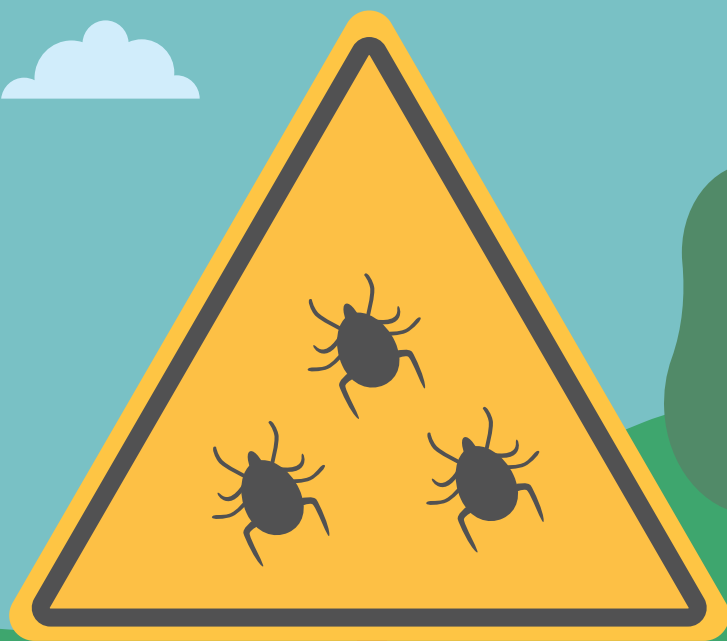




Eurosurveillance

Europe's journal on infectious disease epidemiology, prevention and control



Special edition: Tick-borne encephalitis (TBE)

December 2019

Featuring

- the detection of new endemic focus of TBE virus in England
- new TBE virus hot spot in Northern Zealand, Denmark
- EQA for molecular detection of emerging neurotropic viruses in Europe
- TBE in Europe from 2012 to 2016

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A probable case of tick-borne encephalitis (TBE) acquired in England, July 2019

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Citation style for this article:

Kreusch Teresa M, Holding Maya, Hewson Roger, Harder Thomas, Medlock Jolyon M, Hansford Kayleigh M, Dowall Stuart, Semper Amanda, Brooks Tim, Walsh Amanda, Russell Katherine, Wichmann Ole. A probable case of tick-borne encephalitis (TBE) acquired in England, July 2019. *Euro Surveill.* 2019;24(47):pii=1900679. <https://doi.org/10.2807/1560-7917.ES.2019.24.47.1900679>

Article submitted on 07 Nov 2019 / accepted on 21 Nov 2019 / published on 21 Nov 2019

The United Kingdom (UK) has thus far been considered to be free from tick-borne encephalitis (TBE), yet in July 2019, a German infant developed serologically diagnosed TBE following a tick bite in southern England. This first report of a probable human case together with recent findings of TBE virus in ticks in foci in England suggest that TBE may be acquired in parts of England and should be considered in patients with aetiologically-unexplained neurological manifestations.

End-July 2019, a case of tick-borne encephalitis (TBE) in a 3-month-old infant was notified to the German mandatory surveillance system for infectious diseases. The patient's family, resident in a TBE-non-endemic region in Germany, had holidayed in England during the incubation time. We present the case report based on German surveillance data, information provided by the family, laboratory reports and two hospital discharge summaries, and describe the public health response.

Case report

A German family including a 3-month-old infant spent their holiday in southern England from 1 to 15 July 2019 (Figure 1). The mother was not vaccinated against nor reported past TBE infection. On 6 July, the family picnicked near Woodgreen in the New Forest National Park (Figure 2), where the child laid on a blanket on the grass. An unengorged tick, attached to the infant's neck, was discovered on 7 July. The tick was removed incompletely, using tweezers, and the wound was disinfected. The remaining tick fragments detached 2 days later.

The previously healthy infant developed fever on 17 July, 11 days after the tick bite. Medical history was unremarkable; the infant had thus far received one

hexavalent routine childhood vaccination. Subtracting the maximum incubation period of 28 days [1] from symptom onset, renders 19 June as the earliest possible infection date. The infant reportedly did not visit any other location where a tick bite could have occurred except their home area in Hesse, Germany which is non-endemic for TBE. Each bout of fever was accompanied by focal seizures, lasting ca 1 min. Hospitalisation occurred on 17 July, prompting a series of diagnostic tests (Table). Based on elevated cerebrospinal fluid markers (Table), meningitis was diagnosed and the infant was treated with intravenous cefotaxime, ampicillin and aciclovir. The focal seizures became generalised lasting up to 5 min and were treated with anticonvulsants (clonazepam, midazolam, levetiracetam). The infant was transferred to a specialised hospital on 20 July. Magnetic resonance imaging and repeated electroencephalograms revealed pathological results (Table). Having excluded numerous neurotropic pathogens, TBEV-specific serology tested positive for IgM and IgG (Table) and meningoencephalitis because of TBEV infection was diagnosed by the treating physicians. The infant was discharged 15 days after admission with mild remaining neurological symptoms, which had subsided by the check-up 6 weeks later.

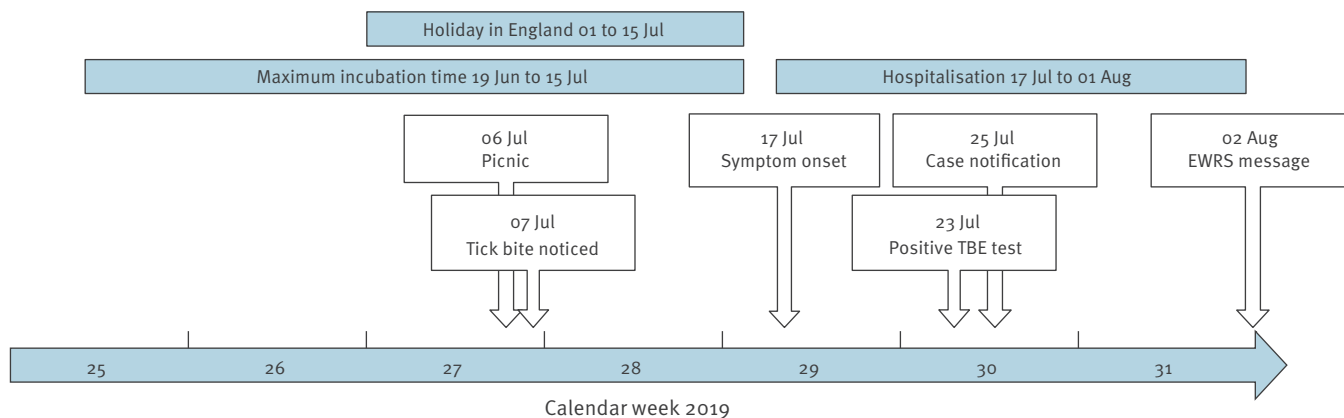
Public health response

Upon receiving the notification on 25 July, the Robert Koch Institute asked the patient's family for their detailed travel history in England. One week later, the event was reported through the European Commission Early Warning and Response System (EWRS) selective exchange to inform United Kingdom (UK) colleagues.

Following TBEV detection in ticks in Thetford Forest in 2019, from samples collected February 2018 to January 2019 [2], enhanced clinical surveillance activities were

FIGURE 1

Timeline of infection, disease progression and public health response to the probable tick-borne encephalitis case in an infant, Germany, July–August 2019



EWRS: Early Warning and Response System; TBE: tick-borne encephalitis.

FIGURE 2

Map of the likely place of infection of tick-borne encephalitis case in a German infant, Woodgreen, New Forest National Park, England, 2019



TBEV: tick-borne encephalitis virus; UA: unitary authority.

Source: Ordnance Survey and National Statistics data for geographical and administrative boundaries.

TABLE

Diagnostic tests performed on the probable case of tick-borne encephalitis (TBE) during hospitalisation, Germany, July–August 2019

Date (2019)	Test (sample type/assay)	Result	Interpretation
17–19 Jul ^a	CSF diagnostics	1,000 cells (norm: 0–5) (40% granulocytes, 60% lymphocytes) 1.5 g protein 59 mg/dL glucose level (norm: 40–80)	Inflammation
17–19 Jul ^a	Blood culture	Negative	Normal
17–19 Jul ^a	CSF culture	Negative	Normal
17–19 Jul ^a	Multiplex PCR (CSF) for: - <i>Escherichia coli</i> , - <i>Haemophilus influenzae</i> , - <i>Listeria monocytogenes</i> , - <i>Neisseria meningitidis</i> , -Streptococcus, -Cytomegalovirus, -Enterovirus, -Herpes Simplex Virus 1 and 2, -Human herpesvirus 6, -Human parechovirus, -Varicella zoster virus, -Cryptococcus	Negative	Normal
17–19 Jul ^a	Enterovirus (stool)	Negative	Normal
20 Jul	MRSA and MRGN screening	Negative	Normal
23 Jul	TBEV-IgG (serum) ^b	10.3 AE/mL	Positive (cut-off: 0.241)
23 Jul	TBEV-IgM (serum) ^b	12.5 index	Positive (cut-off: 0.234)
25 Jul	<i>Borrelia burgdorferi</i> (IgG-ELISA)	<5.2 U/mL	Negative (cut-off: <7 U/mL)
22 Jul and 25 Jul	Electroencephalography	Slowed activity in right hemisphere; Epileptic activity in right temporal/central and left occipital areas.	Pathological
01 Aug	Magnetic resonance imaging	Leptomeningeal enhancement. No sign of parenchymal defect or brain abscess.	Pathological

CSF: cerebrospinal fluid; MDRGN: multidrug resistant Gram-negative bacteria; MRSA: methicillin-resistant *Staphylococcus aureus*; TBEV: tick-borne encephalitis virus.

^a Performed during the first hospital stay. Exact test dates were not given in discharge summary.

^b The test kit used was Enzygnost Anti-TBE virus (IgG, IgM) (Siemens, Marburg, Germany) which determines a specific cut-off for each run (alpha-method).

underway in the east of England, focusing on encephalitis cases without confirmed cause [3]. Following the EWRS message, these activities were extended to areas surrounding the New Forest National Park. TBEV seroprevalence studies in groups at high risk of tick bites and in the general population are also being implemented in both areas. Tick surveillance was already underway around the New Forest National Park following previous findings [2], but additional tick surveys were conducted around Woodgreen on 8 and 23 August 2019. Only 135 ticks (70 nymphs, 25 adult males, 40 adult females) were collected, likely because the peak tick questing season had already passed. Pools of 10 nymphs, five adult males or five adult females were homogenised for RNA extraction and RT-PCR analysis [4]. No TBEV or other TBEV-serocomplex RNA was detected.

Discussion

We report a human TBE case, believed to be the first acquired in the UK. Diagnosis was by serology only, which can be regarded as a limitation. No reserve sample was available for additional testing (TBE-specific PCR or neutralization assay [5]). Because of the lack of therapeutic consequences, no follow-up blood sample was drawn from the infant; therefore it was not possible to test for a rise in IgG titre in paired samples [5]. Several pieces of evidence support the likelihood that this is a true TBE case. First, the tick bite, the clinical symptoms and the incubation time of 11 days, close to the median of 8 days [5], fit the typical picture of TBEV-infection. This patient did not have the biphasic course of TBE, which is observed in 72–87% of TBE cases [5]. Second, as the infant resides in a TBE-non-endemic area in Germany it is highly unlikely that a second tick bite occurred there within the incubation time, went unnoticed and caused the infection. The likelihood of the infection having occurred near Woodgreen is far higher given the known tick bite. Third, the extensive array of differential diagnostics ruled out numerous other neurotrophic pathogens. Fourth, the TBE serological results were far above the cut-offs. Finally, as the mother did not report any past TBE vaccination or infection, it is unlikely that maternal TBE antibody transfer occurred, and certainly not with such high titres.

In 2019, TBEV was reported for the first time in ticks in discrete foci in Thetford Forest, England [2], but the pathogenicity is unknown and no other human cases have yet been identified in the UK. Tick surveys around Woodgreen did not detect any TBEV, however, it must be noted that only a small tick sample was collected. Yet, a pool of questing ticks sampled previously, on the Hampshire/Dorset border in June 2019, tested TBEV-positive, suggesting that TBEV has established itself in the UK [6]. Follow-up tick surveys will be conducted during spring 2020.

Although the clinical presentation and serology are consistent with the European TBE case definition [7], this interpretation has to be considered in light of the

natural endemicity of Louping ill virus (LIV) in the UK. Until recently, LIV was believed to be the only virus of the TBE-serocomplex endemic in the UK [8]. Like TBEV, LIV is also transmitted by *Ixodes ricinus* ticks and mainly occurs in sheep, cattle and red grouse in upland grazing areas of the British Isles [8]. LIV infects humans in rare cases and cross-reacts with TBEV serologically. In the absence of an isolate or sequence data from acute phase samples, the exact aetiology in the case presented here remains uncertain. However, LIV is most prevalent in upland areas, which are located mostly in the north and west of the UK, and less than 50 human clinical LIV cases have been reported since 1934 [8], with one in England reported as recently as 2011 [9]. The likelihood of LIV thus is low in our case and we believe that it is a true TBEV-infection.

This first probable human TBEV-infection in England and the detection of TBEV in ticks stand in accordance with the patchy spread of TBEV to new areas observed in parts of Europe. In Germany, the number of TBE-endemic districts increased from 129 in 2007 to 161 in 2019 [10]. The first TBE cases from the Netherlands were reported in 2016 [11,12]; and a new focus was recently discovered in Denmark following three human TBE cases in summer 2019 [13]. TBEV can spread to new areas through mammalian hosts or migratory birds infested with TBEV-carrying ticks [14]. This may either lead to sporadic infections, or sometimes to the establishment of new foci, if local climatic conditions are favourable to the transmission cycles between ticks and their rodent hosts [5].

In England, the public health authorities currently assess the risk of TBEV infection as very low for the general population and low for those who may be bitten by ticks in areas where infected ticks are located [15]. Seroprevalence studies in groups at high risk of tick bites and in the general population, tick sampling and enhanced surveillance of human encephalitis cases without confirmed cause are underway to better understand the human infection risk in areas where TBEV was detected in ticks or wildlife. Public Health England continues to promote tick awareness for those spending time outdoors. The public health risks from TBEV in England will be dynamically reviewed as new findings come to light.

Acknowledgements

We are grateful to Achim Brumm at the local health authority in Hesse, Germany, for his support in communicating with the family and in collecting extended surveillance data including hospital discharge summaries. Tick collection was supported by Public Health England's Medical Entomology group. Maya Holding, Roger Hewson, Stuart Dowall, Jolyon Medlock, Tim Brooks and Amanda Semper are affiliated to the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Emerging and Zoonotic Infections at University of Liverpool in partnership with Public Health England (PHE), in collaboration with Liverpool School of Tropical Medicine. Maya Holding, Roger Hewson, Stuart Dowall, Jolyon Medlock, Tim Brooks and Amanda

Semper are based at Public Health England. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health and Social Care or Public Health England.

Conflict of interest

None declared.

Authors' contributions

Teresa Kreuzsch, Thomas Harder and Ole Wichmann were involved in the public health response in Germany, collated and reviewed the relevant data from the case, and wrote the first draft. Tim Brooks, Amanda Semper, Amanda Walsh, and Katherine Russell were involved in the public health response in the UK. Maya Holding, Roger Hewson, Stuart Dowall, Kayleigh Hansford and Jolyon Medlock were involved in the tick collection and testing near Woodgreen. All authors discussed the article's content and approved of the final version.

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Detection of new endemic focus of tick-borne encephalitis virus (TBEV), Hampshire/Dorset border, England, September 2019

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Citation style for this article:

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Article submitted on 30 Oct 2019 / accepted on 21 Nov 2019 / published on 21 Nov 2019

The presence of tick-borne encephalitis virus (TBEV) was detected in a questing tick pool in southern England in September 2019. Hitherto, TBEV had only been detected in a limited area in eastern England. This southern English viral genome sequence is distinct from TBEV-UK, being most similar to TBEV-NL. The new location of TBEV presence highlights that the diagnosis of tick-borne encephalitis should be considered in encephalitic patients in areas of the United Kingdom outside eastern England.

