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### Summary

### Zusammenfassung

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## Evaluation of serological tests for detecting tick-borne encephalitis virus (TBEV) antibodies in animals

### *Vergleich serologischer Tests zur Bestimmung von Antikörpern gegen das Frühsommer-Meningoenzephalitis-Virus (FSMEV) bei Tieren*

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Tick-borne encephalitis (TBE) in animals is not well understood yet. TBE virus (TBEV) serology in several host species could be valuable for epidemiological analyses in the field as well as for the detection of clinical cases. However, performance and suitability of the available test systems are not well assessed. Therefore, we evaluated two commercial TBEV-ELISA kits in a pilot study and compared them for their suitability in veterinary applications. For this purpose, we tested 163 field collected goat sera and evaluated the results by serum neutralization test (SNT) as "gold standard". Twenty-eight SNT positive sera (17.2%) were detected. The best suited ELISA kit was used for determination of a species-specific cut-off for horses, cattle, sheep, goats, pigs, mice, dogs, rabbits and monkeys with defined sera from animals without known or with improbable contact to TBEV. The level of non-specific ELISA results does not only differ between animal species but may also be influenced by the age of the tested animals. The number of sera which tested false positive by ELISA was higher in older than in young sheep. In order to obtain defined polyclonal sera as references, two dogs, cattle, goats, sheep, rabbits and pigs each, as well as one horse and 90 mice were immunized four times with a commercially available TBEV vaccine. In conclusion, our results demonstrated that commercial TBEV-ELISA kits are suitable for application in veterinary medicine for both, verification of clinical TBE cases and epidemiological screening. However, positive ELISA results should be verified by SNT. Only a very low number of false negative ELISA-results were found.

**Keywords:** seroprevalence, ELISA, serum neutralization test

Zur Frühsommer-Meningoenzephalitis (FSME) bei Tieren ist bisher wenig bekannt. Die FSME-Serologie bei verschiedenen Tierspezies kann sowohl für epidemiologische Analysen als auch für die Abklärung klinischer FSME-Fälle beim Tier bedeutsam sein. Bisher sind die verfügbaren Testsysteme für Seren von Tieren nicht gut evaluiert. Deshalb wurden zwei kommerziell erhältliche FSME-ELISA kits auf ihre Verwendbarkeit in der Veterinärmedizin verglichen. Dazu wurden 163 Ziegenserum aus Baden-Württemberg verwendet. Alle ELISA Ergebnisse wurden mittels Serum-Neutralisationstest (SNT) als „Gold Standard“ überprüft und 28 positive Seren (17,2 %) gefunden.

Das am besten geeignete ELISA Kit wurde zur Bestimmung eines tierartspezifischen Cut-Offs für Pferde, Rinder, Schafe, Ziegen, Schweine, Mäuse, Hunde, Kaninchen und Affen herangezogen. Die Seren stammten von Tieren, die mit sehr hoher Wahrscheinlichkeit keinen Kontakt zu FSME-Viren (FSMEV) hatten. Um definierte polyklonale Seren als Referenzmaterial zu erhalten, wurden je zwei Hunde, Rinder, Ziegen, Schafe, Kaninchen, Schweine, ein Pferd und 90 Mäuse mit

einem kommerziell erhältlichen Impfstoff immunisiert. Die Anzahl der unspezifischen ELISA-Reaktionen scheint nicht nur von der Tierart, sondern auch vom Alter der Tiere beeinflusst zu werden. Bei Schafen war die Zahl der im ELISA falsch positiv getesteten Tiere bei älteren Tieren deutlich höher, als bei jüngeren Tieren. Die Ergebnisse zeigen, dass die Verwendung der kommerziellen FSMEV-ELISA-Kits in der Veterinärmedizin möglich ist, sowohl zur Abklärung klinischer FSME-Fälle als auch als Screening-Test für epidemiologische Fragestellungen. Jedoch sollten die positiven ELISA Ergebnisse immer mittels SNT verifiziert werden. Es trat eine geringe Zahl an falsch negativen ELISA-Ergebnissen auf.

