Review Article

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Journal of Zoonotic Diseases and Public Health

2017
Vol. 1 No. 1: 4

Comparative Tick-Borne Encephalitis (Virus) Surveillance in Belgium 2009-2015: Experiences with Diagnostic Tests, Sentinel Species and Surveillance Designs

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Abstract

When it is not overtly affecting human beings, the Tick-Borne Encephalitis Flavivirus (TBEV) remains mostly unnoticed during its enzootic cycles within vectors and unaffected animal species. Until recently, Belgium was "presumed" free of this important neuro-pathogenic virus without any scientific substantiation. Nonetheless, Belgium is clearly at risk of Tick-Borne Encephalitis (TBE) emergence and incursions from endemic zones in the neighboring countries.

This comparative review paper describes 5 Belgian veterinary serological studies with enzyme-linked immunosorbent assays and seroneutralisation tests (ELISA/SNT), in which several surveillance schemes were used (active/passive, risk-/laboratory-/range-based) in classic TBE sentinel species (dogs, cattle, roe deer, wild boar). Additionally, passive syndromic surveillance in two medical laboratories resulted in inconclusive medical data. Details are given on the scientists' experiences with available first/second line diagnostic tests and with the different surveillance methods/survey designs.

Each of the veterinary studies clearly demonstrated the presence of TBEV-specific antibodies in Belgian sentinels, sometimes even at high seroneutralisation (SNT) titers, while the medical data remain so far inconclusive, despite positive reactions of some patients in some TBEV-tests. These results have substantiated our suspicion of TBEV-presence in Belgium from 2010 onwards and have allowed sentinel comparisons based on "suitability criteria". Furthermore, the studies have highlighted the need for further veterinary validation of commercial ELISA-tests in comparison to the gold standard SNT.

Keywords: Tick-borne encephalitis (virus); Wild boar; Roe deer; Sentinel species; Seroneutralisation test

Received: December 14, 2016; **Accepted:** July 19, 2017; **Published:** July 26, 2017

Citation: Roelandt S, Suin V, Gucht SV, Stede YV, Roels S (2017) Comparative Tick-Borne Encephalitis (Virus) Surveillance in Belgium 2009-2015: Experiences with Diagnostic Tests, Sentinel Species and Surveillance Designs. J Zoonotic Dis Public Health. 1 No. 1:4

Introduction

The Western/European subtype of Ticktick-Borne Encephalitis Virus (W/Eu-TBEV) or Frühsommer Meningoenzephalitis (FSME) has been the most important, highly pathogenic and neurotropic arthropod-borne flavivirus in Europe for a long time [1-4]. This flavivirus is carried by tick vectors: Mostly by sheep ticks Ixodes ricinus [5,6] and to some extent by the ornate dog tick *Dermacentor reticulatus* [7].

Tick-Borne Encephalitis (TBE) has become a considerable public health risk in many European countries with currently

on average 3,000 hospitalized encephalitis-meningitis-myelitis cases per year, and with long-term sequelae and disability in up to 60% of patients [3-4,8]. The strong increases (1970-2000) and fluctuations (>2000) in human TBE incidence in Central and Eastern European countries and the emergence of the disease in Scandinavian countries and France (>2000), have sparked international concern and research [1,9-16].

In general, domestic and wild animals appear to be relatively less frequently infected and affected by TBEV. Although in most of the infected animal species there is viremia and seroconversion without clinical signs [17-24], TBEV can nevertheless cause general and multifocal neurological clinical signs in dogs and horses [25-31]. Additionally, domestic animals may carry infected ticks from endemic to non-endemic areas and into close vicinity of humans [17,20,27,32-36].

The zoonotic iceberg analogy, as proposed by Randolph and Sumilo [37], allows capturing the complexity of real-life TBEV epidemiology and surveillance **(Figure 1)**. It has been amply shown in several countries that the majority of human TBEV exposures do not lead to clinical signs; hence the confirmed cases represent only the very tip of the zoonotic iceberg [37,38]. When it is not overtly affecting human beings, the TBE-flavivirus remains mostly unnoticed during its enzootic cycles within its vectors and unaffected animal species [37,38]. This "bulk" of the iceberg may be much larger and may involve a large variety of domestic and wild animal species, all known to be I. ricinus hosts [20,24].

As a natural result, there can exist discordance between clinical case prevalence in humans and the prevalence of TBEV in ticks and sentinels in an endemic area [39-44]. For these eco-epidemiological reasons, it is stated by international bodies and many scientists that medical TBE case reporting alone is unreliable to characterize a geographical area or public health risk, even in regions where TBE is highly endemic [4,8,45].

Consequently, both veterinary and tick studies have clearly proven their added value in known TBE endemic areas and specifically in areas or countries with few or no human cases and/or few suspected/endemic areas, as an early warning for suspected endemic areas, such as in Norway, Denmark, Japan, the Netherlands, Luxembourg, Spain. Veterinary TBEV sentinel surveillance studies were recently reviewed [29,46,47]. Appendix A for a reproduction of the review tables in Roelandt, 2016 [47].

Despite a total lack of confirmed human TBE cases in Belgium, here are strong suspicions in the Belgian medical-scientific community that some cases may have occurred in the last decade

