9763	n.s.

[a] CI, confidence interval; association test, asymptotic linear-by-linear association test (evaluation by kit); n.s., non-significant. Euroimmun Vienna IgG, anti-FSME/TBE virus ELISA "Vienna" IgG, Euroimmun; Euroimmun IgM, anti-FSME/TBE virus ELISA IgM kit, Euroimmun; RIDASCREEN IgG, RIDASCREEN® FSME/TBE IgG kit, R-Biopharm; RIDASCREEN IgM, RIDASCREEN® FSME/TBE IgM kit, R-Biopharm; Serion IgG, SERION ELISA *classic* FSME Virus/TBE Virus IgG kit, Virion\Serion; Serion IgM, SERION ELISA *classic* FSME Virus/TBE Virus IgM kit, Virion\Serion.

Table 4
Anti-TBEV-IgG and IgM qualitative ELISA test results of discrepant samples in relation to serum neutralization testing results.

Kit	number of false-positive test results[a]	number of false-negative test results[b]
IgG		
Euroimmun Vienna IgG	4 (7)/21	0/21
Serion IgG	0 (1)/21	4 (5)/21
RIDASCREEN IgG	4/21	1/21
IgM		
Euroimmun Vienna IgM	0 (2)/6	0 (1)/6
Serion IgM	0/6	0 (1)/6
RIDASCREEN IgM	0/6	3/6

[a] The number of false-positive results when regarding an equivalent result as "false-positive" is given in brackets.

[b] The number of false-negative results when regarding an equivalent result as "false-negative" is given in brackets.

and standardization of the different assays in order to improve comparability of the different diagnostic tests.

37/251 (14.7 %) of the tested samples yielded discrepant results in IgG and/or IgM testing. Using SNT (25 samples) or results provided by EQA organizers (2 samples), reference values (positive or negative test result) were established for 27 of these samples. In relation to these values, false-positive results were observed mainly for Euroimmun Vienna IgG and RIDASCREEN IgG, whereas false-negative results were primarily observed for Virion\Serion IgG and RIDASCREEN IgM kits (Table 4). Consistent with these findings, both Euroimmun Vienna IgG and RIDASCREEN IgG significantly more often yielded positive results compared to Virion\Serion IgG, whereas RIDASCREEN IgM significantly more often yielded negative results compared to Euroimmun IgM in the overall study population (Tables 2 and 3). Interestingly, in the limited set of samples analysed, both Euroimmun Vienna IgG and Euroimmun IgM tended to show a higher cross-reactivity with samples previously tested positive for IgM against other pathogens (*B. burgdorferi*, CMV; n = 3 for anti-TBEV IgG and n = 2 for anti-TBEV IgM, including equivalent results) than did the RIDASCREEN (CMV, n = 1 for IgG) and Virion\Serion (n = 0) assays (Table 5). Taken together, the test kits have some limitations in sensitivity (Virion\Serion IgG, RIDASCREEN IgM) or specificity (Euroimmun Vienna IgG, RIDASCREEN IgG), respectively. The reasons for this are not entirely clear. Differences in viral strains used for antigen production (Table 1) might explain the dissimilar kit performances. However, the degree of variation between different strains is known to be low. Even for strains belonging to the different subtypes, a maximum degree of amino acid variation of 2.2 % for the major antigenic determinant, the viral envelope protein, has been shown (Ecker et al., 1999). Also, sensitivity and specificity constraints did not to the same extent affect IgG and IgM kits of one company; since the companies use just one strain for both IgG and IgM ELISA antigen preparation, the viral isolate itself is an unlikely source of variation in kit performance. Irrespective of the viral isolate, full virus lysate antigens containing high amounts of epitopes conserved among different flaviviruses are used by many manufacturers, and these may reduce specificity of the assays. Interestingly, the Virion\Serion ELISA classic TBE IgG and IgM are based on a preparation of the envelope protein of TBEV strain Moscow B4 instead of full virus lysates as antigen. The use of this antigen preparation is supposed to increase specificity, possibly going along with a reduced sensitivity of the assay. In our study, Virion\Serion ELISAs yielded the lowest number of false-positive results (IgG and IgM) but the highest number of false-negative results in IgG but not IgM testing (Table 4), which is in agreement with the supposed ELISA antigen properties. Besides the used antigen, suboptimal settings of cut-off values could account for false-positive or false-negative test results. However, there was no obvious difference in ELISA IgG or IgM titers from false-positive to true-positive or false-negative to true-negative results (data not shown).

Five selected samples with IgG results concordantly positive for all evaluated ELISAs were tested using SNT. From these, four tested positive and one negative for neutralizing antibodies against TBEV. This result is likely due to the presence of cross-reactive antibodies. The antigenic structure is highly cross-reactive among all flaviviruses, and nearly all ELISA assays for TBEV also detect cross-reactive antibodies against other flaviviruses (Litzba et al., 2014), especially for IgG (Lindquist, 2014). As indicated above, the clinical and/or vaccination history for TBEV and other flaviviruses is not known for our study population. In Switzerland, anti-flavivirus antibodies with specificities other than anti-TBEV are detected primarily in association with subjects traveling to areas endemic for other flaviviruses or being vaccinated against Yellow fever virus. We expect a more or less high proportion of concordantly positive results in our study to be a consequence of cross-reactive antibodies. For reasons of costs and the amount of work required, not all sera were tested using SNT. However, our findings support the fact that cross-reactivity remains a major limitation in serological diagnosis of TBE when using ELISAs (Lindquist, 2014).