**Schlüsselwörter:** Seroprävalenz, ELISA, Serum-Neutralisationstest

## Introduction

Tick-borne encephalitis (TBE) is the most important viral tick-borne zoonosis in Europe and in some parts of Asia (Süss et al., 2010). TBE virus (TBEV), a flavivirus, circulates between the virus-transmitting ticks, *Ixodes ricinus* mainly in Western Europe and *Ixodes persulcatus* in Eastern Europe and Siberia, and competent hosts in geographically strictly limited natural foci, whose size can range from large to very small. The reason for the patchwork-like spread of TBEV is not quite clear, and is in contrast to that of *Borrelia burgdorferi* s. l., which is endemic in central Europe and more or less distributed in all areas where *Ixodes ricinus* occurs.

In Germany, TBE in humans is a notifiable disease based on the infection protection bill of 2001, and all autochthonous human cases are evaluated and registered by the Robert-Koch-Institute, Berlin, Germany. Subsequently, districts are defined as "TBE risk area" based on the incidence of autochthonous human cases according to the guidelines of the Robert-Koch-Institute (Robert-Koch-Institute, 2007). An update on autochthonous human TBE cases and risk areas in Germany is published every year (Robert-Koch-Institute, 2010).

According to Süss et al. (2010) a TBEV endemic area is defined as an area where TBEV circulates between ticks and vertebrate hosts, inferred by the detection of TBEV or the occurrence of autochthonous infections in animals or humans within the last 20 years.

In the past, clinical cases of TBE in veterinary medicine were rarely detected in dogs, horses and monkeys (Waldvogel et al., 1981; Leschnik et al., 2002; Süss et al., 2007, 2008; Klaus et al., 2010a), severe clinical TBE cases particularly occurred in dogs and monkeys. The fact that TBE is rarely diagnosed in animals may partially be the result of a lack of diagnostic tools. Due to the very low incidence in animals the number of methods for veterinary use is very limited (Müller, 1997; Leschnik et al., 2002; Leutloff et al., 2006). In contrast, diagnostic tools for TBE in humans are well established. Adaptation of these tools for veterinary application instead of the development of in house methods may be a possibility to improve the situation.

It is well known that goats, sheep and cattle are hosts for *Ixodes ricinus* stages. These animals only develop low-level TBEV viraemia for about two to five days (van Tongeren, 1955; Grešíková, 1958a; Grešíková and Calisher, 1988), and do not show any clinical signs, but seroconversion can be observed after infection with TBEV (Rieger et al., 1997; Labuda et al., 2002). So far however, only a limited number of validated data is available on the course of TBEV viraemia in domestic animals.

TBEV antibodies in animals might serve as a tool for clinical veterinary diagnostics as well as for describing a natural TBEV focus if detected with a validated serological detection system as described here.

## Material and Methods

### ELISA procedures

Two TBEV-ELISA kits were tested: Immunozyzm FSME IgG all species kit (Progen GmbH, Germany) classified as an "all species kit" for veterinary application and Immunozyzm FSME IgM kit (Progen GmbH, Germany) validated for IgM detection in human routine TBE diagnostics. Both kits are two-step ELISAs and differ especially in the included conjugate as described in the manufacturer's instructions. In both kits the ELISA test strips are coated with inactivated TBEV, strain Neudoerfl and the protein G peroxidase conjugate acts as a marker for the bound anti-TBEV antibodies during the second incubation phase after serum incubation. The Immunozyzm FSME IgM kit includes a more TBEV specific protein G peroxidase conjugate, developed by the manufacturer. In both kits, in the third incubation phase the substrate reaction takes place using hydrogen peroxide as substrate. To stop the reaction, sulphuric acid is added and the yellow colour intensity is measured.

The Immunozyzm FSME IgG all species kit for veterinary diagnostic was used according to the manufacturer's instructions. For the Immunozyzm FSME IgG all species kit, the results were determined according to the manufacturer's instructions by using lyophilized human calibration sera included in the kit and expressed in Vienna units/ml (VIE/ml).