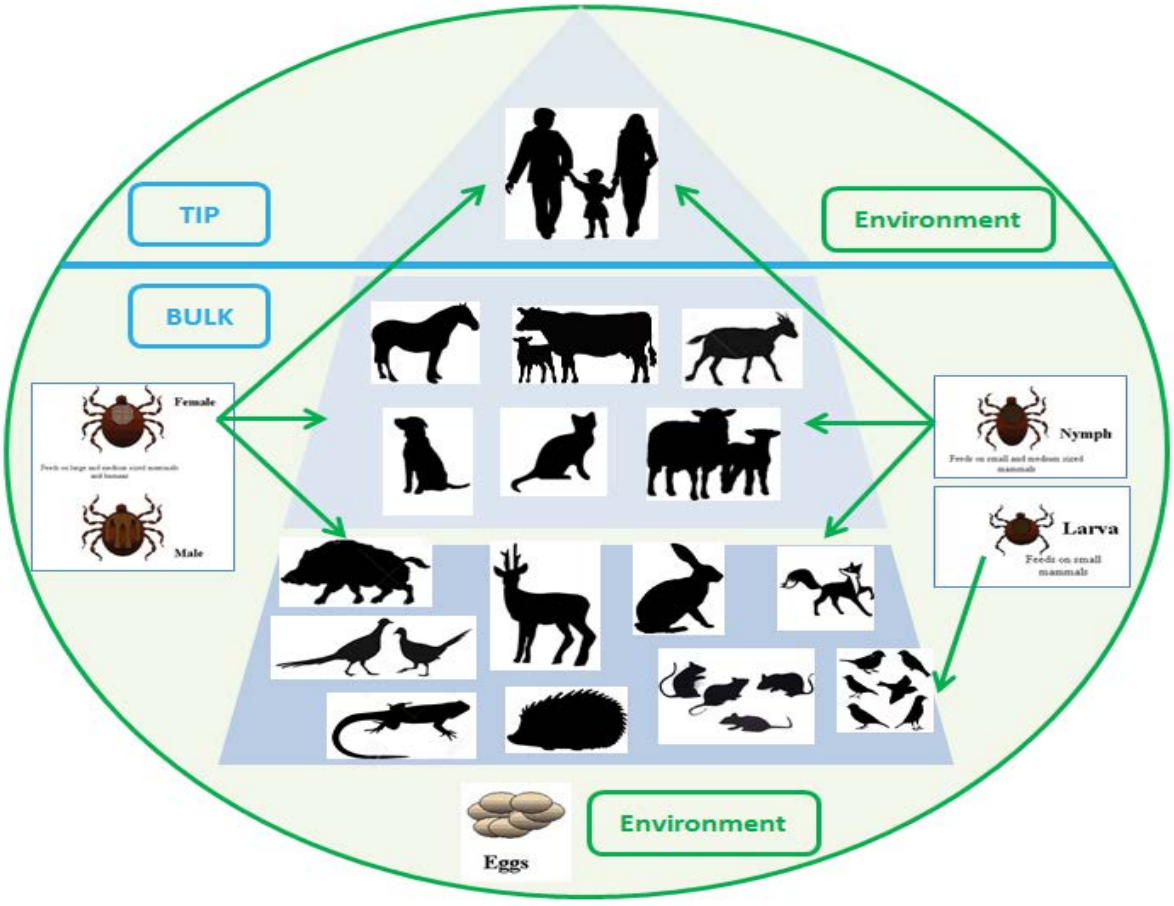

Figure 1 The Zoonotic Iceberg Analogy for TBE epidemiology created based on Randolph and Sumilo; Drelich et al. [37,48].

(pers. comms.) and favorable environmental/climatological (a) biotic conditions for Ixodid ticks and TBEV are present. Belgium is clearly at risk of TBE emergence and incursions from endemic zones in neighboring countries Germany and France [48,49].

The aim of this paper is to review the TBEV surveys performed by different Belgian research institutes in humans and in sentinel animal species between 2000 and 2016. The goal of this comparative paper is to draw some lessons from these studies to enable the design of a potential future national one health TBE(V) surveillance and to estimate the public health risk of this emerging tick-borne disease for Belgium.

Materials, Medical and Veterinary Belgian TBEV Studies*

In the following subsections, the diagnostic tests and survey designs of each of the five Belgian TBEV-studies will be briefly described. These survey characteristics are then summarized in **Table 1**. There are restrictions on the availability of raw materials or information from the studies to which the authors did not participate… *Any materials, raw data and protocols associated with each original publication should be requested from the respective contact authors.

Medical passive surveillance in Humans

During 2000-2016, no autochthonous TBE cases were reported and the virus was not considered to be endemic in Belgium [8,50-52]. In this time frame, there was only one published paper which tried to identify TBE in four human patients with a viral CNS infection of unknown etiology from Belgium. None of the four Belgian patients included in this study were confirmed as a TBE case [53]. During this whole period, passive medical surveillance was performed at only one laboratory at a time: Queen Astrid Military Hospital, i.e. QAMH (QAMH, 2000-2011), the Belgian Institute of Public Health (IPH, 2010-2015) and the Institute of Tropical Medicine, i.e. ITM (ITM, 2014-ongoing) **(Table 1)**.

The QAMH (Brussels) performed the first serological first-line screening with the Virotech® IgG/IgM ELISA (Sekisui Diagnostics®-Genzyme Virotech) in 359 suspect tick bite and neurology patients. Of these, 55 tested IgG positive (15.32%) and 19 tested IgM positive (5.29%). However, none of these results could be confirmed in SNT and patient histories are unknown.

Since 2010, the TBE National Reference Centre (NRC) at the Institute of Public Health, i.e., IPH (Brussels) has used the European case definition [8,54] and has offered serological and Polymerase Chain Reaction (PCR) screening to the medical sector as part of a diagnostic service of the referral laboratory and a limited passive surveillance system with voluntary reporting of Central Nervous System (CNS) cases 8. During 2010-2016, the Belgian TBE-NRC (WIV-ISP) has been using Progen Immunozym FSME/TBEV IgM and IgG kits to screen human patients [55,56] and the TBEV-SNT as a confirmation test [57]. SNT-results from ≥1/10 onwards are considered sufficiently protective against clinical TBE, but titers are usually much higher after full vaccination [58-60]. Additionally, comparative immunofluorescence assay IFA Biochips (Mosaic 3, Euroimmun®, Germany) [61] and qRT-PCR been available at the IPH and current best practices in TBE-diagnostics are followed.

As such, TBE tests have been performed on patients suspected of neuroborelliosis and on cases that were sent by general practitioners or hospitals based on direct TBE clinical suspicion. In 60 samples from 2009, 10 reacted in IgG ELISA (borderline or positive), one was positive in IgM-ELISA and seven reacted positive in SNT. In 2014, 53 suspected patients were tested and a total of 18 samples were IgG-ELISA-positive or -borderline, while three were SNT-positive, but none were IgM-ELISA-positive.

No samples were positive for all three tests together, and since convalescent (paired) samples were not available and the TBE/flavivirus vaccination status of the patients is unknown, the interpretation of these results remains inconclusive. So far, six imported cases of human TBE imported from Scandinavia, Austria, Kyrgyzstan and Slovenia have been confirmed by the IPH.

Currently, the ITM (Antwerp) is hosting the TBEV-NRC. None of the Belgian medical samples submitted in between 2014-16 were positive in serology (n=40) or PCR (n=25), while occasional imported travel related cases (approximately 1 per year) continue to be diagnosed. This is more or less the expected number, considering that ECDC and other data sources reported a total of only 38 travel-related cases for the whole EU for 2012.

Veterinary surveys in Belgian dogs, cattle and wild boar

In the three studies by Roelandt and others [62-64], a commercially available enzyme-linked immunosorbent assay

Table 1 Summary of sampling strategies in Belgian medical surveys. Strategy, sample sizes, selection criteria and potential TBEV-seroprevalence detection limits if TBEV is present.