Although SNT is regarded as the gold standard for the detection of TBEV-neutralizing antibodies (Holzmann, 2003), it has recently been shown that some protocols have problems in both sensitivity and specificity (Litzba et al., 2014). Since we have included sera positive for antibodies against other flaviviruses in our assay validation process and were able to detect TBEV-neutralizing antibodies in weak-positive/equivocal EQA samples, sensitivity and specificity problems are of minor concern with our protocol.

A significantly elevated proportion of equivocal test results could not be established for any of the kits (IgG: $p > 0.05$, CMH test; IgM: $p = .04$, CMH test, but $p > 0.05$ for all pairwise comparisons using McNemar's test). An elevated proportion of equivocal results for one kit would have indicated a comparatively large equivocal zone. However, based on our study, there is no need for narrowing the equivocal zone

Table 5
Detailed data on testing of samples yielding discrepant ELISA test results for a) anti-TBEV IgG and b) anti-TBEV IgM testing. c) shows the results of SNT testing for 5 samples with concordant positive anti-TBEV IgG ELISA results.

a)

Nr.	Category	Euroimmun Vienna IgG		Ridascreen IgG		Virion\Serion IgG		SNT		Evaluation[a]
		Conc. (U/ml)	Interp.	Conc. (U/ml)	Interp.	Conc. (U/ml)	Interp.	Titer	Interp.	
1	routine testing TBEV IgG pos.	0	neg.	158.0	pos.	37.4	neg.	1: < 8	neg.	Ridascreen false-pos.
15	routine testing TBEV IgG pos.	275.8	pos.	364.4	pos.	120.9	equ.	1:45	pos.	(Serion false-neg.)
19	routine testing TBEV IgM pos.	37.5	neg.	112.6	equ.	8.9	neg.	1: < 8	neg.	(Ridascreen false-pos.)
27	routine testing TBEV IgG pos.	830.5	pos.	306.0	pos.	91.0	neg.	EQA IgG pos.		Serion false-neg.
64	routine testing TBEV IgG pos.	847.2	pos.	303.1	pos.	95.2	neg.	EQA IgG pos.		Serion false-neg.
73	routine testing TBEV IgG pos.	292.3	pos.	281.7	pos.	115.6	equ.	1:32	pos.	(Serion false-neg.)
138	routine testing TBEV IgG neg.	187.7	pos.	12.0	neg.	7.0	neg.	1: < 8	neg.	Euroimmun false-pos.
139	routine testing TBEV IgG neg.	241.5	pos.	67.0	neg.	17.6	neg.	1:11	neg.	Euroimmun false-pos.
147	routine testing TBEV IgG neg.	148.0	equ.	232.8	pos.	86.7	neg.	1:16	pos.	Serion false-neg.
149	routine testing TBEV IgG neg.	271.8	pos.	85.9	neg.	33.6	neg.	1:32	pos.	Ridascreen, Serion false-neg.
152	routine testing TBEV IgG neg.	254.7	pos.	31.8	neg.	9.3	neg.	1: < 8	neg.	Euroimmun false-pos.
153	routine testing TBEV IgG neg.	256.2	pos.	168.7	pos.	76.3	neg.	1:16	pos.	Serion false-neg.
168	routine testing TBEV IgG neg.	224.7	pos.	67.5	neg.	20.1	neg.	1: < 8	neg.	Euroimmun false-pos.
171	routine testing TBEV IgG neg.	163.1	equ.	49.5	neg.	7.1	neg.	1: < 8	neg.	(Euroimmun false-pos.)
189	routine testing TBEV IgG neg.	100.7	neg.	192.9	pos.	106.4	equ.	1:11	neg.	Ridascreen (Serion) false-pos.
190	routine testing TBEV IgG neg.	61.6	neg.	167.5	pos.	32.6	neg.	1: < 8	neg.	Ridascreen false-pos.
203	routine testing Borrelia IgM pos	161.5	equ.	30.1	neg.	7.1	neg.	1: < 8	neg.	(Euroimmun false-pos.)
215	routine testing Borrelia IgM pos.	271.9	pos.	396.2	pos.	126.6	equ.	1:16	pos.	(Serion false-neg.)
221	routine testing CMV IgM pos.	70.1	neg.	171.9	pos.	2.4	neg.	1: < 8	neg.	Ridascreen false-pos.
222	routine testing CMV IgM pos.	124.8	equ.	14.5	neg.	1.0	neg.	1: < 8	neg.	(Euroimmun false-pos.)
231	routine testing EBV IgM pos.	187.1	pos.	315.7	pos.	1.8	neg.	1:16	pos.	Serion false-neg.

b)