For veterinary samples the Immunozyzm FSME IgM kit was used in the modified version by Müller (1997). The most important difference to the manufacturer's instructions was that the Immunozyzm FSME IgM kit was used without the IgG blocking step in order to determine the total immunoglobuline as it was also done with the other kit. For the Immunozyzm FSME IgM kit, the value of the units/l (U/l) was adapted for veterinary purposes by testing and evaluating a number of dog sera in the past (Müller, 1997), the results were documented as units/l and classified for dogs as < 5 U/l negative (-), 5–7.5 U/l borderline (?) and > 7.5 positive (7.5–14 +, > 14–50 ++ and > 50 +++).

### Serum neutralization test

All serum samples which were tested positive or close to borderline in the ELISAs were re-tested by SNT according to Holzmann et al. (1996) in the described modified

version (Klaus et al., 2010a) as gold standard to avoid false positive results: For the SNT, the low pathogenic strain “Langat” was used with about 100 TCID<sub>50</sub>/well. The virus titre used was confirmed by back-titrations. Serum samples were titrated in duplicates starting at a dilution of 1:5 in MEM Earle’s medium (Biochrom AG, Germany), and a BHK-21 cell suspension was added to the virus-serum-sample and incubated at 37°C for four days. Subsequently, virus replication in the wells was detected by immunofluorescence analysis using a TBEV-specific rabbit antiserum. Titres were expressed as the reciprocal of dilutions that neutralized the infection of the cell culture in 50% of the test wells (neutralizing dose 50% = ND<sub>50</sub>), and were calculated according to the method of Spearman and Kärber (Mayr et al., 1977).

**Field-collected sera**

Between 2007 and 2008 163 sera from goats grazing in TBE risk areas in Baden-Wuerttemberg were collected and examined to compare the two ELISA kits. In former investigations, TBEV antibody-positive sera have been found in sheep and monkeys in a TBE risk area in Baden-Wuerttemberg (Klaus et al., 2010a).

All the 163 goat sera which were tested with both described ELISA kits were re-tested by SNT to determine the sensitivity and specificity of the two ELISA kits.

**Determination of cut-off values**

In order to determine species-specific cut-offs calculated as the doubled standard deviation of the mean value of a negative sample pool, sera from animals without known contact or with improbable contact to TBEV were collected and sera were selected as follows:

**Horses**

52 sera were collected over the years 2006, 2007 and 2009; 50 from 3 barns in Thuringia (Meura, Weimar and Bad Langensalza) without known human or animal TBE cases in the surroundings, and two sera from horses owned by the Friedrich-Loeffler-Institut which had not left the institute for several years.

**Cattle**

52 sera were collected; 50 from a dairy farm in Buttstedt, district Weimarer Land in Thuringia in 2006, so far a TBE non-risk area, but with some single autochthonous human cases according to the guidelines of the Robert-Koch-Institute. The animals were held in animal houses and not on meadows. Two animals were owned by the Friedrich-Loeffler-Institut in 2009 and had never left the institute since then.

**Goats**

In 2009, 52 sera were collected, 50 from a flock in Eichelborn, district Weimarer Land, and two goats owned by the Friedrich-Loeffler-Institut, bought from a flock in Greußen, district Sömmerda, in January 2009. Both Thuringian districts are TBE non-risk areas.

**Pigs**

In 2009, 50 sera from eleven-week-old pigs were collected from a pig farm in Hermstedt, district Weimarer Land in Thuringia, a TBE non-risk area. The pigs were kept in a pigpen and spent their whole life inside.

**Mice**

In 2009, 50 sera were collected from mice owned by the Friedrich-Loeffler-Institut.

**Dogs**

In 2009, 52 sera were collected by veterinarians in different TBE non-risk areas in Germany.

**Rabbits**

In 2009 and 2010, 34 sera were collected. Sixteen rabbits were owned by the Friedrich-Loeffler-Institut and 18 rabbits were held in a housing unit of the Friedrich-Schiller-University Jena.