Medical Surveillance	QAMH	IPH	ITM
Year Sampled	2000-2009	2009-2014	2014-2016
Sampling	Hospitals	Lyme Referral Hospital	Hospitals
Strategy	Passive (catchment area)	Passive/Enhanced (catchment area)	Passive (catchment area)
Criteria Humans	Tick bite Lyme or TBE suspected Clinical CNS	Lyme or TBE suspected Clinical CNS + Lyme tests negative	Lyme or TBE suspected Clinical CNS
Sample Size	n=359	n=113	n=40
Detection Limit 95% confidence	≥0.85%	≥2.50%	≥7.50%

(Immunozym FSME/TBE IgG All Species-ELISA®, Progen Biotechnik GmbH, Heidelberg, Germany) was used for first-line detection of TBEV-specific IgG-antibodies. This non-competitive indirect assay uses horseradish peroxidase–Protein G conjugate to detect IgG against whole TBE-virus. Using standard curves, sample concentrations were read in Vienna Units per ml (VIEU/ml). Sera with <53 VIEU/ml were negative, sera with >126 VIEU/ml were positive and those between 53 VIEU/ml and 126 VIEU/ml were classified borderline **(Table 2)**.

The seroneutralisation test TBEV-SNT is the gold standard for TBEV diagnosis [65] and was always used following the "rapid fluorescent focus inhibition test" protocol: RFFIT in microtiter plates using Vienna units per ml (VIEU/ml) [57]. SNT test panels are necessary in order to distinguish the cross-reacting flaviviral antibodies (other SNT tests), so that one can estimate true TBEV-seroprevalence in each species and can evaluate veterinary ELISA screening accuracy. The latter can be performed with accepted gold standard evaluation techniques, including two-by-two table parameters and ROC-curves [66]. As much as possible, the relevant combinations of species serum+flavivirus exposure control samples (positive/negative) were used for each test [47].

In the Belgian canine study, serum samples of Belgian dogs were obtained from three diagnostic laboratories from Northern (n=688) and Southern Belgium (n=192). Since the true diagnostic sensitivity and specificity of the ELISA kit were unknown for dogs, we lowered the cut-off by 15% compared to the kit cut-off (>53 → >45 VIEU/ml). ELISA-positive (>126 VIEU/ml), borderline (>53 VIEU/ml) and near-borderline (>45 VIEU/ml) samples were subjected to TBEV-SNT. One dog was confirmed TBEV seropositive. Several ELISA-positive and (near)borderline sera underwent seroneutralisation and hemagglutinin inhibition tests to rule out West Nile and Louping Ill viruses, but tested negative. The clinical history of the seropositive dog could not explain beyond doubt where and when TBEV infection was acquired [62].

In the Belgian cattle study a targeted, risk-based subselection (age >2 years, pasture access, Eastern provinces) of a cross-sectional sampling design was used to perform serological screening on Belgian cattle (n=650), selected from the 2010 Belgian national cattle winterscreening surveillance serum bank. All samples were subjected to the gold standard TBEV Seroneutralisation Test (SNT). Seventeen bovines were seropositive (titer>1/15) and six had borderline results (1/10<titer<1/15). The accuracy of the SNT was confirmed in a mouse inoculation test. The overall bovine TBEV-seroprevalence in the targeted area was estimated between 2.61 and 4.29%. This confirms the presence of infected foci in Belgium for the first time. Further surveillance in cattle, other sentinels, ticks and humans at risk is recommended to further determine the location and size of endemic foci and the risk for public health [63].

In the Flemish wild boar study, TBEV serological screening was performed on sera from (Sus scrofa; n=238), within the frame of a Flemish wildlife surveillance. These sera were collected in 2013 throughout the whole Flemish wild boar population range (northern Belgium) by hunters and vets. All samples were subjected to gold standard TBEV seroneutralisation (SNT). Seven wild boars were seropositive and showed moderate to high SNT-titers-three had borderline results. Seroprevalence was estimated around 4.20% (95% CI: 1.65-6.75%). Other Flaviviridae (Classical Swine Fever, West Nile Fever, Louping Ill viruses) were ruled out and thirteen available tonsils tested negative in TBEV RT-PCR [47,64].

In Walloon roe deer study [67], 498 hunted roe deer (*Capreolus capreolus*) sera were collected in Wallonia (southern Belgium) between 2007 and 2009, through an active surveillance program [68]. The animals originated from 28 forest districts uniformly distributed in the four provinces of southern Belgium (no map available). The sera were tested using the Immunozym FSME TBE IgG All Species ELISA (Progen Biotechnik) and 62 sera (12.4%) were ELISA-positive. Five highly ELISA-reactive sera were analyzed further in SNT and two displayed a significant SNT-titretiter (1:20 and 1:160). The two SNT-positive roe deer were sampled in 2008 and 2009 and originated from two different forest districts. Larger-scale screenings are being carried out by the University of Liège to evaluate the potential TBE risk areas in this region [67].

In the Flemish roe deer study [69], hunted roe deer sera were collected in Flanders (northern Belgium) between 2008 and 2013. A total of n=98 samples were examined for specific TBEV IgG antibodies using RFFIT-SNT. An antibody prevalence of 4.90% was found (95% CI: 1.61-11.70%) and the two TBEV-seropositive samples presented with relatively low titers (1/16). These

Table 2 Summary of sampling strategies in Belgian veterinary sentinel strategies. Strategy, sample sizes, selection criteria and potential TBEV-seroprevalence detection limits if TBEV is present.

Sentinel Animals	Dogs Belgium	Cattle Belgium	Wild boar Flanders	Roe Deer Wallonia	Roe Deer Flanders
Year Sampled	2008	2010	2013	2007-2009	2007-2013
Sampling	Laboratories 2 FL+1 W	Winterscreening Cross-sectional	FL range: LIM+ANT-WFL Disease Surveillance	W range: Surveillance Network	FL range: Volunteer Network
Strategy	Convenience (Catchment)	Risk ~ Lyme/TBEV 4 Provinces East BE	Convenience (Hunters–Vets)	Convenience (Hunters)	Convenience (Hunters)
Criteria Animals	None	≥2 years old (pasture access)	Representative of population and range	Shot	Shot or found dead
Sample Size	n=880 dogs from 293 communities	n=650 cattle from 44 herds/communities	n=238 wild boar from 14 communities	n=498 from 28 forest districts	n=98 from 7 hunting areas
Detection Limit 95% confidence	≥0.35%	≥0.55%	≥1.25%	≥0.60%	≥3.00%

results resembled those of other European studies and would suggest potential presence of TBEV in Flemish roe deer. It was concluded that roe deer, being omnipresent and increasing in abundance, offer good possibilities for comparative European sero-surveillance of several ruminant and zoonotic, including TBEV [69].