The geographical spread of tick-borne encephalitis virus (TBEV) is expanding in Europe [1]. In the Netherlands, the first human cases of TBE were recorded in 2016 [1]. In the UK, TBEV was detected in ticks removed from deer in the Thetford Forest area of East Anglia in eastern England in May 2019 [2,3]. Here we report findings of further investigations in Hampshire and its bordering areas in southern England.

Detection of tick-borne encephalitis virus using deer as sentinels

TBEV is a member of the flavivirus family, causing tick-borne encephalitis (TBE), a neurologic encephalitic disease of humans. Five subtypes of TBEV are known: European (TBEV-Eu), Far Eastern (TBEV-FE), Siberian (TBEV-Sib), Baikalian (TBEV-Blk) and Himalayan (TBEV-Him) [4]. *Ixodes ricinus* is the main tick vector of TBEV-Eu, the predominant subtype in western Europe [5]. Louping ill virus (LIV), vectored by the same tick species, is a member of the TBEV serocomplex that is endemic in areas of the UK where it causes disease in sheep, and on rare occasions, also in humans [6]. The close genetic homology between LIV and TBEV results

in cross-reactivity in standard serological assays, therefore the detection of viral nucleic acid is necessary to differentiate between the two viruses.

Between February 2018 and January 2019, 1,309 deer serum samples were collected from culled deer in England and Scotland as part of a research study; 4% of samples were ELISA-positive for the TBEV serocomplex [2]. Our seroprevalence data highlighted two key geographic areas of interest (Figure 1) that showed evidence of flavivirus seropositivity in deer. Notably, these areas, Thetford Forest on the Norfolk/Suffolk border in eastern England and Hampshire in southern England, have not reported LIV in livestock [7,8]. This raised suspicion that another flavivirus may be present and follow-up investigations were conducted.

Questing tick sampling

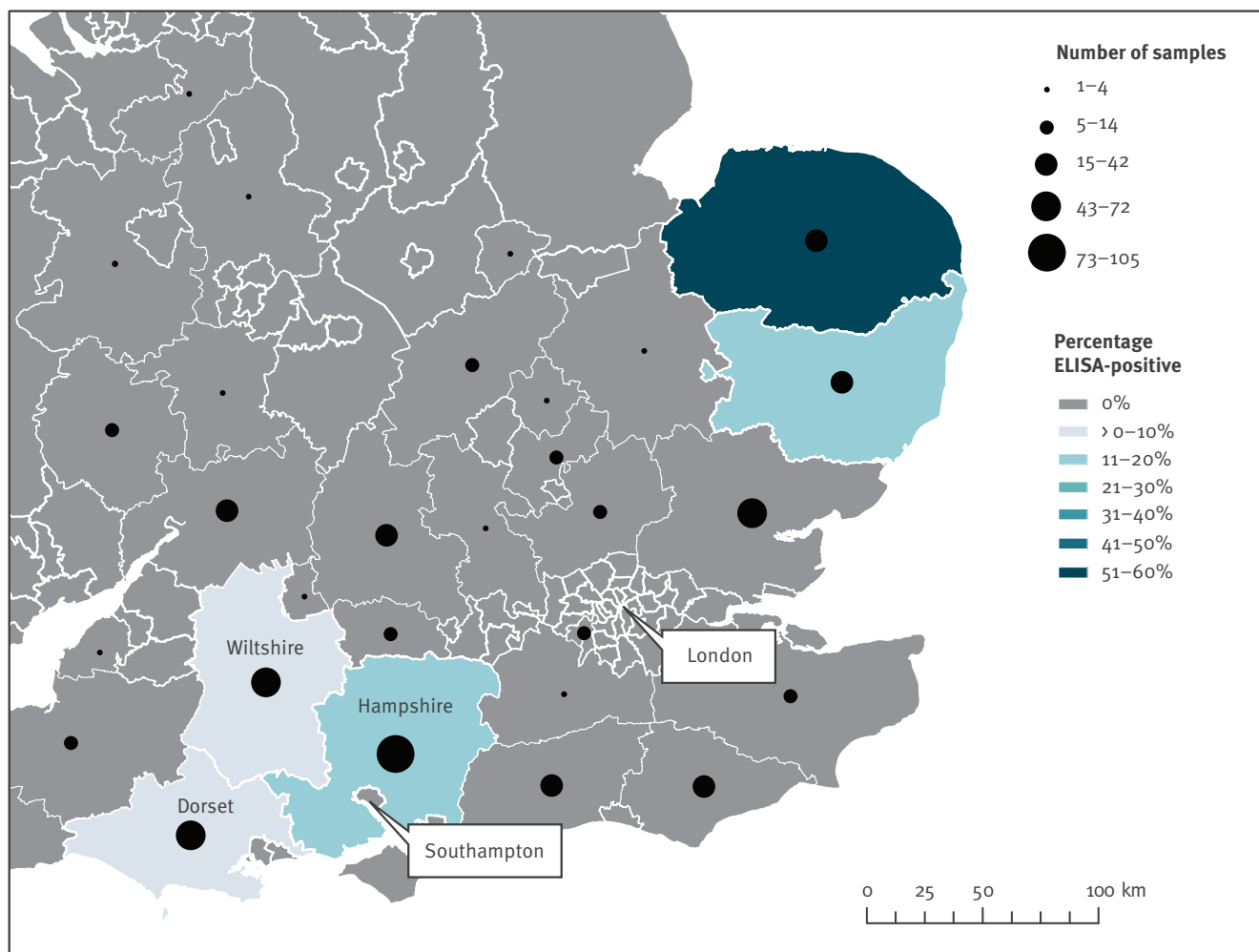
Questing tick surveys were conducted at four sites during July and August 2018 (Table): (i) one on the Hampshire/Dorset border (site 1A) (ii) two in Hampshire (sites 2 and 3), and (iii) one on the Hampshire/Wiltshire border (site 4). The four sites were selected as areas where at least one seropositive deer was previously identified. Additional sampling was conducted on site 1 during June 2019 because this location had the highest concentration of seropositive deer (50%) within Hampshire and its bordering counties in the previous year. Three localities were surveyed at site 1 (1A, 1B and 1C), where 915 ticks were collected and tested during 2018 and 2,155 in 2019.

Detection of viral RNA

During September 2019, after all tick samples had been collected, ticks were morphologically identified

FIGURE 1

Number of deer samples tested for exposure to tick-borne encephalitis virus serocomplex^a and relative percentage of positives, eastern, southern and central England, February 2018–January 2019



TBEV: tick-borne encephalitis virus.

Source: Ordnance Survey and National Statistics data for geographical and administrative boundaries. Adapted from version found in [2].

^a Commercial TBEV ELISA was used to determine if samples were positive for antibodies to TBEV serocomplex [2].

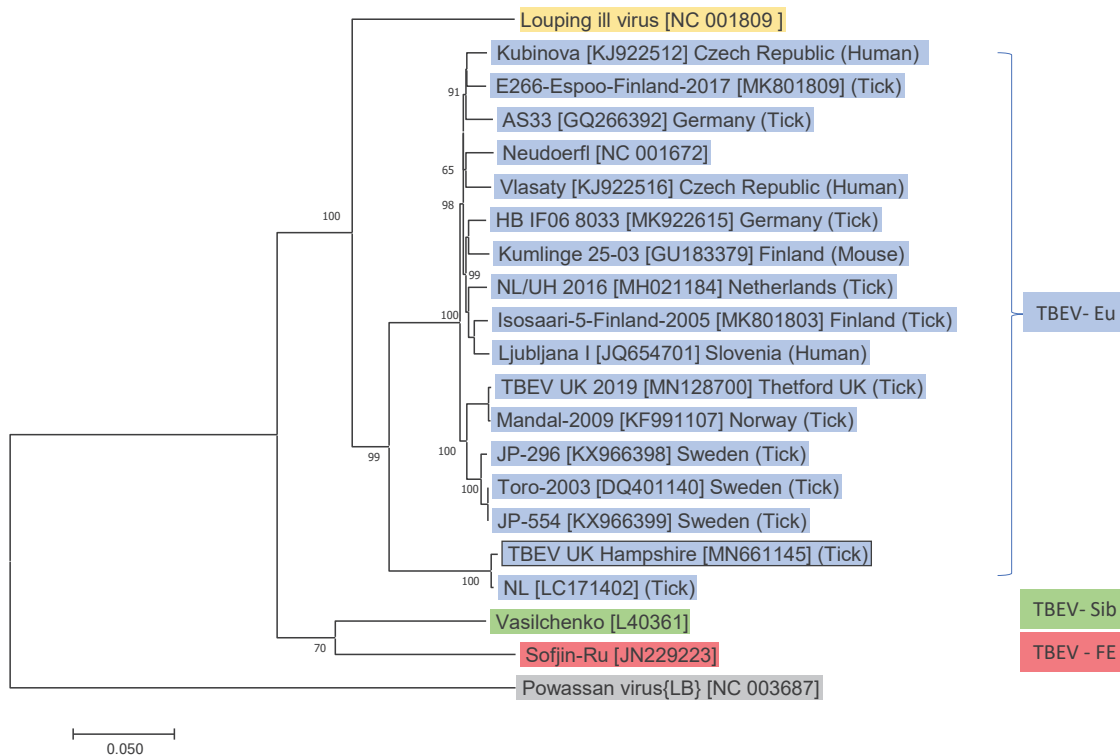
TABLE

Number of questing ticks tested by site, Hampshire and its borders, England, United Kingdom, 2018 and 2019

Month and year	Site	Area	Nymphs (n)	Adult males (n)	Adult females (n)	Total ticks (n)
July and August 2018	1A	Hampshire/Dorset border	420	25	35	480
	2	Hampshire	160	10	30	200
	3	Hampshire	100	15	20	135
	4	Hampshire/Wiltshire border	90	5	5	100
June 2019	1A	Hampshire/Dorset border	870	100	110	1,080
	1B	Hampshire/Dorset border	430	65	80	575
	1C	Hampshire/Dorset border	340	75	85	500

FIGURE 2

Phylogenetic relationship of contemporary strains of tick-borne encephalitis virus (TBEV) and TBEV-UK Hampshire, England, United Kingdom, 2019



The box highlights the ticks collected in Hampshire in 2019. The tree was constructed with a maximum-likelihood analysis of full length genomes and is rooted with the tick-borne Powassan virus. European TBEV strains are highlighted in blue, Siberian TBEV in green, Far Eastern TBEV in pink, and Louping ill virus in yellow. Strains are identified with the name, GenBank accession numbers, country location and host.

as *Ixodes ricinus* [9] and grouped into pools of 10 nymphs or 5 adult males or 5 adult females. Pooled ticks were homogenised in 300 µl buffer RLT in MK28-R Precellys tissue homogenising tubes using a Precellys 24 homogeniser (Bertin, Montigny-le-Bretonneux, France) [2]. Samples were then passed through a QIAshredder (Qiagen, Hilden, Germany) and extracted using the BioSprint 96 One-For-All Vet Kit (Qiagen) [2]. All tick pools were tested with the LIV/TBEV real-time RT-PCR assay developed by Schwaiger and Cassinotti [10]. RNA was amplified in 20 µL real-time RT-PCR mix containing 0.8 µL Invitrogen SuperScript III with Platinum Taq Mix (ThermoFisher, Waltham, United States), 10 µL Invitrogen 2X Reaction Mix, 1.6 µL of 50 mM MgSO₄, 1 µL of 1 µM forward primer (F-TBE 1), 1 µL of 18 µM reverse primer (R-TBE 1), 0.2 µL of 25 µM probe (TBE-Probe WT), 5 µL template and 0.4 µL molecular-grade water. One positive pool of a total of 373 pools tested, was detected in an adult female group (Ct 16.12), collected from site 1B on the Hampshire/Dorset border. The minimum infection rate of ticks infected with TBEV in site 1B was estimated as 0.17% [11].

Genome sequencing and phylogenetic analysis

The one pool positive for TBEV RNA was sequenced metagenomically using the Oxford Nanopore GridION [12] and the complete TBEV coding sequence was obtained: TBEV-UK Hampshire, GenBank accession number MN661145. Data was compiled with a range of other published TBEV genomes circulating in Europe, together with reference genomes from other TBEV subtypes to infer the evolutionary history. Figure 2 shows this phylogenetic relationship and indicates that TBEV-UK Hampshire is most closely related to TBEV-NL (LC171402.1), a strain of TBEV detected in ticks in the Netherlands in 2017 [3]. When compared with the TBEV-NL strain, TBEV-UK2 Hampshire contains 49 single nt polymorphisms leading to 12 amino acid substitutions within the coding sequence.

Discussion and conclusion

Our findings indicate that TBEV prevalence in ticks is not limited to the Thetford Forest area in eastern England, but also includes the Hampshire/Dorset

border in southern England. The viral genome sequence obtained from ticks in southern England is most similar to a virus identified in 2017 in the Netherlands [3] and is distinct from the TBEV-UK discovered in the Thetford Forest area in May 2019 [2]. The identification of two distinct TBEV-Eu genomes in the UK provides compelling evidence of two separate importation events into the UK. Birds such as thrushes transport large numbers of ticks over great distances during autumn migration, when many travel to the UK from TBEV-endemic areas in northern and western Europe, including the Netherlands [13,14]. Factoring bird migration routes, the locality of the TBEV-UK Hampshire genome detection in southern England and its close homology to the Netherlands genome suggests that importation of TBEV-UK Hampshire to the UK may have occurred through the transport of infected ticks carried on migratory birds.

Additionally, the presence of TBEV in questing ticks indicates an established enzootic cycle involving ticks and other wildlife hosts, supporting the hypothesis that TBEV is established in the UK and is being maintained in enzootic cycles.

The estimated prevalence of 0.17% in this identified focus is relatively low when compared with some other reports from mainland Europe [15]. As TBEV foci comprise of defined boundaries, a possible explanation could be that the centre of this focus was not detected on this sampling occasion [16]. Follow-up investigations will be conducted to identify the exact location and boundaries of the endemic focus.

The risk of TBEV to the general population in the UK is currently assessed to be very low [17], and there have been no autochthonous confirmed cases of TBE in the UK to date. However, a probable case diagnosed through serology alone has been traced back to a tick bite received at a location in Hampshire close to where the TBEV-positive tick pool was collected [18]. These data reinforce the need to consider TBEV infection as a potential diagnosis in encephalitis patients, particularly those with history of tick bite. However, confirmation of TBE in the UK is complicated by the circulation of LIV, which is cross-reactive in standard serological tests. Further work is required to identify risk areas in the UK where climatic and ecological conditions may support the maintenance of TBEV.

Acknowledgements

This research was funded by Public Health England and by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Emerging and Zoonotic Infections at University of Liverpool in partnership with Public Health England (PHE), in collaboration with Liverpool School of Tropical Medicine. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, the Department of Health or Public Health England.

Conflict of interest

None declared.

Authors' contributions

RH, JMM and MB conceived and developed the study. MH and RV liaised with UK deer management organisations. MH, LM and KMH conducted field work. MH, MCF, JC and SDD carried out tick testing. DPC, STP and RH conducted the genome sequencing and analysis. MH, RH and SDD wrote the first draft of this article. All authors have contributed to editing versions of the manuscript and approved the final version before submission.