Nr.	Category	Euroimmun IgM		Ridascreen IgM		Virion\Serion IgM		SNT		Evaluation[a]
		Conc. (Ratio)	Interp.	Conc. (U/ml)	Interp.	Conc. (U/ml)	Interp.	Titer	Interp.	
23	routine testing TBEV IgM pos.	1.1	equ.	93.7	neg.	69.1	pos.	1:45	pos.	Ridascreen (Euroimmun) false-neg
24	routine testing TBEV IgM pos.	1.6	pos.	25.4	neg.	24.3	pos.	1:64	pos.	Ridascreen false-neg.
26	routine testing TBEV IgM pos.	1.4	pos.	41.5	neg.	16.4	pos.	1: > 1024	pos.	Ridascreen false-neg.
48	routine testing TBEV IgM pos.	3.3	pos.	130.8	pos.	14.2	equ.	1:128	pos.	Serion false-neg.
226	routine testing CMV IgM pos.	1.0	equ.	17.9	neg.	5.4	neg.	1: < 8	neg.	(Euroimmun false-pos.)
253	routine testing EBV IgM pos.	1.1	equ.	9.7	neg.	2.0	neg.	1: < 8	neg.	(Euroimmun false-pos.)

c)

Nr.	Category	Euroimmun Vienna IgG		Ridascreen IgG		Virion\Serion IgG		SNT		Evaluation[a]
		Conc. (U/ml)	Interp.	Conc. (U/ml)	Interp.	Conc. (U/ml)	Interp.	Titer	Interp.	
6	routine testing TBEV IgG pos.	827.9	pos.	595.5	pos.	320.8	pos.	1:64	pos.	all true-pos.
39	routine testing TBEV IgG pos.	> 1000	pos.	770.1	pos.	1497.1	pos.	1: > 1024	pos.	all true-pos.
55	routine testing TBEV IgG pos.	> 1000	pos.	876.7	pos.	2215.2	pos.	1:181	pos.	all true-pos.
62	routine testing TBEV IgG pos.	526.8	pos.	347.5	pos.	361.9	pos.	1: < 8	neg.	all false-pos.
69	routine testing TBEV IgG pos.	868.8	pos.	302.9	pos.	153.9	pos.	1:91	pos.	all true-pos.

[a] The evaluation when regarding an equivalent result as “false-negative” or “false-positive” is given in brackets.

for any of the evaluated kits.

Quantitative test results pronouncedly varied between the different kits and did not even show a very strong monotonically increasing relationship, with Spearman’s rank correlation coefficient ranging from 0.78 to 0.81 for IgG and 0.64–0.72 for IgM. As a consequence, direct comparison of quantitative data from different kits is not appropriate. Importantly, parallel titration is indispensable when an increase in antibody titer has to be documented. Furthermore, the fairly weak rank correlation of quantitative test results accentuates the uncertainty in defining cut-offs for protective immunity. For assays using the so-called Vienna standard (e.g. anti-FSME/TBE virus ELISA “Vienna” IgG, Euroimmun), cut-offs between 127 and 201 Vienna Units/ml are rated as protective (Holzmann et al., 1996; Kunz, 2003). These cut-offs are only valid for vaccinated persons shortly after basic immunization and are not suitable for defining protective immunity in longer intervals following immunization (Webpage Robert Koch Institute; FAQs on vaccination against TBEV). In our study, test results in the range of these cut-offs (between about 120 and 250 U/ml for the Euroimmun Vienna IgG test using Vienna standard) showed a high degree of variation. Therefore, the proposed cut-offs for assays using the Vienna standard must not be applied for test systems using other standards.

5. Conclusions

In our study comparing three commercially available anti-TBEV IgG and IgM ELISA kits (Euroimmun, Virion\Serion, R-Biopharm), we have observed significant differences for both qualitative and quantitative data. In laboratory diagnosis of TBE, qualitative and quantitative test results therefore depend on the test system used in the laboratory carrying out the analysis, indicating the need of standardization of the different assays. SNT results available for a subset of samples revealed problems in both sensitivity (Virion\Serion IgG, RIDASCREEN IgM) and specificity (Euroimmun Vienna IgG, RIDASCREEN IgG) of the evaluated kits. In routine diagnostics, sensitivity problems may be overcome by

testing a follow-up sample to detect seroconversion or rises in titers. In contrast, specificity problems are of major relevance. In case of suspected cross-reactivity, false-positive results can be ruled out using SNT, which is the most type-specific serological test.

Funding

This work was supported by the Swiss Federal Office for Public Health and in-kind contributions. The kits used in this evaluation study were provided free of charge by the manufacturers.

Acknowledgements

We thank Euroimmun, R-Biopharm and Virion\Serion for their interest in our evaluation work and for offering the kits used in this evaluation study. Special thanks go to Michael Vock, Institute of Mathematical Statistics and Actuarial Science, University of Berne, for his very competent support in statistical analyses. Finally, we thank Olivier Engler and Marc Strasser for their support and helpful discussions as well as Johanna Signer and Carine Groux for their excellent laboratory assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ttbdis.2018.03.031.

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