Results and Discussion

Sampling strategies and detection limits

Tables 1 and 2 summarize and compare the sampling strategies and detection limits to be expected from the given sample size. Passive studies, such as the medical ones in this case, often lack the sensitivity to detect rare and emerging diseases [70]. Passive and convenience active sampling are per definition opportunistic and quite cost-effective, but the results are not always representative of larger general populations, due to potential selection bias [71]. Risk-based targeted sampling equally does not allow study results to be extrapolated to non/low-risk areas but gives the worst case scenario and increases probability to detect an emerging disease early, i.e. even at very low prevalence. On the other hand, a fully randomized national sampling for an emerging disease in a very low prevalence situation (such as Belgium) may be cost-prohibitive due to unrealistically high sample sizes that would be required.

As follows from general epidemiological theory and for any sampling strategy (even convenience), a larger the sample size will result in a lower the detection limit **(Table 2)** and a higher precision in the estimates **(Table 3)**. As such, especially the current human passive surveillance (ITM: n=40) and Flemish roe deer sample (n=98) theoretically needed a relatively higher presence of >3-5% for emerging TBEV to be detectable in the respective Belgian populations. A 100% negative survey may indicate true absence or a hidden presence below the detection limit [72]. The detection limits were thus generally lower for the larger animal studies and in the face of a higher expected exposure lower on the iceberg: this is a win-win situation with more potential precision in the seroprevalence estimates.

Prevalence and freedom estimates

Prevalence and freedom calculations were performed similarly for all Belgian studies and are summarized in **Table 3**. Confidence intervals (95% Wald if n>100 or Agresti-Coul if n<100) were calculated on the results and the probabilities of freedom (Prevalence=0.00%) were calculated in EpiTools (Survey Toolbox® AusVet1.04 and http://epitools.ausvet.com.au) [73] and WinEpiscope®2.0 [74], according to the Cameron and Baldock probability formulae [73,75], with a range of design prevalences (cattle: 0.1-50%; wild boar: 0.1-10%; dogs: 0.1-1%; humans: 0.1-35%; Roe deer 0.1-15%) and with the conservative or progressive (SNT-borderline samples as negatives or positives) number of TBE-reactors when applicable. In all species, the observed number of positive reactors (IgG and/or SNT) was always too high to substantiate freedom of TBEV for the targeted population/geographical area (p-values <0.05), at the diverse retrospective design prevalences.

At first sight, the studies seem to contradict each other (TBEV Freedom: yes/no?). However, those that seem to indicate "Freedom" (Yes) seem to suffer from a low sample size and high detection limit (ITM), or from incomplete/no verification of test results with SNT. These studies are not able to distinguish between a truly free population and a population with prevalence below the detection limit. On the other hand, the IPH human study cannot be considered a real "No freedom" either, as the SNT and ELISA results was always conflicting in individual patients and relevant patient history was incomplete.

Veterinary ELISA evaluation against gold standard SNT

In Dogs, several false positive samples were observed in ELISA (~1%). It was also clear that the canine cut-off of this ELISA kit

Table 3 Available TBEV seroprevalence and freedom data in Belgium, anno 2016.

Species	Sample Size	ELISA+	SNT_{pos}%+SNT_{NI}%	Belgium Free of TBE ~SNT/ ELISA?	Original Study, Year
Human	n=359	IgG: 15.32% IgM: 5.29%	-	No, and Prev <10-15% ($P_{disease}$: <0.0001)	QAMH, pers. comm., 2015
Human	n=113	IgG: 24.78% IgM: 0.88%	8.12%+0.90% Prev: 9.02% (3.61-14.09%)	No, and Prev <8-35% ($P_{disease}$: <0.0001)	IPH, pers. comm., 2015
Human	n=40	IgG: 0.00% IgM: 0.00%	0.00%+0.00% Prev: 0.00%	Yes, or Prev <7% ($P_{disease}$: 0.0547)	ITM, pers. comm., 2016
Dog	n=880	IgG: 1.13%	0.11%+0.00% Prev: 0.11% (0.0-0.3%)	Yes, or Prev <0.55% ($P_{disease}$: 0.0842)	Roelandt et al., 2011
Roe Deer	n=498	IgG: 12.4%°	0.4%+0.00% Prev: 0.4% (0.00 -0.95%)*	Yes, or Prev <1.25-15% ($P_{disease}$: 1.000)	Linden et al., 2012
Cattle	n=650	IgG: 3.85%	2.61%+0.92% Prev: 3.45% (2.12-4.96%)	No, and Prev <3.5% ($P_{disease}$ <0.05)	Roelandt et al., 2014
Roe Deer	n=98	-	5.1%+0.00% Prev: 5.1% (2.20-11.39%)	No, and Prev <7% ($P_{disease}$: 1.000)	Tavernier et al., 2015
Wild Boar	n=238	IgG: 5.46%	2.91% (2.56 8.31%)	No: Prev ≥2.5% (P_{free} <0.001)	Roelandt et al., 2016

SNT: Sero Neutralisation Test (+: Positive result; +/-: Doubtful); ELISA: Enzyme-Linked Immunosorbent Assay; n: Sample Size; (values): Wald 95% Confidence Interval; Prev: Prevalence; *Underestimation as not all ELISA-positives were confirmed/tested in SNT [47]

needs further evaluation, especially to improve sensitivity and to avoid false negatives, since the SNT-positive sample was only borderline in ELISA, where we used a lower cut-off of 45 VIEU/ml **(Table 4)** [47].

In Cattle, the IgG protocol of the Progen ELISA® seemed to have an extremely low relative sensitivity DSe in cattle (DSe<20%), combined with a fairly reasonable relative specificity (DSp ≥90%), both compared to SNT at sensitive (1/10 DIL_{50}) or specific (1/15 DIL_{50}) serial dilution thresholds. The precision, predictive values, Cohen's kappa and Youden index also followed similar trends, indicating an overall low capacity of this test/protocol to distinguish and correctly classify TBEV seropositive and negative cattle. When inspecting the cattle ROC-curves (area under the curve: AUC=54-55%), it was felt that no great improvement could be made to this particular protocol by changing the cut-off in this species. A traditionally calculated cut-off based on the negative sample population (c=μneg+2*SDneg with μ=mean and SD=standard deviation) would still have led to a large amount of miss-classification [47].