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New tick-borne encephalitis virus hot spot in Northern Zealand, Denmark, October 2019

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Citation style for this article:

Agergaard Charlotte N, Rosenstjerne Maiken W, Bødker René, Rasmussen Morten, Andersen Peter H S, Fomsgaard Anders. New tick-borne encephalitis virus hot spot in Northern Zealand, Denmark, October 2019. *Euro Surveill.* 2019;24(43):pii=1900639. <https://doi.org/10.2807/1560-7917.ES.2019.24.43.1900639>

Article submitted on 17 Oct 2019 / accepted on 24 Oct 2019 / published on 24 Oct 2019

During summer 2019, three patients residing by Tisvilde Hegn, Denmark were hospitalised with tick-borne encephalitis (TBE) after tick bites. A new TBE virus (TBEV) micro-focus was identified in tick nymphs collected around a playground in Tisvilde Hegn forest. Estimated TBEV prevalence was 8%, higher than in endemic areas around Europe. Whole genome sequencing showed clustering to a TBEV strain from Norway. This is the second time TBEV is found in *Ixodes ricinus* outside Bornholm, Denmark.

Tick-borne encephalitis virus (TBEV), a member of the family *Flaviviridae*, genus flavivirus, causes tick-borne encephalitis (TBE). In Denmark, TBE is endemic only on the island Bornholm, with an incidence of 4 per 100,000 inhabitants per year [1,2]. Here we report three clinical cases of TBE in patients hospitalised within a month and all residing at the boundary of the same forest, Tisvilde Hegn, in Northern Zealand.

Clinical cases and virology analysis

Case 1

Early July 2019, a man in his late 50s, was hospitalised with meningoencephalitis. He lives in a house ca 2.2 km from the Tisvilde Hegn forest border where he sometimes walks, and noticed a tick bite perhaps from his own garden. He developed typical two-phased disease, with 5 days of fever and gastrointestinal symptoms followed by 2 days of recovery, before developing meningoencephalitis. Serum and cerebrospinal fluid (CSF) samples were analysed at Statens Serum Institute, Copenhagen, Denmark. Serum samples from the day of hospitalisation were positive for anti-TBEV IgM and IgG (Enzygnost ELISA, Siemens, Erlangen, Germany) [3]. CSF showed elevated leukocyte count ($48 \times 10^9/L$; norm: 0 cells/L), increased protein (0.9 g/L, norm: 0.15–0.50) and was positive for anti-TBEV IgM and IgG (Table 1). It was negative in RT-qPCRs for TBEV and flavivirus.

Case 2

Late June 2019, a man in his late 60s developed fever, influenza-like symptoms and increasing fatigue. The patient lives in a house with a garden bordering the same forest as Case 1. He uses the forest recreationally and experiences daily tick bites. About 4 weeks later, at the end of July, he was hospitalised with symptoms of meningitis in terms of nausea, vomiting, headache, photophobia, and pain from the neck and the back. CSF was analysed at Statens Serum Institute, Copenhagen, Denmark and showed pleocytosis (mononuclear leukocytes of $70 \times 10^9/L$; norm: 0 cells/L), elevated protein level (1.46 g/L, norm: 0.15–0.50) and positive anti-TBEV IgM and IgG titres, and negative in RT-qPCRs for flavivirus and TBEV. Serum samples were positive for anti-TBEV IgM and IgG (Table 1).

Case 3

Late June 2019, a woman in her 30s was hospitalised with meningoencephalitis manifesting as headache, nausea, fatigue and photophobia. She presented with fever and dehydration, and was in a poor general condition. Blood samples at admission had a low platelet and leukocyte count, and liver parameters were elevated. Ten days earlier, at her summer cottage 3 km from the same forest, she spent time at a playground in the eastern part of the forest, where she noticed a tick bite on her thigh. About 5 days later, she developed back pain and fever, followed by influenza-like symptoms, loose stools and increasing fatigue. She had a few days of recovery, before hospitalisation for a week with clinical signs of meningoencephalitis. Unfortunately, no lumbar puncture or tests for TBE were initially performed. About 1 month later, in August 2019, she still suffered from fatigue and quick excitability and was seen at a hospital outpatient clinic. The patient asked to be tested for TBE and serology was strongly positive for anti-TBEV IgM and IgG. A serum sample 1 month later was still IgM and IgG positive (Table 1).

TABLE 1

TBEV-specific IgG and IgM antibody detection in tick-borne encephalitis cases by time of sampling, Northern Zealand, Denmark, 2019 (n = 3)

Case	Material	Day of sampling ^a	TBEV IgG (U/ml) ^b	TBEV IgM (Index) ^c
1	Serum	0	28.4	10.0
	CSF	5	29.1	3.2
	Serum	10	22.8	8.2
2	CSF	0	43.7	1.1
	Serum	0	106.8	6.9
	Serum	2	118.5	6.6
	Serum	10	73.5	5.8
3	Serum	42	118.5	3.2
	Serum	74	154.0	1.8

CSF: cerebrospinal fluid; TBEV: tick-borne encephalitis virus.

^a Day 0 = day of hospital admission.

^b The IgG antibody concentrations (U/ml) were interpreted according to the manufacturer's instructions (range 7–700 U/ml).

^c The specific IgM antibody concentrations are presented as indices according to manufacturer's instructions (index 1.0–1.4 weakly positive and index >1.4 positive).

Field sampling and whole genome sequencing of tick-borne encephalitis virus

Ticks were collected by flagging, i.e. dragging of a 1x1 m white cloth through the grass, at Tisvilde Hegn in September and October 2019. The initial flagging took place at five different neighbouring sites, site 1–5, in a part of the forest bordering the forest playground on the eastern side where Case 3 received a tick bite (Figure 1).

As of 26 September, a total of 725 ticks were collected, 626 nymphs and 99 adults, and divided into 24 pools (Table 2).

RNA was extracted using MagNA Pure Large Volume kit on a MagNA Pure 96 instrument (Roche Diagnostics, Risch-Rotkreuz, Switzerland), and a TBEV-specific RT-qPCR [4,5] was run in a quality-controlled routine diagnostic reference laboratory. TBEV prevalence in individual ticks were estimated from the pooled samples using an online calculator for variable pool sizes while assuming a perfect diagnostic test [6].

Three pools containing nymphs from site 3, bordering the playground, were all positive and of these, two pools were strongly positive (ct values: 17 and 20). Furthermore, one pool containing nymphs from site 5, ca 50–100 m from the playground, was also positive (ct value: 35) (Table 2).

To further localise the TBEV micro-focus, the areas directly bordering the forest playground were divided into three smaller subsites and flagged once more in October 2019 (Figure 1). A total of 368 ticks, 348 nymphs and 20 adults, were collected and divided into 41 pools (Table 2). Four of 11 pools from site 3A, two

of 19 pools from site 3B and all five pools from site 4A contained nymphs positive for TBEV. No TBEV was found in pools of adult ticks. Sites 3A and 4A, the two sites forming a 20 m wide belt along the eastern side of the playground were strongly positive (ct values: 15 and 18), as compared with the more distant site 3B. The joint prevalence of TBEV in sites 3A and 4A was estimated to 8% (95% confidence interval (CI): 4–14%) (Table 2).

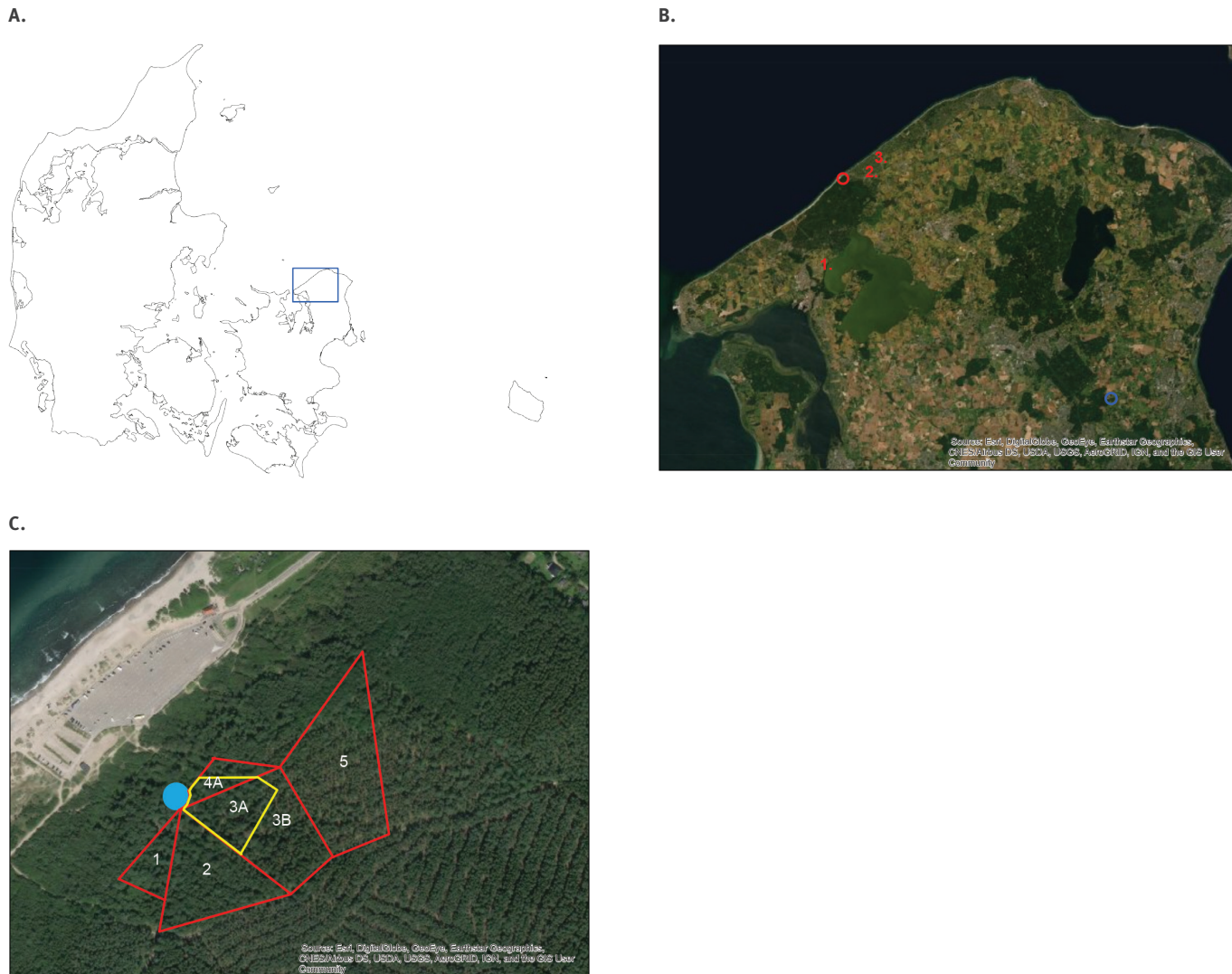
Metagenomic whole genome sequencing of nine of the positive tick pools were performed using the Nextera XT DNA Library Prep Kit (Illumina Inc., San Diego, United States) and the Illumina MiSeq platform. For sequence comparison, the TBEV PCR-positive tick pool from Tokkekøb Hegn in 2009 was also full-genome sequenced and included in the analysis. Four complete whole genome sequences, three from Tisvilde Hegn and one from Tokkekøb Hegn, with an average coverage >100x was obtained. Phylogenetic and molecular evolutionary analyses using MEGA X [7] of the full-length genome sequences from Tisvilde Hegn showed that all three were identical and grouped closely with a TBEV strain from Mandal, Norway (Figure 2). In contrast, the TBEV sequence from Tokkekøb Hegn grouped with TBEV strains from Sweden (Figure 2). The sequences have been deposited in GenBank.

Discussion

The incidence of TBE has been increasing in Denmark, in its neighbouring countries as well and in the rest of Europe in recent years, which mirrors the increased abundance of ticks, the increased geographic spread and potentially climate changes [8–11]. The vector for the European virus subtype, TBEV-Eu, is *Ixodes ricinus*, which is prevalent in most of Europe and the dominant tick species in Denmark (>90%) [12]. In 2009, two

FIGURE 1

Map of Denmark, Northern Zealand and Tisvilde Hegn, 2019



A. shows a map of Denmark and B. shows a map of Northern Zealand with the new tick-borne encephalitis (TBE) micro-focus in Tisvilde Hegn (red ring). It also shows a previous TBE micro-focus at Tokkekøb Hegn (blue ring) and the residence of the three human cases (in red). C. illustrates the tick flagging sites, the forest playground, marked with a blue dot, and the five initial flagging areas, sites 1–5. Areas 3 and 4 were divided into two new areas, of which 3A, 3B and 4A were flagged separately. Map source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, AeroGRID, IGN, and the GIS User Community.

TABLE 2

TBEV-specific RT-qPCR and estimated virus prevalence, Northern Zealand, Denmark, 2019

Sample site	Sampling date (2019)	Tick stage	Number of ticks	Number of pools	Pool size (Number of ticks)	TBEV RT-qPCR	RT-qPCR (ct)	Estimated prevalence (%) (CI 95%)
1	19 September	Nymphs	124	3	40, 41, 43	0/3	No ct	All negative
		Adults	26	2	18 ^a , 8 ^b	0/2	No ct	
2	19 September	Nymphs	91	2	45, 46	0/2	No ct	All negative
		Adults	15	2	4 ^a , 11 ^b	0/2	No ct	
3	26 September	Nymphs	216	5	40, 41, 44, 45, 46	3/5	17, 20, 35	2.0
		Adults	33	2	18 ^a , 15 ^b	0/2	No ct	(1.0–6.0)
4	26 September	Nymphs	23	1	23	0/1	No ct	All negative
		Adults	2	1	1 ^a	0/1	No ct	
5	26 September	Nymphs	172	4	41, 41, 43, 47	1/4	35	1.0
		Adults	23	2	11 ^a , 12 ^b	0/2	No ct	(0.0–29.0)
3A	4 October	Nymphs	112	11	10 ^c , 12	4/11	16, 16, 31, 34	4.0
		Adults	4	2	1 ^a , 3 ^b	0/2	No ct	(1.0–10.0)
3B	4 October	Nymphs	192	19	10 ^c , 12	2/19	34, 39	1.0
		Adults	15	3	4 ^a , 5 ^b , 6 ^b	0/3	No ct	(0.0–3.0)
4A	4 October	Nymphs	44	5	10 ^c , 4	5/5	15, 18, 31, 32, 35	All positive
		Adults	1	1	1 ^a	0/1	No ct	
3A and 4A jointly	4 October	Nymphs	156	16	10 ^c , 12, 4	9/16	NA	8.0 (4.0–14.0)

CI: confidence interval; ct: cycle threshold; NA: not applicable; TBEV: tick-borne encephalitis virus.

^a Adult female ticks.

^b Adult male ticks.

^c All pools contained 10 nymphs unless otherwise indicated.

clinical cases of TBE were reported outside Bornholm and TBEV was detected in Northern Zealand in ticks collected in the forest of Tokkekøb Hegn, which is 40 km south-east of Tisvilde Hegn, in 2009, 2010 and 2011 [4,5]. Surprisingly, TBEV was no longer detected in the same area in Tokkekøb Hegn during 2016 and 2017 [13]. In 2018, another two human cases of TBE outside Bornholm were identified on the Island of Funen and in Jutland, respectively, but no new micro foci of TBEV has been localized [14], (data not shown).