**Monkeys**

In general it is very difficult to obtain sera from monkeys, especially defined sera from TBE non-risk areas. For cut-off calculation 52 sera collected from monkeys (*Macaca sylvanus*) at the monkey mountain Salem (Germany, district Bodenseekreis, 47°45’44.75”N 9°14’40.48”E) in 1998 were examined. During that year no clinical signs of TBE in monkeys were seen. In the following years in contrast, a clinical case of TBE after natural exposure was described in a monkey (Süss et al., 2007, 2008) and we had been also able to isolate a TBEV strain from the brain tissue of this animal and to detect TBEV positive sera in several other monkeys (Klaus et al., 2010a).

**Sheep**

102 sera were collected in 2009; 50 from a flock in Artern, district Kyffhäuserkreis, 50 from a flock in Craula, district Unstrut-Hainich-Kreis in Thuringia, both TBE non-risk areas, and two from sheep owned by the Friedrich-Loeffler-Institut that had never left the institute.

In addition to that, in order to determine a possible influence of the animals’ age on the ELISA results, TBEV titres were determined and compared between 15 younger (up to one year) and 50 older (two to five years) sheep in a flock grazing in a TBE non-risk area, where only negative TBEV titres were expected.

**Preparation of species-specific polyclonal antisera**

In general, for evaluation of the ELISA kits, defined TBEV positive sera are highly recommended as a reference. However, in most cases they are difficult to obtain, and for TBEV antibody screening only sera collected in the field from TBEV risk areas were available. Thus, we decided to vaccinate a small number of animals (two dogs, two cattle, two sheep, two goats, one horse, two rabbits, two pigs, 90 NMRI-mice) experimentally with a vaccine used in human medicine (FSME-IMMUN Erwachsene, Baxter Deutschland GmbH, Germany) four times at weeks 0, 1, 3 and 11. The vaccine contains inactivated TBEV, strain Neudoerfl. At each immunization

**TABLE 1:** Comparison of two ELISA kits for TBEV antibody detection (SNT as a gold standard) in 163 goat sera

Kit	ELISA positive	ELISA borderline	ELISA negative
Immunozytm FSME IgM (adapted)	29	8	126
SNT results	23 SNT pos. 6 SNT neg.	2 SNT pos. 6 SNT neg.	3 SNT pos. 123 SNT neg.
Immunozytm FSME IgG all species	9	7	147
SNT results	9 SNT pos. 0 SNT neg.	7 SNT pos. 0 SNT neg.	12 SNT pos. 135 SNT neg.

\* SNT positive.

**TABLE 2:** ELISA cut offs determined for different animal species with sera from animals without known TBEV contact

	Number of sera	Units/l (mean value)	Standard dev.	Cut off (calculated)
monkeys	52	0.09	0.34	0.68
dogs	52	0.07	1.10	2.20
horses	52	1.05	1.80	3.61
cattle	52	1.35	1.89	3.77
mice	50	0.69	3.82	7.64
rabbits	34	1.88	5.24	10.49
goats	52	2.27	6.78	13.55
sheep	102	4.18	6.80	13.61
pigs	50	4.72	7.02	14.04

one dose of 0.5 ml containing 2.4 µg inactivated virus antigen was injected subcutaneously per goat, sheep and pig. This is the dose that is also used routinely for adult humans according to the manufacturer's vaccination recommendations. Horses and cattle were given two doses per immunization. Dogs and rabbits received one dose of 0.5 ml four times containing 1.2 µg inactivated virus antigen per immunization (FSME-IMMUN Junior, Baxter Deutschland GmbH, Germany). This dose is regularly used for children. Mice received one dose of 0.3 ml four times containing 0.12 µg inactivated virus antigen per immunization (FSME-IMMUN Junior, Baxter Deutschland GmbH, Germany, diluted to obtain the desired concentration).

Sera were collected and examined for TBEV-specific antibodies by ELISA (Immunozytm FSME IgM kit, Progen GmbH, Germany) over a period of 14 to 24 weeks after the first immunization. All animals were observed for clinical signs of illness or allergic reactions as well as for a potential increase of body temperature at least two days before and two days after immunization. Mice were only observed clinically. All vaccinations and sera collections in animals were permitted by government authorities. SNT was done before immunization and two weeks after the third and fourth immunization (week 5 and 13).