In Wild Boar, using the manufacturer's cut-offs and an alternately positive or negative interpretation of SNT-borderline results, the IgG protocol of this ELISA showed low diagnostic sensitivity and good diagnostic specificity: DSe min-max: 40-57% and DSp min-max: 91-92%, with when SNT-borderlines were assumed to be true positives (min) or true negatives (max) respectively. ELISA agreement (kappa) with the SNT was judged only "slight to fair" (0.18-0.22). Currently, the ELISA overall discriminatory ability (area under the curve=AUC) was only 59% or 69% in wild boar at 1/15 or 1/10 SNT thresholds respectively. Additionally, receiver operating curve (ROC)-analysis showed that for early detection screening purposes with SNT follow-up, the ELISA cut-off might be placed as low as 35 Vienna-units: this would result in improved DSe (70-71%) at the cost of DSp (64.04-69.74%) [47].

In humans, the IgG All Species-ELISA® kit can theoretically be used for TBEV testing in all species, including humans. In humans, this ELISA has a reported diagnostic sensitivity of 97% and analytical specificity of 99% for IgG [76]. Considering the SNT as a gold standard, IgG was not as accurate as predicted by the firm, with a DSe of around 90% and a DSp of around 82%.

In all datasets, we should bear in mind that due to the relatively small "positive" sample sizes available, the ELISA DSe estimates are not very precise and may not even be completely accurate, by chance. Whereas in animals the (sometimes extremely) low DSe seemed to be the major problem, in humans DSp was relatively lower, indicating relatively more false positives in the IgG All Species ELISA. The flaviviral cross-reactions interfering with ELISA-DSp are well known from the literature, are also present in haemagglutination inhibition testing (HIT), and can be resolved by confirmation testing with batteries of SNT and IFAT-tests. The causes for the unsatisfactory veterinary DSe results [47], on the other hand, are largely unknown as this problem has not been described yet.

What is clear for TBEV is that veterinary ELISA's have not been validated in large published European field studies or proficiency tests except the study from Reed and Muench [77]. Manufacturer's studies remain unpublished and do not seem to have included sufficient numbers of (low/high) seropositive/negative control samples from low prevalence areas, such as the Low Countries. As a result, the kit-protocols may actually not be fit for this new particular screening purpose/area **(Table 4)** [78].

Alternatively, there may be E-protein mutations present changing the antigenicity of the local Belgian TBE-virus, which has not been found, characterized yet. The hosts (animals) may also be the cause, through short antibody longevity (months rather than years?) or through bad serum quality, or they may undergo a very/too low TBEV-exposure from a (very) low tick-prevalence environment [47].

Table 4 Diagnostic Test Accuracy of the Progen All-species IgG-ELISA. Adapted from (Roelandt, 2016) ELISA accuracy parameters as compared to TBEV Seroneutralisation Test (SNT) as gold standard test. DIL50: 50% endpoint titer cut-off: Titer causing 50% reduction in fluorescent foci [78]. DSe/DSp: Relative diagnostic sensitivity or specificity; AUC ROC: Area under the receiver operating curve; Min: With borderline SNT as true positives; Max: With borderline SNT as true negatives.

Diagnostic Test Accuracy of the Progen All-species IgG-ELISA				
Species Total Sample	SNT DIL_{50} Threshold	ELISA Parameter	Results	Sample Size Used for Calculation
Humans (n=113)	≥1/15	DSe	0.90 (0.55-1.00)	n=10
		DSp	0.82 (0.74-0.89)	n=101
Dogs n=880	≥1/5	DSe	1.00 (2.50-1.00)	n=1
		DSp	0.99 (0.98–1.00)	n=879
Cattle (n=650)	-	DSe	Min: 0.13 (0.00-0.27) Max: 0.17 (0.00-0.21)	n=18-23
	Min: ≥1/15 Max: ≥1/10	DSp	Min: 0.89 (0.87-0.92) Max: 0.97 (0.96-0.98)	n=627
	-	AUC ROC	Min: 0.5489 (0.51-0.59) Max: 0.5535 (0.51-0.59)	n=650
Wild Boar (n=238)	-	DSe	Min: 0.40 (0.12-0.74) Max: 0.57 (0.18-0.90)	n=7-10
	Min: ≥1/15 Max: ≥1/10	DSp	Min: 0.91 (0.86-0.94) Max: 0.92 (0.88-0.95)	n=228
	-	AUC ROC	Min: 0.60 (0.33-0.87) Max: 0.69 (0.37–1.00)	n=238

Confirmation testing and validation

In the human-based studies, flaviviral confirmation testing was not performed, despite the fact that–considering Spatio-temporal tick exposure, travel and vaccination history-cross-reactions are possible between Tick-Borne Encephalitis Virus (TBEV)/West Nile Virus (WNV)/Japanese Encephalitis Virus (JEV)/Yellow Fever Virus (YFV)/Dengue Viruses (DENV1-4) and very few false positives are to be expected in TBEV-SNT [61,79-81]. This is currently a major gap in the medical diagnosis of aseptic meningitis, for which up to 75% of cases remain etiologically undiagnosed, even in 2016 [51,82-85].

The veterinary studies on dogs, cattle and wild boar documented some of the very common flaviviral cross-reactions in first/second line testing. One of the dogs reacted more strongly to Louping Ill virus haemagglutination inhibition testing (LIV-HIT) than to TBEV-SNT, and some of the wild boar showed a relatively lower reaction in LIV-HIT and/or high reaction in Usutu Virus (USUV) and West Nile Virus (WNV)-SNT **(Table 5)**. In flaviviral research, cross-reactions in ELISA kits are so common that we might hypothesize that almost "any" veterinary flaviviral ELISA kit could potentially be used as a screening test for TBEV-exposure, e.g. a WNV kit in horses 21. In the veterinary studies on roe deer, cross-reactions were not ruled out [67], or no comparison was performed [69].

Despite being an absolute necessity for robust results, SNT-confirmation testing against other possibly cross-reacting flaviviruses is not always straightforward. The selected SNT for a combination of species and flavivirus is not always readily available in Belgium/Europe (e.g. LIV/USUV) or not validated for the species under study so that quality of the assays could not be guaranteed (e.g. WNV in wild boar). For the confirmation testing we preferably selected genetically related (LIV) and geographically relevant viruses (USUV, WNV, LIV).

However, our sample panels came from non-target species for each respective test, e.g. wild boar, dog and cattle versus birds, horses, or small ruminants. Hence, control sera of the correct species-flavivirus combinations were often not available. Moreover, the SNT is a delicate test and poor quality samples necessitated kaolin sample treatments for the wild boar and cattle sera besides the usual pre-heating and treatment of stable cell cultures with antibiotics.

Occasionally, we had to opt for "second choice" confirmation tests such as ELISA, HIT or IFAT [47]. Nonetheless, despite the potential cross-reactions, in medical diagnosis HIT and IFA are generally known as reasonably sensitive tests [86], that may also be specific in skilled hands through repetition and titer comparisons [87], and that may agree well with SNT [61].