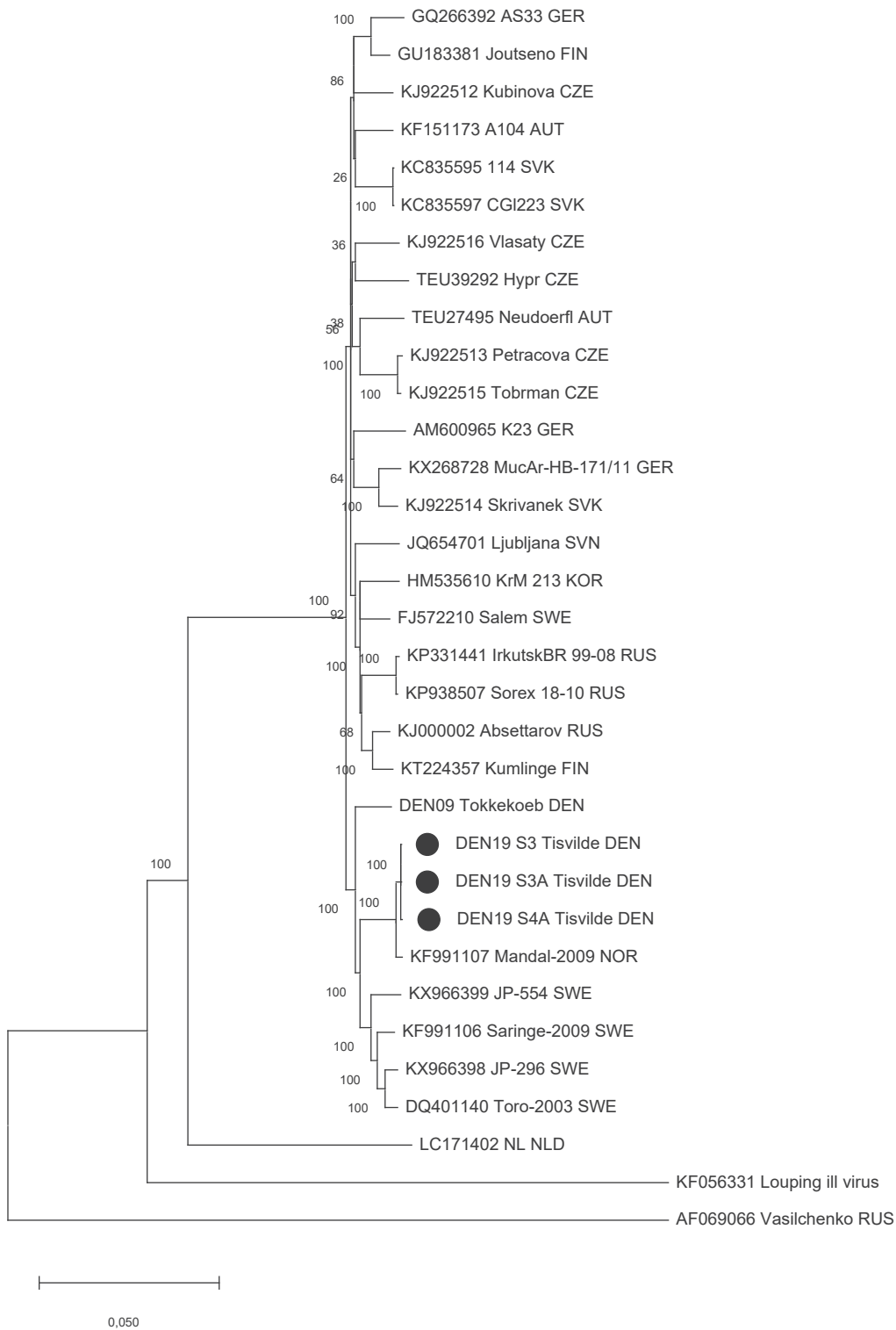
All three patients presented here live close to Tisvilde Hegn in Northern Zealand, and had typical biphasic disease starting with fever, gastro-intestinal or influenza-like symptoms and fatigue, followed by a few days of recovery before clinical meningitis/meningoencephalitis at hospitalisation and neurologic sequelae in terms of primarily fatigue and dizziness.

Subsequent collection of *I. ricinus* ticks from a part of Tisvilde Hegn surrounding a well-visited forest playground, where Case 3 recalled a tick bite, identified a specific area adjacent to the playground to be an acute, new, high-risk TBEV micro-focus in Northern

Zealand. The estimated high prevalence of TBEV is 8% at the centre of the focus which exceeds recent prevalence estimates of 0.6% from endemic Bornholm, as well as Denmark's neighbouring countries and most European countries [4,5,8,10,11,13,15]. The presence of the virus in nymphs, but not adult ticks, and the molecular evolutionary analyses of the homogeneous TBEV sequences suggests a single TBEV introduction in 2019, probably by migrating birds from Norway. Tisvilde Hegn and the forest playground is well-visited by Danish and international tourists, and containment measures such as fencing, grass cutting and signage along the playground's eastern side have been made in order to minimise the risk of further infections and spreading.

FIGURE 2

Maximum-likelihood phylogenetic tree of TBEV full genome sequences, Northern Zealand, Denmark, 2019



TBEV: tick-borne encephalitis virus.

Bootstrapping with 1,000 iterations was implemented for statistical support. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The three full-length TBEV sequences obtained from Tisvilde Hegn are marked. The TBEV sequence from Tokkekøb Hegn is shown, in addition to other TBEV full genome sequences from GenBank.

Acknowledgements

We thank Susanne Lopez Rasmussen and Veronica Christensen for technical assistance and Lene Jung Kjær for generating the maps.

Funding statement: Collection of ticks were carried out by the National Vector Surveillance Program at University of Copenhagen funded by the Danish Veterinary and Food Administration.

Conflict of interest

None declared.

Authors' contributions

Charlotte N Agergaard: patient contact, clinical data collection, preparation of the manuscript.

Maiken W Rosenstjerne: RT-qPCR analysis, revision of the manuscript.

René Bødker: flagging ticks, prevalence calculations, revision of the manuscript.

Morten Rasmussen: whole genome sequencing, NGS data analysis, revision of the manuscript.

Peter H. S. Andersen: epidemiological investigations, manuscript revision.

Anders Fomsgaard: patients/hospital contact, manuscript revision.

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Detection of novel tick-borne pathogen, Alongshan virus, in *Ixodes ricinus* ticks, south-eastern Finland, 2019

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Citation style for this article:

Kuivanen Suvi, Levanov Lev, Kareinen Lauri, Sironen Tarja, Jääskeläinen Anne J., Plyusnin Ilya, Zakham Fathiah, Emmerich Petra, Schmidt-Chanasit Jonas, Hepojoki Jussi, Smura Teemu, Vapalahti Olli. Detection of novel tick-borne pathogen, Alongshan virus, in *Ixodes ricinus* ticks, south-eastern Finland, 2019. Euro Surveill. 2019;24(27):pii=1900394. <https://doi.org/10.2807/1560-7917.ES.2019.24.27.1900394>

Article submitted on 19 Jun 2019 / accepted on 03 Jul 2019 / published on 04 Jul 2019

The newly identified tick-borne Alongshan virus (ALSV), a segmented jingmen virus group flavivirus, was recently associated with human disease in China. We report the detection of ALSV RNA in *Ixodes ricinus* ticks in south-eastern Finland. Screening of sera from patients suspected for tick-borne encephalitis for jingmen tick virus-like virus RNA and antibodies revealed no human cases. The presence of ALSV in common European ticks warrants further investigations on its role as a human pathogen.

Recent reports have associated two members of jingmen virus group, Alongshan virus (ALSV) and jingmen tick virus (JMTV), to febrile disease in humans [1,2]. Here we report the presence and genetic characterisation of ALSV in *Ixodes ricinus* ticks in Kotka archipelago, south-eastern Finland.

In 2010, a novel segmented tick-borne RNA virus, JMTV, was detected in *Rhipicephalus microplus* ticks in Hubei Province, China [3]. Subsequently, similar viruses have been identified in *R. microplus* and cattle in Brazil, i.e. the Mogiana tick virus (MGTV) [4-6]; human Crimean-Congo haemorrhagic fever (CCHF) cases in Kosovo* [7]; *Amblyomma javanense*, *Dermacentor silvarium* and *I. persulcatus* ticks as well as humans in China [1]; and a red colobus monkey in Uganda [8]. Recent reports associate novel JMTV strains from China with human disease [1,2]. A retrospective study conducted by Jia et al. reported identification of JMTV from skin biopsies and blood of febrile patients [1]. Meanwhile, ALSV was detected from *I. persulcatus* and isolated from febrile patient sera in Heilongjiang Province [2].

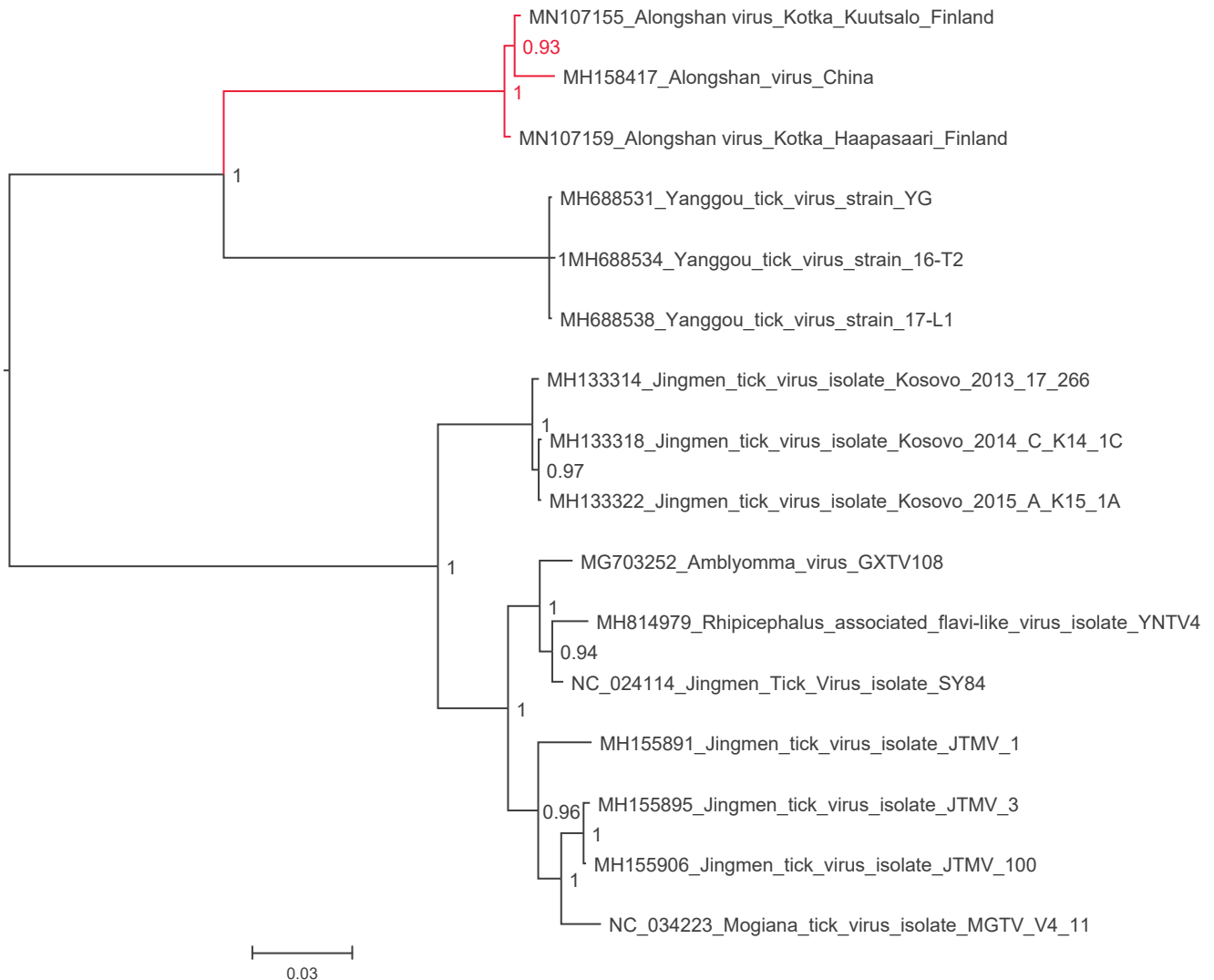
These viruses share the genome organisation of four segments, two of which show similarity to the NS3 and NS5 proteins of non-segmented RNA viruses in the genus *Flavivirus*. The other two segments appear to originate from an unknown ancestor. Together, the viruses form a separate and diverse group tentatively called the jingmen virus group in the family *Flaviviridae* [9].

Detection of jingmen-like virus in Kotka archipelago

In 2019, while performing a metatranscriptomic analysis of ticks collected in 2011 from Haapasaari island, Kotka archipelago, south-eastern Finland, we detected a full genome of JMTV-like virus together with tick-borne encephalitis virus (TBEV) genome. Thereafter, we used RT-PCR to screen 198 *I. ricinus* ticks collected from the Kotka archipelago in 2017 and 2018 for the presence of JMTV-like RNA. We found another positive tick from a neighbouring Kuutsalo island in the Kotka archipelago, and obtained the full genome using next-generation sequencing. The viruses (GenBank accession numbers MN107153 to MN107160) cluster together with ALSV (MH158415 to MH158418) from Heilongjiang Province, China, and form a cluster distinct from the other members JMTV group, including the strains found in Kosovo (MH133313 to MH133324) [2,7] (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5). Nucleotide and amino acid identities between the Finnish strains and the other tick-borne JMTV-like viruses are shown in Table 1. The virus isolation trials in Vero, SK-N-SH and CRL-2088 cells were unsuccessful.

FIGURE 1

The phylogenetic tree of NS3 segment of JMTV-like viruses



JMTV: Jingmen tick virus.

The NS₃ segment amino acid sequences of all available tick-borne Jingmen tick virus-like viruses were aligned using the ClustalW algorithm. The phylogenetic tree was constructed using the Bayesian Markov chain Monte Carlo (MCMC) method, implemented in MrBayes version 3.2 [19]. The GenBank accession number of the strains sequenced in this study are MN107155 and MN107159.

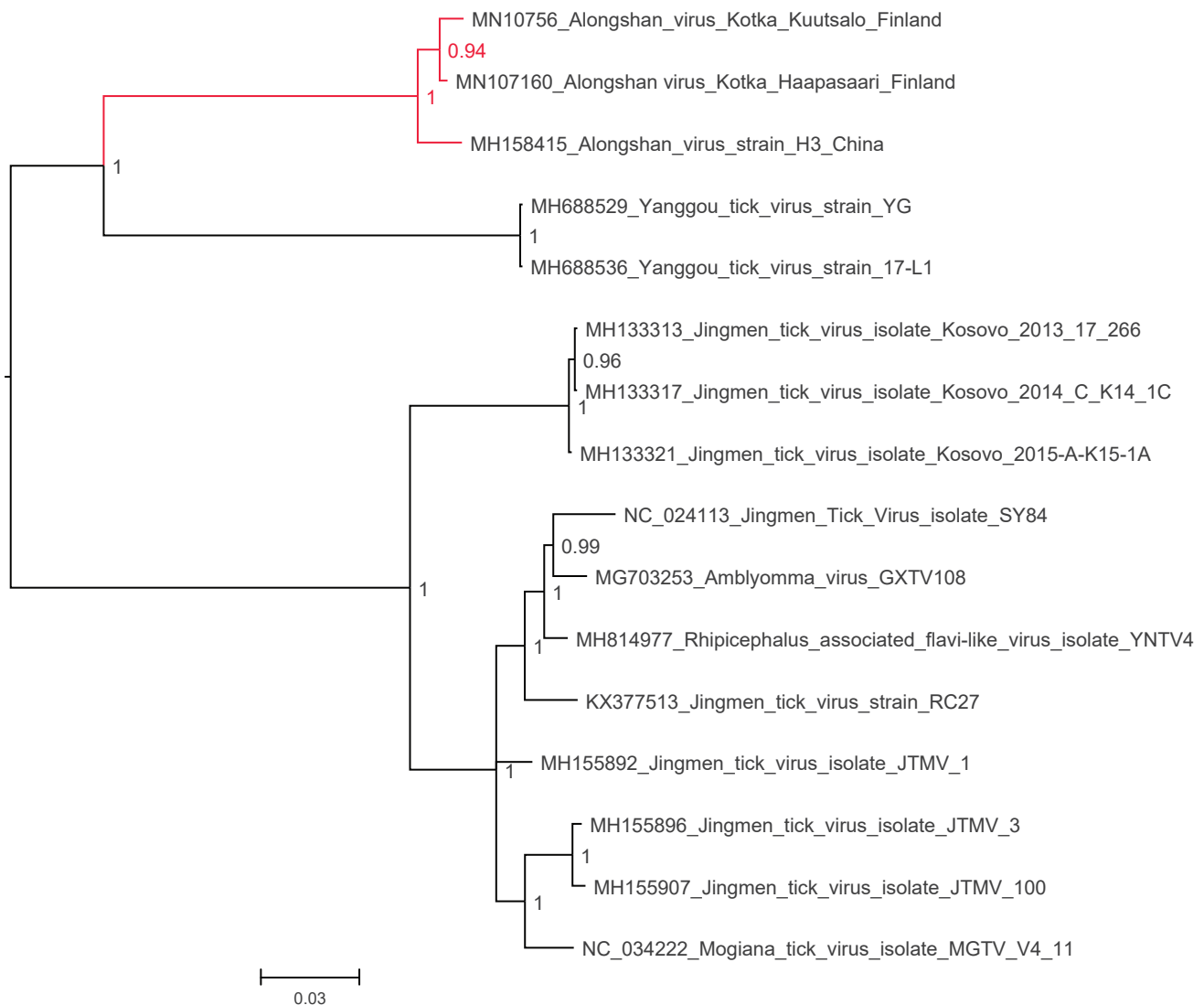
Human and tick samples

The emerging reports on the association of JMTV-like viruses with human disease in China [1,2] led us to investigate sera of TBE-suspected cases for JMTV-like virus RNA or antibodies against recombinant proteins of ALSV in 2019. The sera panel included 974 serum samples from 879 individuals. These samples were originally sent for TBEV antibody testing to Helsinki University Hospital laboratory (Helsinki, Finland) from May to November 2018. All samples were tested for JMTV RNA by RT-PCR, with 304 from 283 individuals (median age: 48 years, range: 1–88 years) for antibodies to JMTV VP1a, VP1b, membrane and capsid proteins.