## Results

### Comparison of ELISA kits

All 163 goat sera were tested with both ELISA kits and re-tested by SNT in order to determine sensitivity and specificity (Tab. 1).

By using the Immunozytm FSME IgG all species kit produced for veterinary purposes, all sera classified as positive (nine sera) or borderline (seven sera) were confirmed to be positive by SNT, but twelve ELISA-negative sera were positive in the SNT and were not detected in the ELISA test. By using the Immunozytm FSME IgM kit in the modified version, 23 of the 29 ELISA positive sera were also confirmed by SNT, and two of eight sera with borderline results were confirmed by SNT. Only three sera scoring negative in both ELISAs were found to be positive by SNT.

Therefore, the sensitivity of the Immunozytm FSME IgG all species kit was 57% in classifying all positive and borderline ELISA samples as positive, and only 32% taking all positive ELISA samples into account. In contrast, the sensitivity of the Immunozytm FSME IgM kit was 89% in classifying all positive and borderline ELISA samples as positive, and 82% regarding the ELISA positive samples only. The specificity of the Immunozytm FSME IgG all species kit was 100%, and of the Immunozytm FSME IgM kit 91% when classifying all positive and borderline ELISA samples as positive, and 95% when classifying ELISA positive samples as positive and ELISA borderline results as negative.

Altogether, 28 of 163 goat sera were found to be positive by SNT (17.2%), and only three sera were not detected by ELISA with the Immunozytm FSME IgM kit, which indicates that this two-step diagnostic system with a screening ELISA and a confirmatory SNT might be suitable for epidemiological investigations in TBE risk areas.

### Determination of cut-off values

Table 2 shows the determined cut-offs of the sera collected from different animal species, and will help to reduce the number of non-specific positive results in the ELISA screening of sera. However, the number of only 34 to 102 tested sera per species allows only a recommendation for cut-offs, and has to be redefined by examination of more sera in the future.

The age of the tested animals can also influence the number of false positive results. In the group of older sheep (two to five years old), more false positive (non-specific) ELISA results were determined in comparison to the younger group (up to one year old), Table 5.

### Species-specific polyclonal antisera

TBEV ELISA antibodies increased to variable titres upon experimental immunization in all experimentally immu-

**TABLE 3:** TBEV antibody titres of immunized animals (U/l), immunization in week 0, 1, 3 and 11

week*	dog 1	dog 2	cattle 55	cattle 57	sheep 87	sheep 90	goat 11**	goat 12**	horse	rabbit 13	rabbit 14	pig 50	pig 52
0	0.1 (< 5)	0 (< 5)	0.1 (< 5)	0.1 (< 5)	1.4 (< 5)	3.3 (< 5)	0.7 (< 5)	0.6 (< 5)	2.4 (< 5)	0.6 (< 5)	0.6 (< 5)	-0.2 (< 5)	0.1 (< 5)
1	0.7	0	0.2	0.3	2.3	9.1	0.6	0.7	8.5	82.8	79.8	19.5	12
3	58	0.3	2.2	7.9	12	23.7	5.3	24	26.3	30	56.4	12.5	9.5
5	26.6 (10)	1.2 (< 5)	11.6 (60)	7 (15)	7.7 (< 5)	17.1 (< 5)	5.4 (< 5)	66.6 (10)	6.6 (5)	34.7 (20)	70 (120)	8.2 (5)	11.5 (7.5)
6	13.5	1.1	9.3	7.2	5.5	15.9	4.7	47.8	4.6	35.8	72.5	5.8	10.8
11	0.8	0.8	5	3.4	4.4	11.3	8	45.7	13.3	56.6	80.8	2.6	4.9
12	7.6	11.1	22.9	26.3	20.8	37.4	60.6	81.8	7	68.7	80.3	17.6	8.3
13	24.7 (20)	19.7 (7.5)	22.9 (80)	25.1 (320)	28.3 (80)	44 (80)	53.6 (10)	79.2 (60)	10.4 (480)	68.9 (80)	73.5 (240)	24.8 (30)	14.7 (> 40)
14	21.4	16.1	19.7	21.1	18.4	36.2	44.3	64.3	24	66.9	74.3	21.9	13.6
15	n. t.	n. t.	n. t.	n. t.	n. t.	n. t.	42	60.6	n. t.	n. t.	n. t.	n. t.	n. t.
18	8.4	8.9	n. t.	n. t.	6.3	27.2	n. t.	n. t.	36.3	n. t.	n. t.	n. t.	n. t.
21	4.9	4	n. t.	n. t.	31.1	25.4	n. t.	n. t.	26.3	n. t.	n. t.	n. t.	n. t.
24	4	3.4	n. t.	n. t.	15.1	32.8	n. t.	n. t.	n. t.	n. t.	n. t.	n. t.	n. t.