In the wild boar study, we had the opportunity to test the Euroimmun flaviviral IFAT-biochips (www.euroimmun.ch) for medical differential diagnostics (TBEV/WNV/JEV/YFV/DENV1-4 [61], after recalibration with specific primary/secondary conjugates and with porcine TBEV control samples obtained from the Friedrich Loeffler Institute (FLI). This IFAT confirmed only the three strongly SNT-positive/ELISA-positive wild boar and thus currently seems to be a less sensitive test than TBEV-SNT in wild boar [47].

General Discussion

Experiences with sentinel species surveys

The Belgian medical community so far has delivered almost no evidence for TBEV-presence or TBE-cases in Belgium throughout the last 16 years, despite a considerable amount of positive (but contradicting) serology test results, and despite the correct diagnosis of several imported cases with accredited tests following best laboratory practices. So, unless passive medical surveillance is more enhanced in neurological referral centers through creating awareness in the medical community and with projects, and unless active surveillance is added in occupationally at risk groups (e.g. military, foresters), humans remain bad sentinels at the very tip of the iceberg. Medical data is certainly not sufficient to evaluate whole areas and regions for endemicity and case reporting is considered unreliable and incomplete (only the tip of the iceberg) at the best of times 4 [8,37,38,45,52].

As opposed to this, all five Belgian veterinary studies have been able to suspect (dogs) or indirectly confirm (cattle, wild boar, roe deer) TBE-viral presence in Belgium. However, which is the best species to continue Belgian TBEV sentinel surveillance? The "ideal" Sentinel species for TBEV sentinel surveillance has been described as having an adequately limited home range in comparison to TBE focus size, which is often a few m^2 up to 1 km^2. It should be available in large numbers; it should be well dispersed in the surveillance area, and should show a long-lasting detectable response after natural infection [88-90]. Additionally and importantly, one may add that TBEV (sero) prevalence should show a good spatial correlation with human TBE incidence/risk, and that sufficiently frequent tick exposure/infestation is clearly advantageous [46].

Table 5 Flaviviral confirmation testing in 3 of the veterinary studies. As compared to TBEV-SNT as gold standard. DSe/DSp: Diagnostic Sensitivity or Specificity; Min: With borderline SNT as true positives; Max: With borderline SNT as true negatives.

Sentinel Animals	Dogs Belgium	Cattle Belgium	Wild boar Flanders
Confirmation Panel	10/10 reactors from TBEV-ELISA (>53/126 VIEU/ml)+5 negatives	23/23 reactors from TBEV-SNT (1/11-1/30)+10 negatives	7/10 reactors from TBEV-SNT (1/11-1/243)+3 negatives
Confirmation Tests/Results	TBEV-SNT: 1 pos (1/5) LIV-HIT: 1 pos (1/160) WNV-SNT: All negative	Rabies-SNT: All neg. WNV-ELISA/-SNT: All negative (1 NI) Mice are protected	SNT: USUV-WNV-CSFV+ELISA: CSFV/HIT: LIV+IFA:YF-DEN-JE-WN-TBE: Most negative or lower titers+IFA confirmed TBEV-SNT++ +1 strong reaction USUV/WNV

Considering the results of the Belgian surveys, a number of additional practical and epidemiological surveillance suitability criteria may equally be taken into account to set up effective sentinel surveillance **(Table 6)**. Some of these issues may severely limit certain aspects of the study/surveillance, such as the accuracy and confirmation testing (blood volume/quality, test availability), statistical power, detection limit, precision of freedom/prevalence calculations (sample size) and the potential to proceed to modelling/mapping exercises by obtaining sufficient "case" data: e.g. numbers of tick bites, sentinel TBEV-seropositive, TBE cases or TBEV infected ticks [47].

After evaluation of these surveillance suitability criteria for the veterinary sentinels used in Belgium (Appendix B: Veterinary Sentinel Evaluation Tables), it seemed to us that [47]:

1. Dogs are most suited for passive clinical surveillance or local risk-based sero-surveillance at community scale.
2. Cattle are most suited for national randomized sero-surveillance at regional/national scale and for studying localized foodborne TBE point outbreaks.
3. Wild boar is most suited for local risk-based targeted sero-surveillance at community scale within their range of presence (Wallonia and parts of Flanders: locally representative).
4. Roe deer are suited for both national randomized sero-surveillance (range and density is sufficient throughout Belgium UTM squares) as well as for local risk-based targeted sero-surveillance at community scale.
5. None of these hosts are particularly suited for finding the actual virus: for this purpose, rodents and ticks are better study subjects.
6. For mapping and modelling exercises, wild boar, roe deer and cattle seem to be the most promising sentinels to provide enough case data in Belgium. This purpose can potentially be extended to all large tick amplifier mammals.

In the end, we did not identify one single best sentinel species, and concluded that the choice of species depends strongly on the purpose of the survey (e.g. broad screening versus in depth investigations, medical risk, and spatial area description) and on the local epidemiological situation: which hosts are present at which stage of the iceberg? The authors also believe that the local eco-epidemiological situation explains most of the current contradictions in "best sentinel discussions" (e.g. wild boar vs. roe deer) and in the available European risk factor/predictive modelling studies. Many more species have been used on occasion to study TBEV presence or seropositivity (Appendix A: Overview Sentinel Studies) and horses (clinical or active), small ruminants (sheep–goats: National, local or foodborne studies), and other wildlife species such as foxes (e.g. urban settings or Northern Europe) may be better sentinels instead of dogs, cattle and boar/deer under some circumstances.

Experiences with diagnostic tests

Despite good international proficiency test results for SNT in the medical setting, it remains an annoying finding that interpretation of IgM/IgG and gold standard SNT serology [65] did so far never provide a conclusive TBE-diagnosis, as it should have based on the data. The contradictions between the IgG-ELISA, HIT and SNT should not even occur more generally [59,76]. Additionally, a final diagnosis should not only be dependent on IgM positivity, as IgM detects only acute infections **(Figure 2)**, i.e., up to 7 weeks or at the most a few months [65] and IgM-seroconversion may not even develop in some patients [59].

Paired sera that demonstrate a 4-fold increase in IgG ELISA titers, the use of very specific TBEV-SNT (considered ±100%), and a complete travel, tick-bite, vaccination history should be sufficient to solve these interpretation problems in future medical studies. Additional SNT or IFA testing should rule out other flaviviruses if one is still not convinced.