For the RNA detection, we could verify that the RT-PCR detects local Finnish strains of ALSV, but we had no human ALSV positive control samples available for the antibody tests. We also studied three serial samples from two patients positive in an earlier sample for JMTV RNA from Kosovo at dilutions 1:20 and 1:80 for reference. These two patients shown to be infected with Kosovo strains of JMTV (capsid/membrane 63.5–64.0% amino acid identity, glycoprotein 49.9–50.1% amino acid identity) did not exhibit clear reactivity to the ALSV recombinant protein [7]. The 90 and 108 ticks collected in 2017 and 2018, respectively, from Kuutsalo

FIGURE 2

The phylogenetic tree of NS5 segment of JMTV-like viruses



JMTV: Jingmen tick virus.

The NS5 segment amino acid sequences of all available tick-borne Jingmen tick virus-like viruses were aligned using the ClustalW algorithm. The phylogenetic tree was constructed using the Bayesian Markov chain Monte Carlo (MCMC) method, implemented in MrBayes version 3.2 [19]. The GenBank accession number of the strains sequenced in this study are MN107156 and MN107160.

island, Kotka archipelago in south-eastern Finland were tested for JMTV RNA (Table 2).

RNA and antibody detection

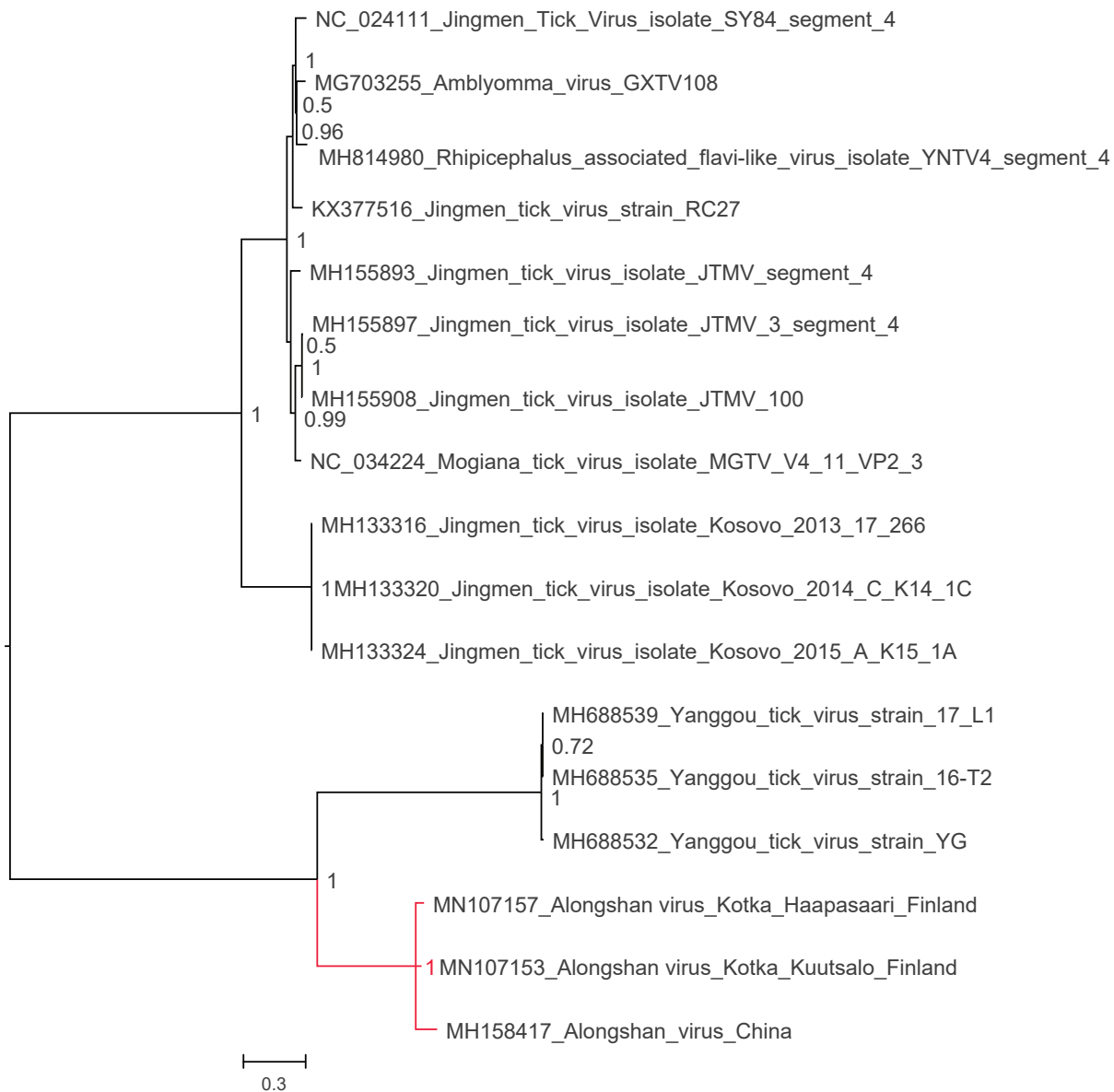
Ticks were homogenised and RNA was extracted as described previously [10]. Total nucleic acids from human serum samples were extracted using MagNa Pure LC 2.0 instrument and Total Nucleic Acid Isolation Kit (Roche, Basel, Switzerland). Viral RNA was detected with real-time or conventional reverse transcription (RT)-PCR targeting the NS5 gene. Primers and the probe were designed based on sequences available to us in August 2018, and we used JM F1312 as the forward primer (5'-TTCGGRGCMTGGCAMSACCT-3'),

JM1548 as the reverse primer (5'-CCKGTTDCCATYTGTTADCCCAT-3'), and JM2 as the probe (FAM-CTCCTAAAGATGTTAAACACTGC-BHQ). Conventional RT-PCR without the probe was initially used for tick samples with SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, California, United States (US)). Patient and tick samples were screened with real-time RT-PCR using the TaqMan Fast Virus 1-Step Master Mix (Thermo Scientific, Waltham, Massachusetts, US). An in vitro transcribed RNA served as the positive control.

Synthetic gene constructs encoding JTMV glycoproteins VP1a, VP1b, membrane and capsid proteins were

FIGURE 3

The phylogenetic tree of putative capsid/membrane segment of JMTV-like viruses



JMTV: Jingmen tick virus.

The putative capsid/membrane segment amino acid sequences of all available tick-borne Jingmen tick virus-like viruses were aligned using the ClustalW algorithm. The phylogenetic tree was constructed using the Bayesian Markov chain Monte Carlo (MCMC) method, implemented in MrBayes version 3.2 [19]. The GenBank accession number of the strains sequenced in this study are MN107153 and MN107157.

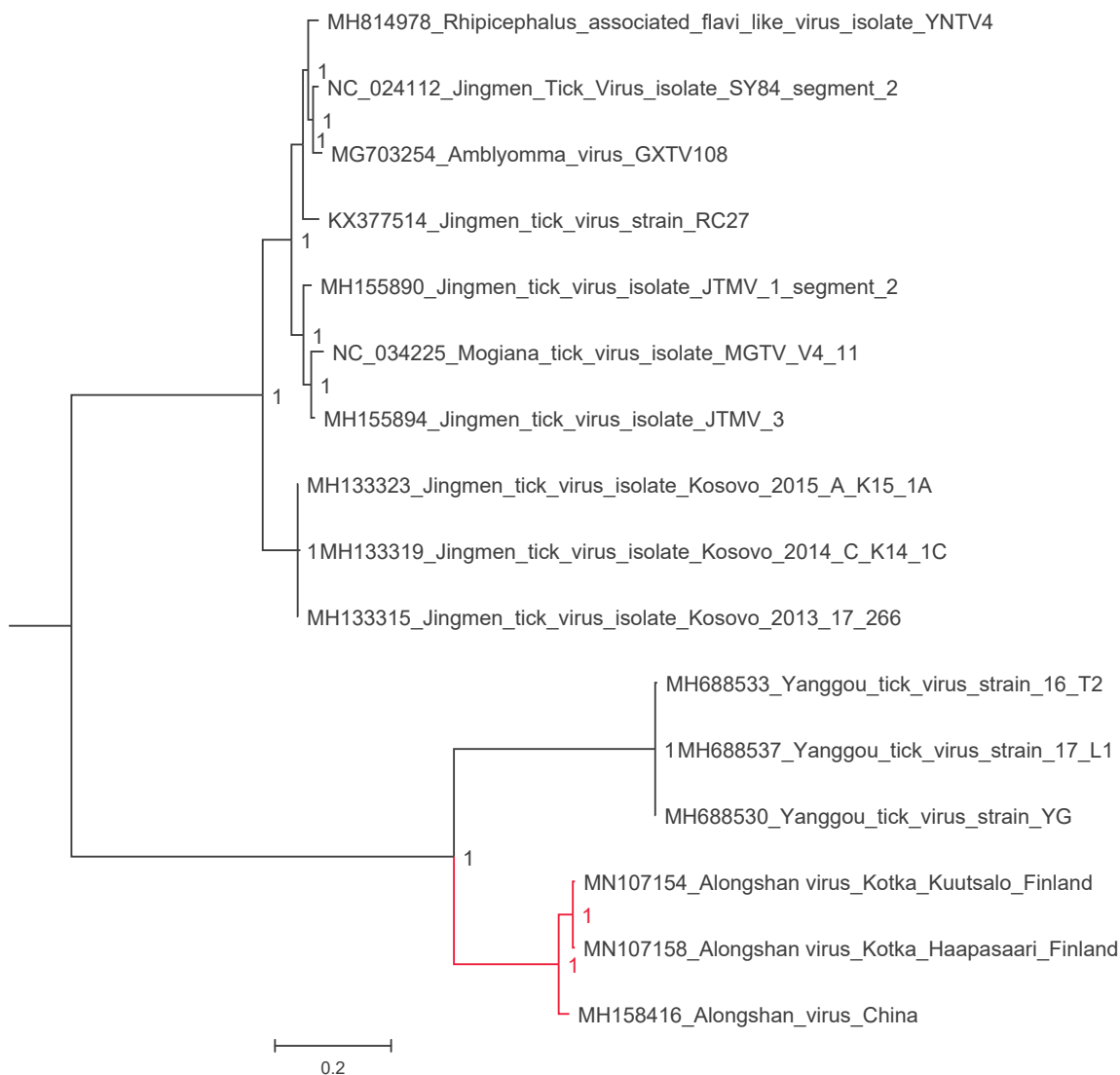
cloned into pCAGGS/MCS [11]. The recombinant and empty plasmids were transfected into Vero E6 cells using Fugene HD according to the manufacturer's instructions. The transfected cells were fixed onto microscopic slides with acetone, serum samples were diluted 1:20 in phosphate-buffered saline and immunofluorescence assay was performed as described previously [12].

Next generation sequencing and phylogenetic analysis

Tick homogenates were treated with a mixture of micrococcal nuclease (New England BioLabs Ipswich, Massachusetts, US) and benzonase (Millipore, Burlington, Massachusetts, US) for 1 hour at 37°C, followed by RNA extraction using TriPure Isolation reagent (Roche, Basel, Switzerland). RRNA was removed using a NEBNext rRNA Depletion Kit (New England BioLabs) according to the manufacturer's protocol. The sequencing library was prepared using a NEBNext Ultra II RNA Library Prep Kit (New England BioLabs). The library fragment sizes were measured using agarose gel

FIGURE 4

The phylogenetic tree of putative glycoprotein segment of JMTV-like viruses



JMTV: Jingmen tick virus.

The putative glycoprotein segment amino acid sequences of all available tick-borne Jingmen tick virus-like viruses were aligned using the ClustalW algorithm. The phylogenetic tree was constructed using the Bayesian Markov chain Monte Carlo (MCMC) method, implemented in MrBayes version 3.2 [19]. The GenBank accession number of the strains sequenced in this study are MN107154 and MN107158.

electrophoresis and the concentrations using Qubit dsDNA BR Assay Kit (Life Technologies, Carlsbad, California, US) and NEBNext Library Quant Kit for Illumina (New England BioLabs). Sequencing was conducted using MiSeq Reagent Kit V2 with 150 bp reads.

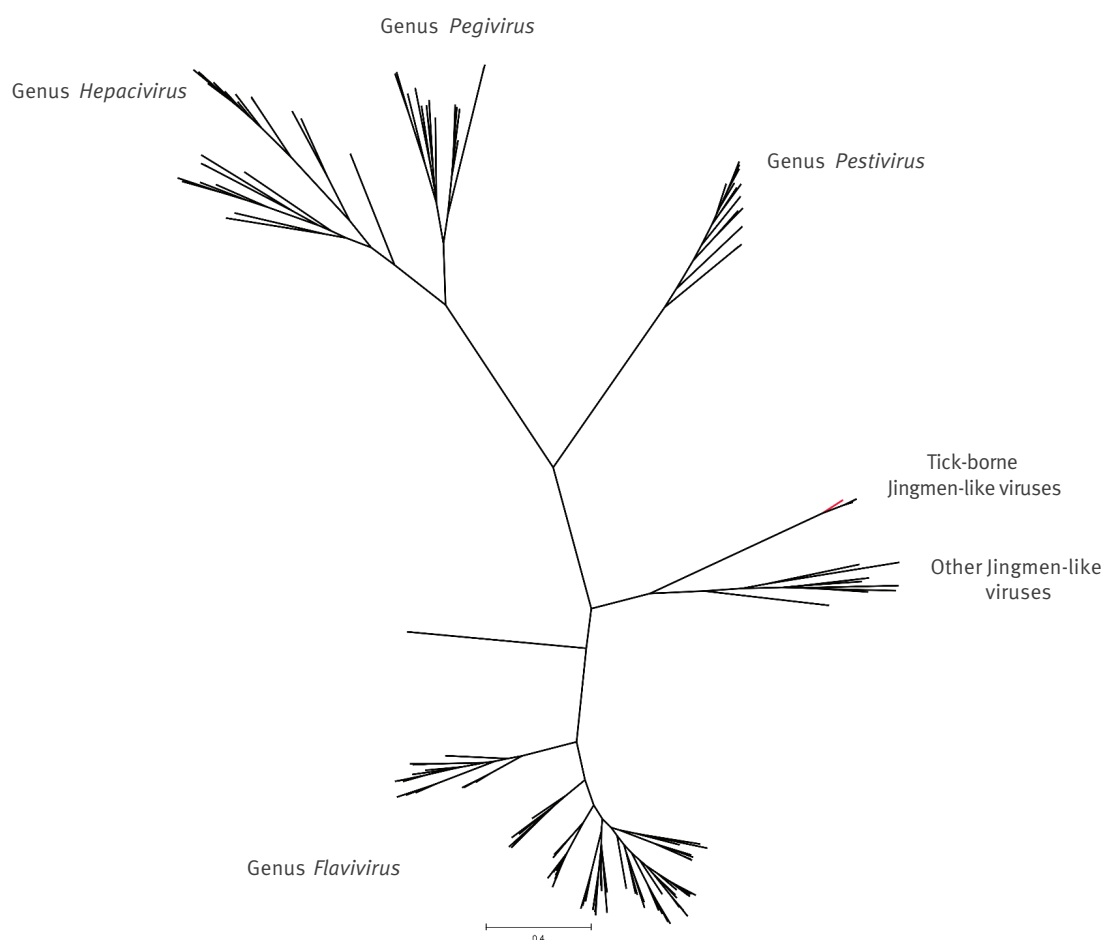
Raw sequence reads were trimmed and low-quality, quality score < 15, and short, < 36 nt, sequences were removed using Trimmomatic [13]. Thereafter, de novo assembly was conducted using MegaHit [14]. Open reading frames were sought using MetaGeneAnnotator [15], followed by taxonomic annotation using SANSparallel [16].