n. t.: not tested, \* week after first immunization, \*\* see also Klaus et al., 2010b, screening with Immunozytm FSME IgM kit, Progen GmbH, Germany, modified by Müller (1997), in week 0, 5, 13 confirmation of positive and borderline results by SNT (in brackets), TBEV specific: => 5, TBEV non specific: < 5.

**TABLE 4:** TBEV antibody titres of 10 immunized mice (U/l), immunization in week 0, 1, 3 and 11

week*	1	2	3	4	5	6	7	8	9	10	mv	sd
0	-0.1 (< 5)	-0.3 (< 5)	-0.2 (< 5)	-0.1 (< 5)	-0.1 (< 7.5**)	-0.2 (< 5)	-0.2 (< 5)	-0.1 (< 5)	0.0 (< 5)	-0.2 (< 5)	-0.15	0.09
1	0.5	0.3	0.1	8.6	0.1	1.4	0.3	1.3	0.8	0.3	1.37	2.57
3	2.6	2.1	1.6	1.7	2.1	1.7	0.5	1.2	0.8	30.3	4.47	9.11
5	10.8 (7.5)	25.9 (40)	18.3 (7.5)	11.8 (7.5)	2.5 (10)	7.4 (10)	10.5 (10)	4.5 (7.5)	17.2 (20)	6.3 (7.5)	11.52	7.16
6	8.9	5.0	16.0	2.3	9.3	3.2	10.0	16.0	2.4	5.6	7.87	5.14
11	0.4	33.4	8.3	2.2	5.9	7.5	10.5	3.4	0.3	11.2	8.31	9.64
12	9.8	4.1	22.1	8.5	7.8	2.1	5.3	24.7	8.9	15.2	14.57	7.53
13	15.1 (> 40)	9.1 (30)	11.8 (20)	13.8 (30)	9.6 (15)	21.1 (15)	22.7 (15)	23.0 (20)	7.3 (30)	12.1 (7.5)	14.57	5.79
14	1.1	2.9	36.3	26.7	13.7	5.5	29.4	12.0	7.8	5.6	14.08	12.34

week\*: week after first immunization, screening with Immunozyzm FSME IgM kit, Progen GmbH, Germany, modified by Müller (1997), in week 0, 5, 13 confirmation of positive and borderline results by SNT (in brackets), TBEV specific: =/> 5, TBEV non specific: < 5, < 7.5\* toxic effect, nonspecific, mv: mean value, sd: standard deviation.

nized animals (Tab. 3). In mice, the mean values and standard deviation of 10 mice per investigation time are shown (Tab. 4).

Compatibility of the immunization was excellent in all animals; no clinical signs of illness or allergic signs, and no increase of body temperature were observed (not measured in mice). In most cases animals developed TBEV-specific antibody titres, however to a different degree. Only in mice, some single animals did not develop any TBEV specific antibodies which resulted therefore in a very high standard deviation. Highest and earliest titres were observed in rabbits, starting one week after the first immunization, and also in horses, but at a lower level. Two weeks after the third immunization, only four animals (dog 2, sheep 87 and 90, goat 11) had not shown specific antibody titres in the SNT, and two weeks after the fourth immunization all animals developed TBEV-specific antibody titres ranging between 14.7 and 79.2 U/l (ELISA) and between 7.5 and 480 (SNT), respectively (Tab. 3 and 4). In most of the immunized animals, a first increase of antibody titres was followed by a short temporary decrease and an increase after the fourth immunization. Some animals showed a delayed reactivity compared to the others and needed four immunizations to develop TBEV specific antibodies.