With the veterinary ELISA accuracy results obtained so far, it seems ill advised to start using any ELISA as a veterinary screening test in a low prevalence area at the fringe of the TBEV geographic distribution, at least not before some further international validation of these tests in multiple species. In general, it is known that measures of accuracy are not fixed indicators of a test performance [66,91] and that test accuracy in the field may be influenced by many factors, including population characteristics, genetic variation in the infectious agent, the sampling, storage and test methodologies and the population prevalence [92,93].

The main reason for this is that Belgium and other low prevalence countries first have to be able to accurately map their endemic

Table 6 Table of suitability criteria for TBEV sentinel surveillance. Compiled from Belgian experiences [47] and literature [46,88-90].

Suitability Criteria for TBEV Sentinel Surveillance	
Clinical Characteristics	• Clinical cases, viraemia and/or lasting antibody response • No flaviviral vaccination or exposure to other flaviviruses than TBEV • Tick exposure, good tick host and lack of preventive actions
Correlation with Spatial Human Risk	• Useful proxy for human risk behaviour, mobility and travel • Spatial presence at national (NUTS 1), regional (NUTS 2), or local (NUTS 3/4) level • Suitable home range (km²) and representative-even distribution in the area
Epidemiological Parameters	• Knowledge of population size, density and sampling frame • Pre-existing surveillance for other pathogens: passive, active or targeted • Sample size sufficient for the surveillance purpose and design prevalence
Practical Parameters	• Organizations/Governments involved and available funding • Serum quality, volume and transport, storage • Available flaviviral diagnostic tests and control samples per spp.-virus combination

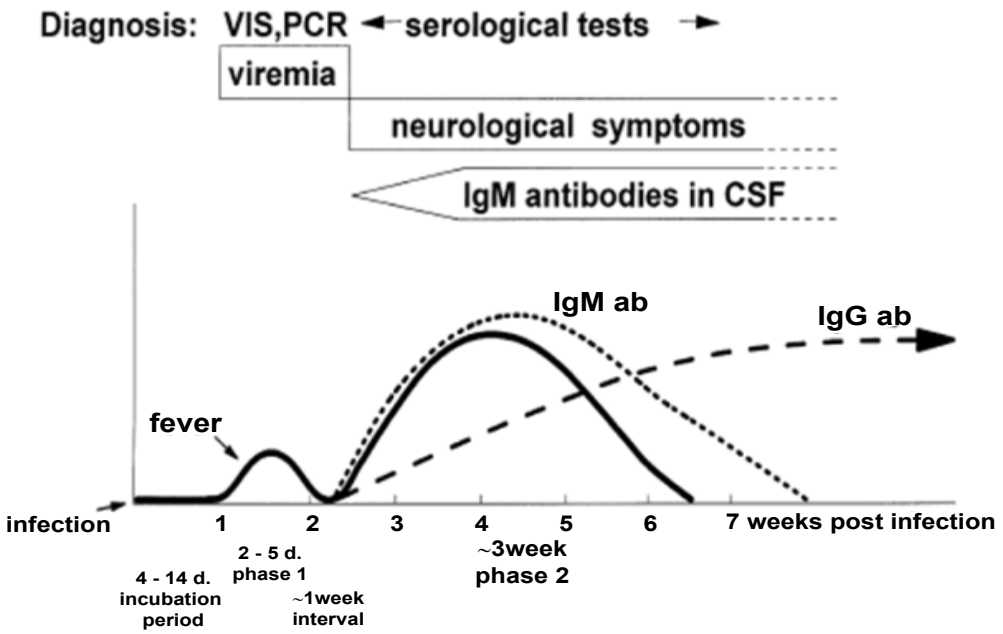

Figure 2 Suitable tests for specific TBE diagnosis. According to biphasic course of a TBEV infection with symptoms and antibody development; PCR: polymerase chain reaction; VIS: virus isolation; IgM ab-IgG ab: Immunoglobulins of class M or G.

risk areas, as opposed to just estimating a true prevalence from an apparent ELISA-prevalence to follow relative trends. For this risk assessment purpose, an unknown proportion of ELISA-false negatives and low sensitivity may constitute a major problem. Clearly, this has never been an issue in the core areas of TBE (V) endemicity where the ELISA-tests were developed, as there the seroprevalence is usually quite high in one or more species and clinical cases are much more common in these areas.

Presently, the IFA and SNT are currently the most accurate veterinary serological tests for the Belgian situation, both for surveillance or diagnostic settings. Even when testing all sera with SNT/IFA tests is not sustainable in veterinary field screening, it should currently be best practice to test all ELISA-positives and -doubtful in SNT/IFA, together with a randomly selected sufficient sample of the ELISA-negatives. In the meantime, research projects should focus on continued (re-) validation and improvement of current ELISA's e.g. sub-viral particle ELISA [94,95] and should (re-)study matrix and species effects on analytical/diagnostic sensitivity/specificity, sample preparation/storage protocols, etc. The goal would be to obtain a more accurate and better characterized screening tool applicable to low prevalence areas for surveillance, risk assessment and trend watching purposes.

Advice on future Belgian One Health TBE(V) surveillance

Medical surveillance: Belgium has always simply been "assumed" to be TBEV-free, though this was based on very little scientific evidence [53]. In 2016, despite five veterinary sentinel publications between 2010-2016, and despite multiple unexplained/inconclusive seropositive human cases, the medical world still considers TBEV as a non-endemic virus and of little importance to Belgium [96].

However, despite improvements in the diagnosis of viral encephalitis since the use of PCR on CSF to try and detect the more common viruses [83,97,98], the etiology of up to 75% of aseptic/viral encephalitis and meningitis cases remains unknown around the world, even in 2016 [51,82-85].

Moreover, TBEV does not feature in the regular diagnostic panel for locally acquired medical encephalitis unless there are very clear anamnestic indications [83,98,99] and medical surveillance has been very passive and limited. The professional awareness in regards with prevention of travel related TBE cases is only beginning to rise now. For the clinician, it is important to try to establish an etiologic diagnosis in all cases of encephalitis/meningitis, even if there are no specific effective treatments, since this may be important for the individual prognosis and counseling of patients and family members [100].

The Belgian veterinary sentinel studies [62-64,67,69] the Dutch ones [72,101] and the recent discovery of a new TBE-virus in the Netherlands on tick samples from September 2015 [101,102] as well as the very first Dutch human TBE case [103] should now prompt Belgian scientists and clinicians to reconsider this situation. Clearly, enhanced medical surveillance and increased awareness among medical professionals are now absolute priorities for the Low Countries, necessary to minimize and assess any potential TBE risk to humans from this uncharacterized strain of TBEV and to guide prophylaxis and public health decisions and measures.