Complete genome sequences of all available tick-borne JMTV-like viruses were downloaded from GenBank (accessed June 2019). The amino acid sequences were aligned using the ClustalW algorithm followed by manual refinement. In addition, NS5 sequences of the representatives of all flavivirus species were retrieved from NCBI Reference Sequence Database (RefSeq) and aligned with MAFFT programme version 7 [17] using E-INS-i algorithm, followed by removal of ambiguously aligned amino acid sites using TrimAl programme [18].

The phylogenetic trees were constructed using the Bayesian Markov chain Monte Carlo (MCMC) method, implemented in MrBayes version 3.2 [19] with two

FIGURE 5

The phylogenetic tree of NS5 of all species in the family *Flaviviridae*



NS5 sequences of the representatives of all flavivirus species were retrieved from NCBI Reference Sequence Database (RefSeq) and aligned with MAFFT programme version 7 [17] using E-INS-i algorithm, followed by removal of ambiguously aligned amino acid sites using TrimAl programme [18]. The phylogenetic tree was constructed using the Bayesian Markov chain Monte Carlo (MCMC) method, implemented in MrBayes version 3.2 [19].

TABLE 1

Nt and amino acid identities between Finnish strains of Alongshan virus and other tick-borne JMTV-like viruses, Finland, 2019

Segment	Nt identity (%)			Amino acid identity (%)		
	ALSV (Finland)	ALSV (China)	Other JMTV-like viruses	ALSV (Finland)	ALSV (China)	Other JMTV-like viruses
Putative capsid/membrane	5.3	9.5–9.7	33.1–37.7	1.4	2.4–2.8	29.0–36.3
Putative glycoprotein	1.5	8.0–8.1	35.9–43.1	0.5	4.4 ^a	38.3–51.2
NS3	4.8	8.8–9.0	28.1–31.1	0.5	1.4–1.6	16.1–21.7
NS5	4.0	10.5–10.6	28.3–32.1	0.9	2.1–2.5	19.8–21.6

ALSV: Alongshan virus; JMTV: Jingmen tick virus; Nt: nucleotide.

^a Both ALSV strains from Finland have the same number of amino acid differences compared to the Chinese strain.

TABLE 2

Summary of JMTV-like virus RT-PCR, NGS and antibody tests of *I. ricinus* ticks and sera from suspected human TBE cases., Finland, 2019

Sample	Year	Sample origin	Number Studied (N)	Number of positives (n)	Method	Sequencing result of positive sample
<i>I. ricinus</i> panel	2011	Haapasaari island, Kotka archipelago	3	1	NGS	Whole genome ALSV
<i>I. ricinus</i> panel	2017	Kuutsalo island, Kotka archipelago	90	1	Conventional RT-PCR	Whole genome ALSV
<i>I. ricinus</i> panel	2018	Kuutsalo island, Kotka archipelago	108	0	Conventional and real-time RT-PCR	NA
Human sera from suspected TBE case	2018	Throughout Finland	974	0	Real-time RT-PCR	NA
Human sera from suspected TBE case	2018	Throughout Finland	304	0	Recombinant JMTV protein IFA	NA

ALSV: Alongshan virus; IFA: immunofluorescence assay; JMTV: Jingmen tick virus; NA: not applicable; NGS: next generation sequencing; TBE: tick-borne encephalitis.

independent runs and four chains per run. The analysis was run for 5 million states and sampled every 5,000 steps.

Conclusion

Our findings show that ALSV, a newly described tick-borne human pathogen, is also present in south-eastern Finland. Notably, ALSV was detected in *I. ricinus* ticks, a tick species that is common across the European continent. Despite apparent ALSV circulation in the south-eastern archipelago of Finland, no ALSV RNA or antibodies to selected recombinant ALSV proteins were found in ca 900 Finnish patients suspected for TBEV infection in recent years. While our results suggest low human infection pressure, further research using other methods, including properly evaluated ALSV antibody tests, and focusing on other geographic areas and patient cohorts beyond meningitis or encephalitis cases is needed.

Note

*This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the International Court of Justice Opinion on the Kosovo Declaration of Independence.

Acknowledgements

We are very grateful to Johanna Martikainen and Mira Utriainen for excellent laboratory assistance.

Funding statement: This work was supported by the Jane and Aatos Erkko Foundation (Jane ja Aatos Erkon Säätiö), the Academy of Finland (Suomen Akatemia), Sigrid Jusélius Foundation (Sigrid Juséliuksen Säätiö) and Helsinki University Central Hospital (Helsingin ja Uudenmaan Sairaanhoidopiiri).

Conflict of interest

None declared.

Authors' contributions

SK drafted the manuscript, and performed RT-PCR and virus isolation. LL produced the antigens and performed the serological screen. LK performed RT-PCR, TS designed the RT-PCRs, AJJ collected the diagnostic data, FZ performed serological screening and JH designed the protein constructs. PE and JSC provided samples, performed serological screen and revised the manuscript. IP and TS performed sequencing and data analysis. TS revised the manuscript, and OV designed the study and revised the manuscript. All authors read and approved the final manuscript.

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Toscana, West Nile, Usutu and tick-borne encephalitis viruses: external quality assessment for molecular detection of emerging neurotropic viruses in Europe, 2017

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Citation style for this article:

Reusken Chantal, Baronti Cecile, Mögling Ramona, Papa Anna, Leitmeyer Katrin, Charrel Remi N. Toscana, West Nile, Usutu and tick-borne encephalitis viruses: external quality assessment for molecular detection of emerging neurotropic viruses in Europe, 2017. *Euro Surveill.* 2019;24(50):pii=1900051. <https://doi.org/10.2807/1560-7917.ES.2019.24.50.1900051>

Article submitted on 13 Jan 2019 / accepted on 13 Jun 2019 / published on 12 Dec 2019

Background: Neurotropic arboviruses are increasingly recognised as causative agents of neurological disease in Europe but underdiagnosis is still suspected. Capability for accurate diagnosis is a prerequisite for adequate clinical and public health response. **Aim:** To improve diagnostic capability in EVD-LabNet laboratories, we organised an external quality assessment (EQA) focusing on molecular detection of Toscana (TOSV), Usutu (USUV), West Nile (WNV) and tick-borne encephalitis viruses (TBEV). **Methods:** Sixty-nine laboratories were invited. The EQA panel included two WNV RNA-positive samples (lineages 1 and 2), two TOSV RNA-positive samples (lineages A and B), one TBEV RNA-positive sample (Western subtype), one USUV RNA-positive sample and four negative samples. The EQA focused on overall capability rather than sensitivity of the used techniques. Only detection of one, clinically relevant, concentration per virus species and lineage was assessed. **Results:** The final EQA analysis included 51 laboratories from 35 countries; 44 of these laboratories were from 28 of 31 countries in the European Union/European Economic Area (EU/EEA). USUV diagnostic capability was lowest (28 laboratories in 18 countries), WNV detection capacity was highest (48 laboratories in 32 countries). Twenty-five laboratories were able to test the whole EQA panel, of which only 11 provided completely correct results. The highest scores were observed for WNV and TOSV (92%), followed by TBEV (86%) and USUV (75%). **Conclusion:** We observed wide variety in extraction methods and RT-PCR tests, showing a profound absence of standardisation across European laboratories. Overall, the

results were not satisfactory; capacity and capability need to be improved in 40 laboratories.

Background

The aetiology of neuro-invasive viral infections remains undetermined in more than 50% of cases [1]. Several viruses can cause infections of the central nervous system (CNS) while, regardless of the causative aetiology, clinical manifestations are often similar, making a confirmed diagnosis dependant on laboratory testing [2]. Neurotropic arboviruses are increasingly recognised as causative agents of neurological disease in Europe but underdiagnosis is still suspected [3]. Confirmed involvement of arboviruses is important for risk communication and risk management strategies, the latter including activities like local vector control, blood safety measures and vaccination campaigns. Four neurotropic arboviruses are emerging and have become endemic in large parts of Europe: Toscana virus (TOSV), Usutu virus (USUV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV).

The TOSV (genus *Phlebovirus*, family *Phenuiviridae*) is transmitted by sandflies of the genus *Phlebotomus* and circulates in Mediterranean countries where it can cause febrile illness and neuroinvasive infections. At least 250 million people are exposed in Europe and neighbouring countries around the Mediterranean basin that are frequently visited by travellers for occupational or leisure purposes [4–7]. In France, Spain and Italy, TOSV is among the three most common agents causing aseptic meningitis and encephalitis, together with enteroviruses and herpesviruses (herpes simplex

TABLE 1

Nucleic acid extraction methods used in the external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 51 laboratories)

Extraction method	Number of laboratories
QIAamp Viral RNA Mini Kit (Qiagen, Hilden)	21
NucliSENSE EasyMag (BioMérieux, Marcy-L'Étoile)	4
EZ1 Virus Mini Kit (Qiagen, Hilden)	3
MagNA Pure 96 DNA and Viral NA kit (Roche, Meylan)	3
RNeasy Mini kit (Qiagen, Hilden)	2
QIAamp MinElute Virus Spin Kit (Qiagen, Hilden)	2
MagNa Pure LC total NA kit (Roche, Meylan)	2
MagNa Pure Compact NA isolation kit (Roche, Meylan)	2
iPrep PureLink Virus Kit (Thermo Fisher, Bourgoin-Jallieu)	2
QIAamp DSP Virus (Qiagen, Hilden)	1
Maxwell RSC Viral Total NA Purification Kit (Promega, Charbonnières-les-Bains)	1
QIAxtractor VX (Qiagen, Hilden)	1
QIAamp RNA Blood Mini Kit (Qiagen, Hilden)	1
MagCore Viral NA extraction kit (RBCBioscience, New Taipei City)	1
TriPure isolation reagent (Sigma-Aldrich, Saint-Louis)	1
High Pure Viral RNA kit (Roche, Meylan)	1
NucleoSpin RNA Virus (Macherey-Nagel, Düren)	1
MagDea NA extraction kit for magLead (PSS-Ltd, Tokyo)	1
RIBO-prep NA extraction kit (AmpliSense, Voisins-Le-Bretonneux)	1

and varicella–zoster viruses) [8]. Viraemia is short-lived (typically 5 days, range: 2–7) and diagnosis is done either by detecting viral RNA in cerebrospinal fluid (CSF) or serum at the acute stage of infection or by detecting IgM in an early serum sample [8]. The currently known circulation of three genetic lineages may be indicative of a wide genetic diversity of this viral species and thus molecular assays are needed to detect genetic variants [8].

WNV (genus *Flavivirus*, family *Flaviviridae*) is transmitted by *Culex* spp. mosquitoes. WNV can cause febrile illness with or without neurological manifestations. During the last decade, WNV activity in Europe has shown a profile similar to that observed in North America, with substantial activity reported every year and with recurring major outbreaks [9,10]. Major recent activity in the eastern Mediterranean region is also a matter of concern for Europe [11].

Lineages 1 and 2 have been identified in human WNV cases in Europe [12]. Severe cases are more frequent in elderly and immunocompromised patients. In the acute stage of disease, WNV RNA can be detected in CSF. WNV viraemia is typically short-lived, but viral RNA can be detected for longer periods in some specimens such as urine and whole blood, and also in fatal cases or immunocompromised patients. The high degree of cross-reactivity with other flaviviruses in serology is problematic. Although a combination of serology and PCR is desirable, the detection of WNV RNA alone is an

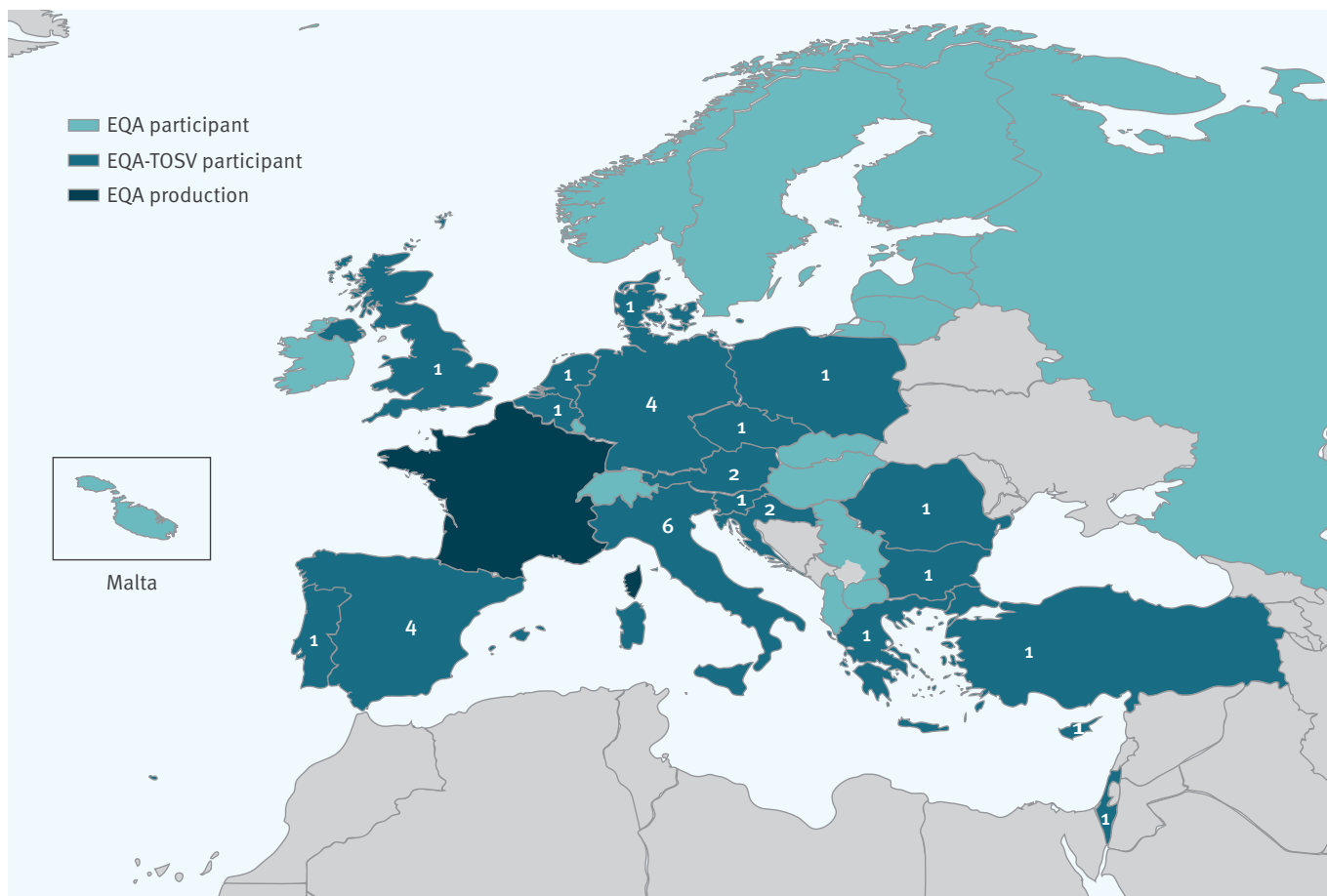
important means of undisputable confirmation of acute infection [13].