## Discussion

In our test comparison study the major aim was to define a system that allows screening of large populations of (livestock) animals for TBEV-specific antibodies. Therefore, two commercially available ELISA kits were compared using the serum neutralization test as “gold standard”. In our study both tested ELISA kits were able to detect TBEV antibodies in goat sera. However, much better sensitivity values could be achieved with the Immunozyzm FSME IgM kit than with the Immunozyzm FSME IgG all species kit. Some TBEV-specific positive goat sera (tested by SNT) were not detected by ELISA-screening with the Immunozyzm FSME IgG all species kit. In contrast, specificity of the Immunozyzm FSME IgG all species kit was higher than that of the Immunozyzm FSME IgM kit. For confirmation, final selection and scoring of both assays was done by investigating all ELISA-positive and borderline sera by SNT. The rationale behind this was that this assay had a very high specificity and a good sensitivity. Sera positive in the ELISA and confirmed by SNT have therefore a very high probability of being correctly scored. For all further tests,

the Immunozyzm FSME IgM kit was used in its adapted version due to the higher sensitivity. Additionally, the titration results of TBEV antibody positive sera (low and high titers) indicated that the adapted Immunozyzm FSME IgM kit is suitable for veterinary application. In general it is not a problem to obtain sera from different animal species without known contact to TBEV, positive sera however are not available in high numbers. In monkeys it is particularly difficult to obtain defined sera from TBE non-risk areas. The sera examined in our study were e. g. collected at the monkey mountain Salem, a TBE risk area. During 1998, the year of sera collection, no clinical signs of TBE were seen in the monkeys. In the following years in contrast, one clinical case of TBE after natural exposure was described in a monkey (Süss et al., 2007, 2008) and we had been able to isolate a TBEV strain from the brain tissue of this monkey and to detect TBEV positive sera in several other monkeys (Klaus et al., 2010a).

Our results in the determination of species-specific cut-offs (screening by ELISA, Immunozyzm FSME IgM kit, Progen GmbH, Germany, and confirmation of all positive and borderline results by SNT) in animals showed that it is recommended to differentiate assay cut-offs between the different animal species in order to prevent a too large number of false positive results. In monkey, dog, horse and cattle sera, it is recommended to classify all samples with a result of more than 5 U/l as positive, in mice all sera of more than 8 U/l, and in rabbits, goats, sheep and pigs all sera of more than 14 U/l. By using a species dependent cut-off, it is possible to make the Immunozyzm IgM kit more suitable for veterinary purposes, but mainly for a first screening, since the specificity values make confirmation of ELISA-positive sera by the SNT necessary. For a more sophisticated validation of the assays, more positive sera are needed. Therefore, a screening in risk areas is planned and with a larger panel of positive samples cut-off values might be recalculated by statistical analyses (e. g. using techniques of test comparison without a gold standard). Nevertheless, we think that the analysis presented here, even having the risk of a bias due to an imperfect gold standard test like the SNT, are reliable, especially for the described

**TABLE 5:** ELISA TBEV antibodies of younger and older sheep in one flock

	Sheep (up to 1 year old), ELISA (Units/l)	SNT positive sera	Sheep (2 to 5 years old), ELISA (Units/l)	SNT positive sera
positive	1	0	8	0
borderline	0		16	0
negative	14	not tested	26	not tested
<b>total</b>	<b>15</b>	<b>0</b>	<b>50</b>	<b>0</b>

Screening with Immunozyzm FSME IgM kit, Progen GmbH, Germany, modified by Müller (1997), confirmation of positive and borderline results by SNT.

cluster detection in different TBEV risk areas. In addition, the selected ELISA and the SNT showed a satisfactory correlation of the observed results.

In conclusion, for the described system it is recommended to pre-screen all samples by the selected ELISA with species-specific sample cut-off values and to confirm all reactive ones by the SNT as “gold standard” (Mayr et al., 1977; Klaus et al., 2010a, 2010b).