Additionally, medical surveillance may lead to explanations for the apparent mismatch between the veterinary findings and the

lack of medical cases. Potential hypotheses to be explored may include: suboptimal diagnostic test quality, timing of (paired) sampling, insufficient testing by clinicians, lack of awareness, a large proportion of asymptomatic or clinically mild exposures, and presence of an atypical low-virulent TBEV virus.

TBE should be made notifiable, as in other European member states [8,52,59]. Next to a more enhanced passive component, it should include an active component to increase the detection sensitivity of the overall surveillance system [70,104] and to improve usefulness, value and cost-effectiveness of the data [105]. As suggested for veterinary surveillance, existing medical surveillance schemes and available tick, sera and risk factor datasets should be exploited.

Virological surveillance

Due to a low detection probability and the considerable resources needed to collect sufficient ticks/samples to obtain confident PCR results, scientists are often not activated to conduct such surveillance activities in low prevalence TBE areas [106]. TBEV does not often cause epidemics, though occasionally one may be confronted with a food-borne outbreak with a few dozen cases [107,108]. This flavivirus remains mostly submerged in the bulk of the iceberg. These sylvatic tick-host cycles are within largely unaffected populations of diverse animal species, where TBEV causes only short viremias and a vast majority of asymptomatic infections.

This makes it very difficult to "catch the virus in action" and the international scientific community still rarely succeeds in isolating TBEV-strains from known human/veterinary cases, or from hosts even in known highly endemic areas. Well characterized and fully sequenced TBEV isolates are scarce throughout the entire Eurasian endemic zone [109-111]. Some of the characterized TBEV strains were isolated from ticks or rodents, and rarely from a human case [4,112-115].

However, the TBE RNA-virus is capable of evolution, mutation and recombination when passaged in the lab through different hosts [110,116,117], in the field throughout the Eurasian continent [118,119], and certainly at the biogeographic edges of its distribution, where it is subjected to a number of ecological constraints [120,121]. In Europe, the closely related Louping Ill Virus (LIV), Spanish Sheep Encephalitis Virus (SSEV), Greek Goat Encephalitis (GGEV) virus and Turkish Sheep Encephalitis Virus (TSEV) [122,123] and the in 2015 characterized Spanish Goat Encephalitis Virus (SGEV) virus are present [124].

Very recently (June 2016), a Dutch-Belgian research team successfully detected TBEV-viral RNA from an unknown TBEV strain [101,102]. This was in two ticks collected in Autumn 2015, obtained in a forested area where six roe deer sera from 2010 were found seropositive. The Dutch isolates were found to cluster within the TBEV-species complex, but not within the three established TBEV subtypes (W-S-FE) nor within the LIV-cluster, implying that it concerns a novel TBEV-subtype [101].

So far, the TBE-virus has not been PCR-amplified or isolated yet in Belgium. The study on 13 wild boar tonsils (all negative) was the first published attempt [64]. Secondly, during 2014-2015, the WIV-ISP has executed a field study in rodents in some of the communities where the Belgian TBEV-seropositive cattle were found earlier [63]. Nonetheless, so far the Belgian rodents have been SNT-negative (n=0/173) and PCR-negative (n=0/308). This could be due to a number of factors, such as large rodent turnover, so they remain only seropositive for a short time, low TBEV tick-prevalence and non-viraemic transmission, or just bad luck with the location of the sites, as TBE endemic foci can be quite small. The sentinel, reservoir and tick research should continue at least until a Belgian TBEV-strain is characterized, as this strain may very well be an atypical "Low Countries" strain, as found in the Netherlands.

Bringing it together: One health epidemiology

In a globalized world with increasing numbers of emerging diseases, an interdisciplinary so-called "one health" approach is indispensable for the prevention and control of vector-borne zoonoses, such as TBE [125]. This approach leads to better preparedness and contingency planning, more effective surveillance and targeted control systems, increased health equity and improved sharing of logistics and costs [126-128].

The presently available veterinary Belgian scientific studies have allowed us to conclude that TBEV is indeed present in Belgium, despite an apparent lack of medical TBE cases and despite previous predictions against emergence. These five humble sentinel studies (often conducted with very limited resources) have at long last made Belgium join the "peloton" of TBE-endemic European countries trying to make sense out of (emerging) TBE(V) eco-epidemiology. The additional (and exciting) Dutch revelations, including the first virus and case detections in 2015-2016 [101-103,129] make us even more confident that the investigations are moving in the right direction.

The (lack of) medical data leaves us somewhat puzzled for now… However, an immediate increase in medical awareness and sustained virological research are now justified for the Low Countries (e.g. Belgium-Netherlands-Luxemburg). This research will be needed to proceed confidently into the "endemic era" by:

1. Catching and characterizing the Belgian TBEV-strain: is it the "Low Countries" strain of the Netherlands or yet something else?
2. Diagnosing and describing the nature of the first Belgian human TBE cases: have they just been missed by the medics up till now, or are they mostly benign and asymptomatic?
3. Supplying necessary data for the competent governments to perform individual and public health risk assessments: is the risk low-medium-high and is there a difference between the general population and professionally at risk groups?
4. Supply sufficient case data to allow statistical risk prediction modeling [130,131] and spatial mapping: where is TBEV (risk) emerging and what are the driving risk factors in the Low Countries?

Spatial mapping and predictive modeling are risk-based methods that have been used particularly in vector-borne diseases to identify the areas and time periods in which surveillance is more likely to successfully detect emerging health threats at an early stage. Such techniques greatly benefit governments and public health agencies to target resources, research and control measures; have been performed in many other endemic countries or multiple country meta-analysis exercises [132-193].

Conclusion

This comparative review paper described five Belgian veterinary serological studies (ELISA/SNT/IFAT/HIT) in which several surveillance schemes were used (active/passive, risk-/laboratory-/range-based) in classic TBE sentinel species (dogs, cattle, roe deer, wild boar). Additionally, passive syndromic surveillance in two medical laboratories resulted in inconclusive medical data. Details were given on the scientists' experiences with available first/second line diagnostic tests and with the different surveillance methods/survey designs.

Each of the veterinary studies clearly demonstrated the presence of TBEV-specific antibodies in Belgian sentinels, sometimes even at high SNT titers, while the medical data remain so far inconclusive, despite positive reactions of some patients in some TBEV-tests. These results have substantiated our suspicion of TBEV-presence in Belgium from 2010 onwards and have allowed sentinel comparisons based on "suitability criteria". Furthermore, the studies have highlighted the need for further veterinary validation of commercial ELISA-tests in comparison to the gold standard SNT.

We have been able to suggest an integrated future TBEV one health surveillance program for Belgium, combining several components at different levels of the TBEV zoonotic iceberg. This should lead to sufficient data collection for predictive modelling and spatial risk mapping in future studies.

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