USUV (genus *Flavivirus*, family *Flaviviridae*) was first isolated in Africa in 1959 [13]. It is a *Culex*-transmitted flavivirus closely related to WNV [13]. The earliest human cases (presenting as neuro-invasive disease) were recorded in 2009 in Italy in two immunocompromised patients having received blood products [13]. Since then evidence of zoonotic transmission accompanied by neurological disease of USUV is accumulating while population studies show the occurrence of asymptomatic infections [13]. Nothing is known about the length of USUV viraemia and the kinetics of antibody production in humans [13]. Based on its close relatedness to WNV, viraemia is expected to be short and low level [13]. At the acute stage of neuroinvasive infection the virus is expected to be detectable by RT-PCR in CSF. A high degree of cross-reactivity with other flaviviruses is seen in serology. For this reason, molecular detection is the preferred method for confirmatory laboratory diagnosis.

TBEV (genus *Flavivirus*, family *Flaviviridae*) is a tick-borne flavivirus; the incidence of TBEV infection in humans and its geographical distribution have increased in Europe [14]. Three subtypes are recognised, of which the Western subtype is endemic in northern, central and eastern Europe. The clinical spectrum of the disease ranges from mild meningitis to severe meningoencephalitis. The course of TBE is often biphasic. The first acute phase typically has

FIGURE 1

Number of laboratories per country that provided results for Toscana virus, external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 32 laboratories)



EQA: external quality assessment; TOSV: Toscana virus.

Blue: countries participating with TOSV RNA testing; dark blue: France (the Unite des Virus Emergents (National Reference Centre for arboviruses) at Aix Marseille University prepared and anonymised the panel); light blue: countries that submitted results for viruses other than TOSV.

non-specific symptoms. It is followed by an asymptomatic interval that precedes the second phase characterised by neuroinvasive disease. Therefore, the window of molecular detection in serum and CSF is often missed as diagnostics are typically requested during the second phase of illness. Once neurological symptoms are manifest, TBEV RNA is rarely detected in blood and CSF. However, in endemic regions, unexplained febrile illness alone can justify molecular testing. As TBEV cases often do not present with typical symptoms, diagnosis often relies on laboratory documentation. As for WNV, viraemia is typically short-lived and low while serology is hampered by extensive cross-reactivity among flaviviruses [15].

To support molecular diagnostic capacity and capability building for these emerging neurotropic viruses in the European Union (EU)/ European Economic Area (EEA) and EU pre-accession countries, an external quality assessment (EQA) was organised for members of the

expert laboratory network EVD-LabNet (<https://www.evd-labnet.eu/>) funded by the European Centre for Disease Prevention and Control. Here, we present this assessment and the inventory of methods for RT-PCR detection of these viruses that are used in European and national reference laboratories.

Methods

EQA scheme organisation

All members of EVD-LabNet (69 laboratories at 1 November 2017) were invited by email to participate through online registration. Fifty-four laboratories from 35 countries registered online.

Panel composition

The EQA panel consisted of 10 samples with six samples positive for one of four different viral species (plasma samples spiked with viruses), and four negative control samples. The panel included two

TABLE 2

RT-PCR methods used for Toscana virus RNA detection, external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 32 laboratories).

Target	Method	Number of laboratories	False-negative ^a
Toscana virus-specific			
TOSV N	Perez-Ruiz et al., 2007 [29] ^b	13	1 (lineage B)
TOSV N	Weidmann et al., 2008 [30] ^b	5	None
TOSV N	Brisbarre et al., 2015 [31] ^b	2	None
TOSV L	Sanchez-Seco et al., 2003 [43] ^c	1	1 (lineage B)
TOSV various	Own design ^d	5	1 (lineage B)
TOSV N	Progenie (commercial) ^b	1	None
Pan-phlebovirus			
Pan-phlebo L/N ^e	Sanchez-Seco et al., 2003 [43]	7	1 (lineage A), 2 (lineage B)
Pan-phlebo N	Lambert and Lanciotti, 2009 [44]	1	1 (lineage B)
Pan-phlebo unknown	Own design ^d	1	None

TOSV: Toscana virus.

^a The missed TOSV lineage is indicated between brackets.

^b Included in statistical analysis as classified as virus-specific real-time RT-PCR.

^c Included in statistical analysis as classified as conventional RT-PCR.

^d Excluded from statistical analysis as no distinction could be made whether it is real-time RT-PCR or conventional RT-PCR.

^e Not all participants indicated which of two pan-Phlebo RT-PCRs in the reference was used.

WNV RNA-positive samples (WNV lineage 1, strain UVE/WNV/2001/FR/DON2001 (ref#001V-02215), 7.2×10^4 RNA copies/0.4 mL [16] and WNV lineage 2, strain B956 source BNI, Hamburg, 4.96×10^5 RNA copies/0.4 mL), two TOSV RNA-positive samples (TOSV lineage A, strain UVE/TOSV/2010/TN/ T152 (ref#001V-02119), 1.57×10^5 RNA copies/0.4 mL [17] and TOSV lineage B, strain UVE/TOSV/2010/FR/4319 (ref#001V-02442), 1.24×10^5 RNA copies/0.4 mL [18]), one TBEV RNA-positive sample (Western subtype, strain UVE/TBEV/1953/CZ/Hypr (ref#001V-EVA134), 5.06×10^4 RNA copies/0.4 mL [19]), one USUV RNA-positive sample (USUV, strain *Turdus merula* NL2016 (ref#011V-02153), 6.34×10^3 RNA copies/0.4 mL [20] and four viral RNA-negative plasma samples. Strains referenced in the European Virus Archive (EVA) can be accessed at <https://www.european-virus-archive.com>. Each sample of the panel was prepared from a batch that consisted of qualified non-therapeutic human plasma obtained from the French blood bank, spiked with virus culture supernatant and heat-inactivated at 60°C for 1 hour. A total of 70 0.4-mL aliquots were prepared and freeze-dried into glass vials. Proper inactivation was confirmed by the absence of cytopathic effect in Vero cells and by undetectable increase of the viral RNA titre in the supernatant 5 days after inoculation. The viral loads per reconstituted sample were quantified with reference to in-house TOSV-, WNV-, TBEV- and USUV-specific synthetic RNA controls; a fragment (ca 500 bp) tagged at the 5' end with the T7 promoter sequence (5'TAATACGACT CACTATAGGG3') and containing the virus-specific TaqMan-targeted sequence was amplified by RT-PCR using the Access RT-PCR

kit (Promega, Charbonnières-les-Bains). The resulting PCR products were purified and transcribed using the T7 Megashort script kit (Ambion, ThermoFisher Scientific, Bourgoin-Jallieu). The obtained RNA was purified with the MegaClear purification kit (Ambion, Bourgoin-Jallieu). RNA concentration was measured using a NanoDrop 1000 (Thermo Scientific, Bourgoin-Jallieu) and translated into copy numbers. Real-time RT-PCR was performed using the Express One-Step Superscript qRT-PCR Kit, universal (Life technologies, Bourgoin-Jallieu) on a QuantStudio 12K Flex Real-Time PCR System. For each EQA sample, the number of copies contained in 0.4 mL of freeze-dried material in the glass vial was calculated by comparison with a dilution series of T7-generated RNA standard containing 10^2 to 10^8 RNA copies.

Result submission, evaluation and EQA scoring

We provided the Laboratories with a link to an online form to submit their EQA results. Laboratories could indicate for which of the four target viruses they had tested the EQA panel and background information of the diagnostic tests that the laboratory assessed with the EQA. Data were collected and analysed in Microsoft Excel 2011. Fisher's exact test (www.socscistatistics.com/tests/fisher/Default2.aspx) was used to compare the rate of false-negative results obtained with virus-specific real-time assays and with other assays for TOSV, USUV, TBEV and WNV. Fisher's exact test was used because the significance of the deviation from a null hypothesis can be calculated exactly, rather than relying on an approximation that becomes exact in the

TABLE 3

Summary of results of laboratories in the external quality assessment on molecular diagnostics of emerging neurotropic viruses, Europe (n = 51)

Sample ID	1 ^a	2	3	4	5	6	7	8	9	10
Virus	TOSV (lineage A)	TOSV (lineage B)	USUV	WNV (lineage 1)	WNV (lineage 2)	TBEV	Negative	Negative	Negative	Negative
Concentration	1.57 x 10 ⁵ RNA cp/o.4mL	1.24 x 10 ⁵ RNA cp/o.4mL	6.34 x 10 ³ RNA cp/o.4mL	7.2 x 10 ⁴ RNA cp/o.4mL	4.96 x 10 ⁵ RNA cp/o.4mL	5.06 x 10 ⁴ RNA cp/o.4mL	n/a	n/a	n/a	n/a
Total correct positive when tested for the specific virus	31/51	28/51	23/51	42/51	46/51	37/51	n/a	n/a	n/a	n/a
Total correct positive when not tested for the specific virus	17/51	18/51	19/51	2/51	2/51	8/51	n/a	n/a	n/a	n/a
Total correct	48/51	46/51	42/51	44/51	48/51	45/51	47/51	48/51	49/51	48/51
Total partially correct: identification at the genus level	0/51	0/51	1/51	2/51	1/51	2/51	n/a	n/a	n/a	n/a
False	4/51	5/51	5/51	5/51	2/51	4/51	3/51	3/51	1/51	3/51
Total sensitivity	31/32	28/32	21/28	42/48	46/48	36/42	n/a	n/a	n/a	n/a

n/a: not applicable; TBEV: tick-borne encephalitis virus; TOSV: Toscana virus; USUV: Usutu virus; WNV: West Nile virus.

^a Number >100% as one laboratory submitted both a correct result (positive for TOSV) and one false result (positive for TBEV) for this sample.

limit as the sample size grows to infinity, as with the chi-squared test.

Results

EQA participation

The final EQA analysis included 51 laboratories from 35 countries: 44 laboratories from 28 EU/EEA countries, four laboratories from four EU pre-accession countries (Albania, North Macedonia, Serbia and Turkey) and three laboratories from three non-EU/EEA countries (Israel, Russia and Switzerland). From the EU/EEA, there was no participation from laboratories in Iceland and France besides the reference laboratory in Marseille that produced the panel. Liechtenstein does not have a reference laboratory participating in EVD-LabNet. From EU pre-accession countries, there was no participation by Bosnia and Herzegovina, Montenegro and Kosovo*, the two latter being not members of EVD-LabNet at the time.

Nucleic acid extraction methodology

The different techniques used for extraction of nucleic acids are presented in the Table 1. Various Qiagen kits were used by 31 laboratories: 21 used the QIAamp Viral RNA Mini Kit (Qiagen, Hilden) and the remaining 10 laboratories used six different Qiagen kits. The extraction kits from Roche (Meylan) were the second most frequently used brand, with eight laboratories using four different types of Roche kits. Because of the high diversity, it was impossible to include the type of RNA purification in the analysis.

Toscana virus

Of the 51 laboratories, 32 laboratories in 19 countries (17 EU/EEA, one EU candidate, one other) tested the

panel for the presence of TOSV RNA (Figure 1). Nineteen laboratories in 19 countries had no TOSV test available. Seven laboratories used a pan-phlebovirus RT-PCR only, 23 laboratories a TOSV-specific RT-PCR only and two laboratories used both type of tests in combination. Some laboratories used more than one TOSV-specific or pan-phlebovirus test (Table 2).

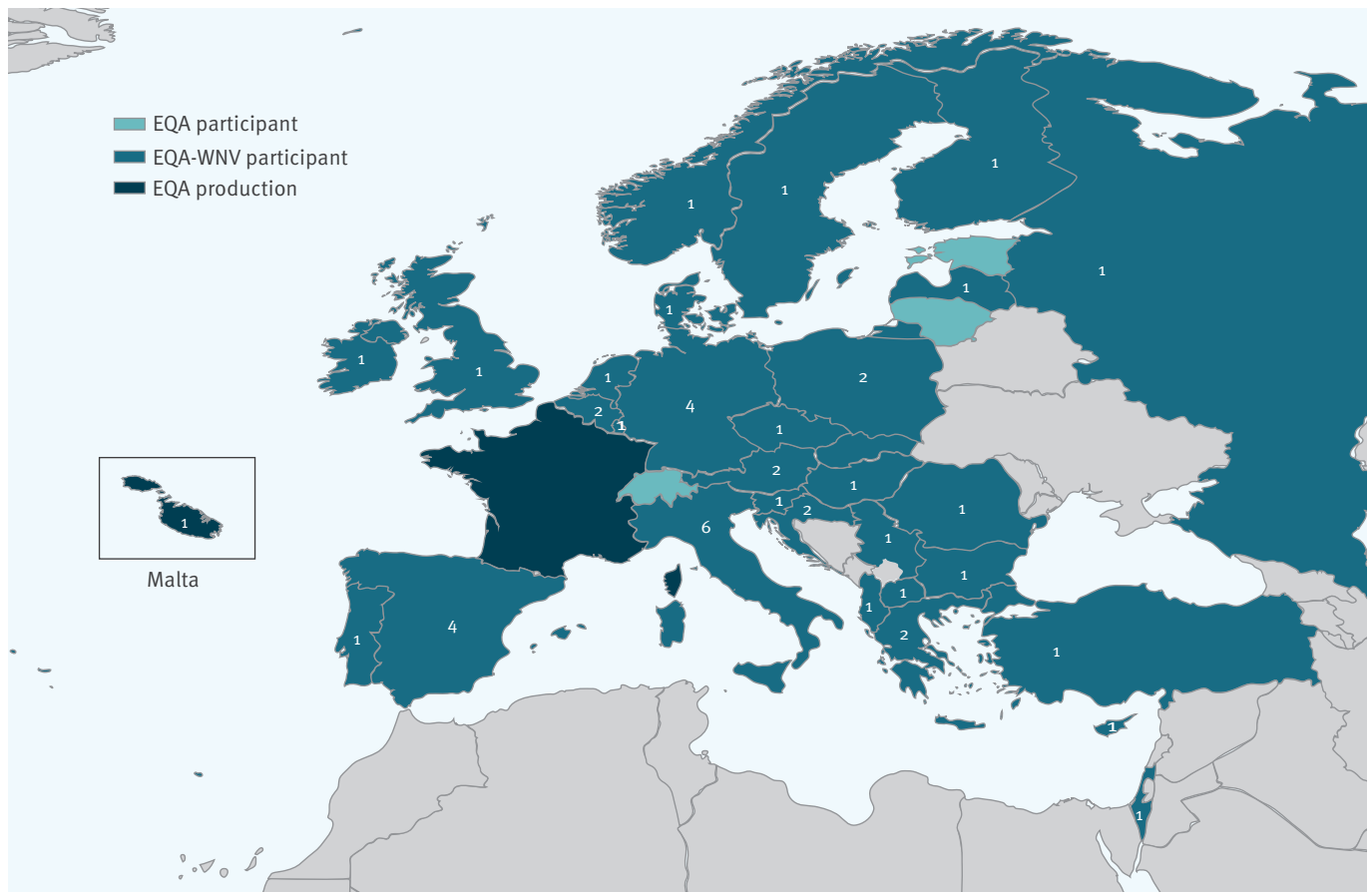
Excluding TOSV-specific assays for which no information was available (n=10), TOSV-specific real-time tests (n=42) provided false-negative results significantly less frequently than all other tests together (pan-phlebo, classic and nested RT-PCR; n=20; p=0.011). Thirty-one of the 32 laboratories detected TOSV RNA correctly in sample #1 (lineage A) and 28 laboratories detected TOSV RNA correctly in sample #2 (lineage B). The RT-PCR tests used by laboratories that missed the presence of TOSV in sample #1 (n=1) or in sample #2 (n=6) are presented in Table 2. One laboratory falsely detected TBEV RNA besides TOSV RNA in sample #1 (Table 3).