It is also possible to develop species-specific ELISA kits by replacing the protein G conjugate with a species-specific conjugate to reduce the number of non-specific reactions. In this case, it is necessary to prepare and validate ELISA tests for each species during the process of quality management in the lab. As there is a huge number of species that can be tested for TBEV antibodies and in some cases the number of sera of one species is small (for example monkeys or dogs with clinical signs of TBE or SNT-confirmed TBEV antibodies), it can be very time consuming and expensive to prepare and validate all species-specific ELISA tests. Thus we decided to use only the commercially available kits, since their use is also realistic under routine testing conditions.

In general, as described above the use of an ELISA kit suitable for all species is a compromise, in our opinion however an acceptable one, since no better system is available at the moment. It allows the use of a commercially available kit with an acceptable sensitivity, which is easy to handle and also suitable for small and large sample numbers of different animal species. In both kits a number of false negative results were found, a problem that cannot be avoided completely. In addition, we also cannot exclude that very few of these samples were also wrongly classified in the SNT due to cross-reactivity with other flaviviruses. However, a commercially available kit with well standardized reagents can be a better diagnostic tool than a less validated species-specific in house ELISA. The sensitivity of the best commercially available kit is in our opinion sufficient for epidemiological investigations if the recommendations for proper use, as suggested in this study, are followed. In addition, the lower specificity of 91% to 95% can be fully compensated by re-testing positive sera in the highly specific SNT. Also here, the cross-reactivity with other flaviviruses must be taken into account and larger discrepancies, e. g. an unexpected high number of “false positives” should be investigated in more detail.

Grazing domestic animals and wildlife animals can be used as sentinels for characterization of TBEV natural foci by means of serosurveillance tests besides virus detection in ticks and the number of autochthonous human cases (Rieger et al., 1997; Rieger et al., 1999; Labuda et al., 2002; Leutloff et al., 2006). For these investigations, the described methods (ELISA and SNT) can be used successfully. Twenty-eight of 163 goat sera that were used for comparing ELISA kits were found to be positive by SNT (17.2%). These sera were collected in four districts in Baden-Württemberg, all classified as “TBE risk areas”. This indicates that goats may be suitable sentinels for classifying the TBE risk of a given area.

The results of two groups of sheep (up to one year and two to five years old, Tab. 5) of a flock grazing in a TBE non risk area indicated that the age of the examined animals might play an important role. It should be taken into account that the number of false positive results increased especially in older animals. It also cannot be fully excluded that this might be due to a false nega-

tive result in the SNT for some of the ELISA-positive samples, which however is not very likely, if we look at the data of the standard sera of the vaccinated animals which show a very high sensitivity of the SNT.

The preparation of defined positive sera in the framework of this study improved the validation of the ELISAs for veterinary purposes. As only a limited number of data for TBEV immunization of animals was available (Grešíková 1958a, 1958b; Müller, 1997), the scheme was adapted according to the fast immunization scheme against TBEV that is recommended for humans. The first antibody increase might be an IgM dominated response (Müller, 1997), followed by a secondary response, in most cases after the fourth immunization. Only in rabbits an earlier secondary response could be observed (two weeks after the second immunization). The delayed development of TBEV-specific antibodies in some single animals, even after repeated contact with the antigen, remains unclear and cannot be explained by these investigations. It may be possible that these cases are an example for the so called “low responder” phenomenon, which is also observed in some humans after immunization against TBEV (Lindquist and Vapalahti, 2008). In general, immunization of animals against TBEV is not well examined up to now and should be investigated further, e. g. to assess the optimal vaccination scheme and the protective effects against TBEV infection.

On the basis of our results, further epidemiological investigations will be carried out especially in a large number of sera from grazing animals serving as sentinels in TBEV endemic or non endemic areas. It is also recommended to evaluate TBEV immunization schedules for veterinary purposes, especially for dogs that live in TBEV risk areas. Also for these studies our data are a valuable basis allowing reliable serological studies. Finally, the future panel of positive sera will be used to re-calculate the cut-off values of the test system to further enhance the correctness of our analysis system.

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