West Nile virus

Forty-eight laboratories in 32 countries (26 EU/EEA, four EU candidates and two other) tested the panel for WNV RNA (Figure 2). One laboratory used a pan-flavi RT-PCR test while 28 laboratories used a WNV-specific RT-PCR. Eighteen laboratories used both a pan-flavi and WNV-specific RT-PCR, but the questionnaire did not allow linking the result with either assay. One laboratory did not report what type of test was used. Some laboratories used more than one RT-PCR test (Table 4). The diversity of WNV-specific tests used was high with a total of 25 different tests. Excluding WNV-specific assays for which no information was available (n=4), there was no statistically significant difference

FIGURE 2

Number of laboratories per country that provided results for West Nile virus, external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 48 laboratories)



EQA: external quality assessment; WNV: West Nile virus.

Blue: countries participating with WNV RNA testing; dark blue: France (the Unite des Virus Emergents (National Reference Centre for arboviruses) at Aix Marseille University prepared and anonymised the panel); light blue: countries that submitted results for viruses other than WNV.

between results provided by WNV-specific real-time tests (n=29) and all other tests together (real-time pan-flavi, classic and nested RT-PCR; n=22; p=0.38). Forty-two of 48 laboratories that tested the panel for the presence of WNV RNA detected WNV RNA correctly in sample #4 (lineage 1), while one laboratory indicated to have detected flavivirus RNA. Five laboratories falsely scored sample #4 as negative. WNV RNA was correctly identified in sample #5 (lineage 2) by 46 of 48 laboratories. Of the two laboratories providing a false-negative result for sample #5, one laboratory reported the presence of TBEV RNA in sample #5; the other reported the presence of flavivirus RNA, although it did not claim to test for WNV (Table 3).

Usutu virus

Twenty-eight laboratories in 18 countries (16 EU/EEA, two other) tested the panel for USUV (Figure 3). Six laboratories used a pan-flavivirus RT-PCR test only, 15 used an USUV-specific RT-PCR only and seven used both a pan-flavivirus and USUV-specific RT-PCR, but it

was impossible to trace which one was used to provide the submitted results. Some laboratories used more than one USUV-specific or pan-flavivirus RT-PCR test. There was a lot of variation in USUV-specific RT-PCRs used, with 17 different test systems (Tables 3 and 5).

Excluding USUV-specific assays for which no information was available (n=4), USUV-specific real-time tests (n=20) provided false negative results significantly less frequently than all other tests together (real-time pan-flavi, classic and nested RT-PCR; n=15; p=0.019). Seven of the 28 laboratories that tested the panel for USUV missed the positive sample #6. The RT-PCR tests used by these laboratories are indicated in Table 5.

Tick-borne encephalitis virus

Forty-two laboratories in 28 countries (25 EU/EEA, three other) tested the panel for TBEV RNA (Figure 4): seven used a pan-flavi RT-PCR test only, 24 used a TBEV-specific RT-PCR only and 11 used both a pan-flavi and TBEV-specific RT-PCR, however, the questionnaire

TABLE 4

RT-PCR methods used for West Nile virus RNA detection, external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 48 laboratories)

Target	Method	Number of laboratories	False-negative ^a
West Nile virus-specific			
WNV 5'-UTR/C	Linke et al., 2007 [45] ^b	11	1 (lineage 2)
WNV NS2A	Eiden et al., 2010 [46] ^b	2	None
WNV 3'-UTR	Tang et al., 2006 [47] ^b	1	None
WNV 3'UTR	Lanciotti et al., 2000 [48] ^b	1	None
WNV E/NS1	Shi et al., 2001 [49] ^b	1	None
WNV NS3	Chaskopoulou et al., 2011 [50]	1	None
WNV various	Own design	3	1 (lineage 1)
WNV unknown	Altona RealStar (commercial) ^b	5	1 (lineage 2)
WNV unknown	Qiagen Artus (commercial) ^b	3	1 (lineage 1)
WNV unknown	Fast Track Tropical fever Core (commercial) ^b	3	1 (lineage 1)
WNV unknown	Sacace (commercial) ^b	1	None
WNV unknown	Amplisense (commercial) ^b	1	None
Pan-flavivirus			
Pan-flavi NS5	Scaramozzino et al., 2001 [51]	7	None
Pan-flavi NS5	Sanchez-Seco et al., 2005 [52]	3	None
Pan-flavi NS5	Moureau et al., 2007 [53]	2	1 (lineage 1)
Pan-flavi NS5	Patel et al., 2013 [54]	2	None
Pan-flavi NS5	Briese et al., 1999 [55]	1	None
Pan-flavi NS5	Vina-Rodriguez et al., 2017 [56]	1	None
Pan-flavi NS5	Vazques et al., 2012 [57]	1	None
Pan-flavi unknown	Own design	2	None
Pan-flavi unknown	Genekam (commercial)	1	None
Pan-flavi unknown	TibMolBiol (commercial)	1	None
Information not provided ^c		1	1 (lineage 1)

WNV: West Nile virus.

^a WNV lineage missed indicated between brackets.

^b Included in statistical analysis as classified as virus-specific real-time RT-PCR.

^c Excluded from statistical analysis since cannot be classified as real-time RT-PCR or conventional RT-PCR.

did not permit to trace submitted results to one or the other assay. Moreover, some laboratories used more than one pan-flavivirus test. Table 6 gives an overview of the different RT-PCRs tests that were used on the EQA panel to detect TBEV RNA.

Excluding TBEV-specific assays for which no information was available (n=4), there was no statistically significant difference between results provided by TBEV-specific real-time tests (n=29) and all other tests together (real-time pan-flavi, classic and nested RT-PCR n=25; p=1).

Thirty-six of 42 laboratories that tested the panel for the presence of TBEV RNA detected TBEV RNA correctly in sample #3. Four laboratories falsely scored sample #3 as negative including those using two commercial tests. One laboratory falsely indicated the presence of WNV RNA. One laboratory scored sample #3 as pan-flavi-positive only (Table 3).

Contamination

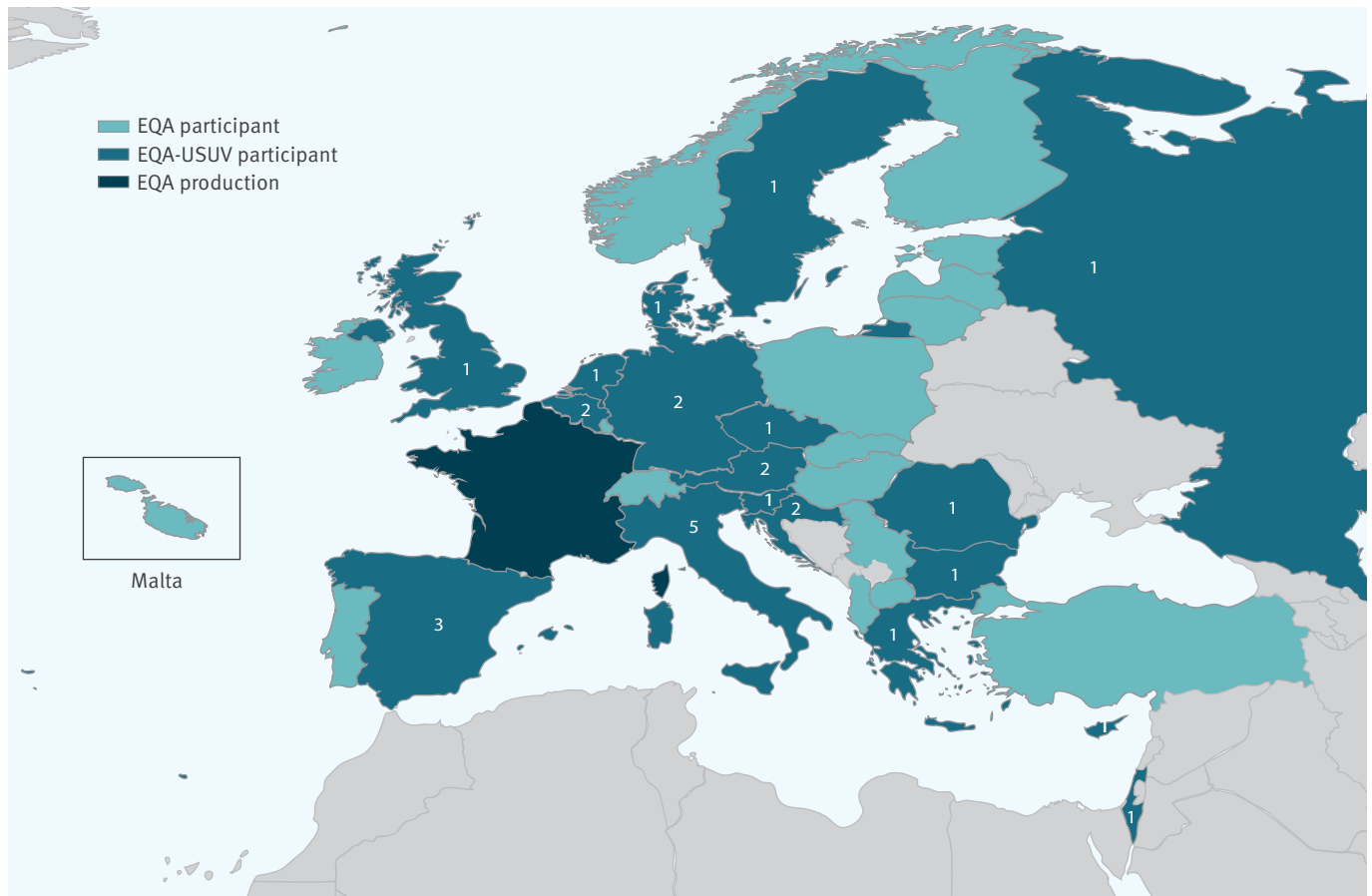
Contamination issues were noticed in six of the 51 participating laboratories. Contamination issues involved detection of flavivirus, Zika virus, WNV or TBEV RNA in the negative control samples or in samples containing other specific viruses.

Discussion

Fifty-one laboratories from 35 countries (28 EU/EEA, four EU pre-accession, three non-EU/EEA) participated in this EQA on molecular detection of emerging neurotropic viruses. Twenty-five laboratories in 16 countries (15 EU/EEA, one non-EU/EEA) reported capacity for testing of all four EQA target viruses. However, only 11 of the 25 scored the panel 100% correct. These 11 laboratories represented 10 EU/EEA countries and one non-EU/EEA country. Overall, the results of the EQA are not satisfactory. The capacity and capability for molecular detection needs to be improved in the vast majority of the participating laboratories because these four viruses demonstrate a growing burden on

FIGURE 3

Number of laboratories per country that provided results for Usutu virus, external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 28 laboratories)



EQA: external quality assessment; USUV: Usutu virus.

Blue: countries participating with USUV RNA testing; dark blue: France (the Unite des Virus Emergents (National Reference Centre for arboviruses) at Aix Marseille University prepared and anonymised the panel); light blue: countries that submitted results for viruses other than USUV.

public health, have sympatric circulation (at least two of them) in several European countries and are indistinguishable clinically. It is important to underline that most of the participating laboratories were not first-line routine laboratories but national reference laboratories [3]. The fact that samples were missed by laboratories is of concern as the samples had RNA loads within the average of clinical relevance and were not intended to be at the detection limit to evaluate sensitivity. Another worrisome observation is the fact that six of 51 participating laboratories scored one or more of the viral RNA-negative samples positive, which is indicative of contamination issues and happened more frequently than in previous EQAs [21-23].

In our study, the total number of panels tested by each RT-PCR test did not allow statistically significant conclusions about specific methods that laboratories should be advised to use. Nevertheless, for TOSV and USUV, methods other than virus-specific real-time assays provided false-negative results more frequently

than virus-specific real-time PCR tests. Although the same trend was not observed for WNV and TBEV, this could be taken into consideration by laboratories to improve the performance of their diagnostic capacity.

Because TOSV is endemic in countries surrounding the Mediterranean Sea, the majority of reference laboratories in Europe deal only with imported TOSV cases [24-28]. The neglected state of TOSV is reflected in the general absence of commercial tests, except for one which was used by one laboratory for the EQA panel. TOSV detection capacity had a geographical and laboratory coverage comparable to USUV, i.e. 32 laboratories in 19 countries which included all participating countries with known TOSV circulation (Croatia, Cyprus, France, Greece, Italy, Portugal and Spain). Bosnia and Herzegovina and Kosovo*, two other European countries with TOSV activity, did not participate in the EQA. Three TOSV lineages circulate in Europe, two of which were represented in the EQA panel, i.e. lineages A

TABLE 5

RT-PCR methods used for Usutu virus RNA detection, external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 28 laboratories)

Target	Method	Number of laboratories	False-negative
Usutu virus-specific			
USUV NS5	Nikolay et al., 2014 [58] ^a	11	1
USUV NS5	Cavrini et al., 2011 [59] ^a	5	none
USUV NS1	Jöst et al., 2011 [60] ^a	2	none
USUV NS5	Weissenböck et al., 2013 [61] ^a	1	none
USUV 3'UTR	Del Amo et al., 2013 [62] ^a	1	none
USUV unknown	Own design ^b	4	none
Pan-flavivirus			
Pan-flavi NS5	Scaramozzino et al., 2001 [51]	5	3
Pan-flavi NS5	Sanchez-Seco et al., 2005 [52]	3	3
Pan-flavi NS5	Patel et al., 2013 [54]	2	none
Pan-flavi NS5	Vina-Rodriguez et al., 2017 [56]	1	1
Pan-flavi NS5	Vazques et al., 2012 [57]	1	1
Pan-flavi unknown	Own design	2	none
Pan-flavi unknown	Genekam (commercial)	1	none

USUV: Usutu virus.

^a Included in statistical analysis as classified as virus-specific real-time RT-PCR.

^b Excluded from statistical analysis since cannot be classified as real-time RT-PCR or conventional RT-PCR.

and B. The third lineage, lineage C, has only recently been discovered in Greece and Croatia and could not be included in the panel because the virus isolate was not available at the time. Of the 32 laboratories that tested for TOSV, four laboratories in four countries missed the TOSV lineage B sample; another laboratory in a fifth country missed the lineage A sample. At RT-PCR test level, lineage A was missed with one test while lineage B was missed six times by five RT-PCR tests of which four were conventional RT-PCR methods, despite the fact that the samples had similar viral loads. Apparently some laboratories used systems that were not sensitive enough for detection of TOSV lineage B strains, although this lineage is geographically most widely spread [4]. TOSV RNA loads provided in this EQA were in line with the virological findings in CSF [29-31,32,33]. The recent discovery of lineage C merits attention and the capacity of currently described assays to detect such strains need to be verified; since virological and genetic characterisation of this lineage is ongoing in Greece, inclusion of this lineage will be possible in future EQAs. At the country level, three of the five laboratories that missed a TOSV RNA-positive sample were located in a country endemic for TOSV. Better insight into the capability of TOSV molecular detection in Europe should be obtained with a dedicated EQA, including all three lineages at different viral loads, designed for a comparative evaluation of the RT-PCR methods described in the literature.

The widest geographical (32 countries) and laboratory (n = 48) coverage was for WNV testing. The WNV lineage 1 sample was missed by five laboratories in five EU/

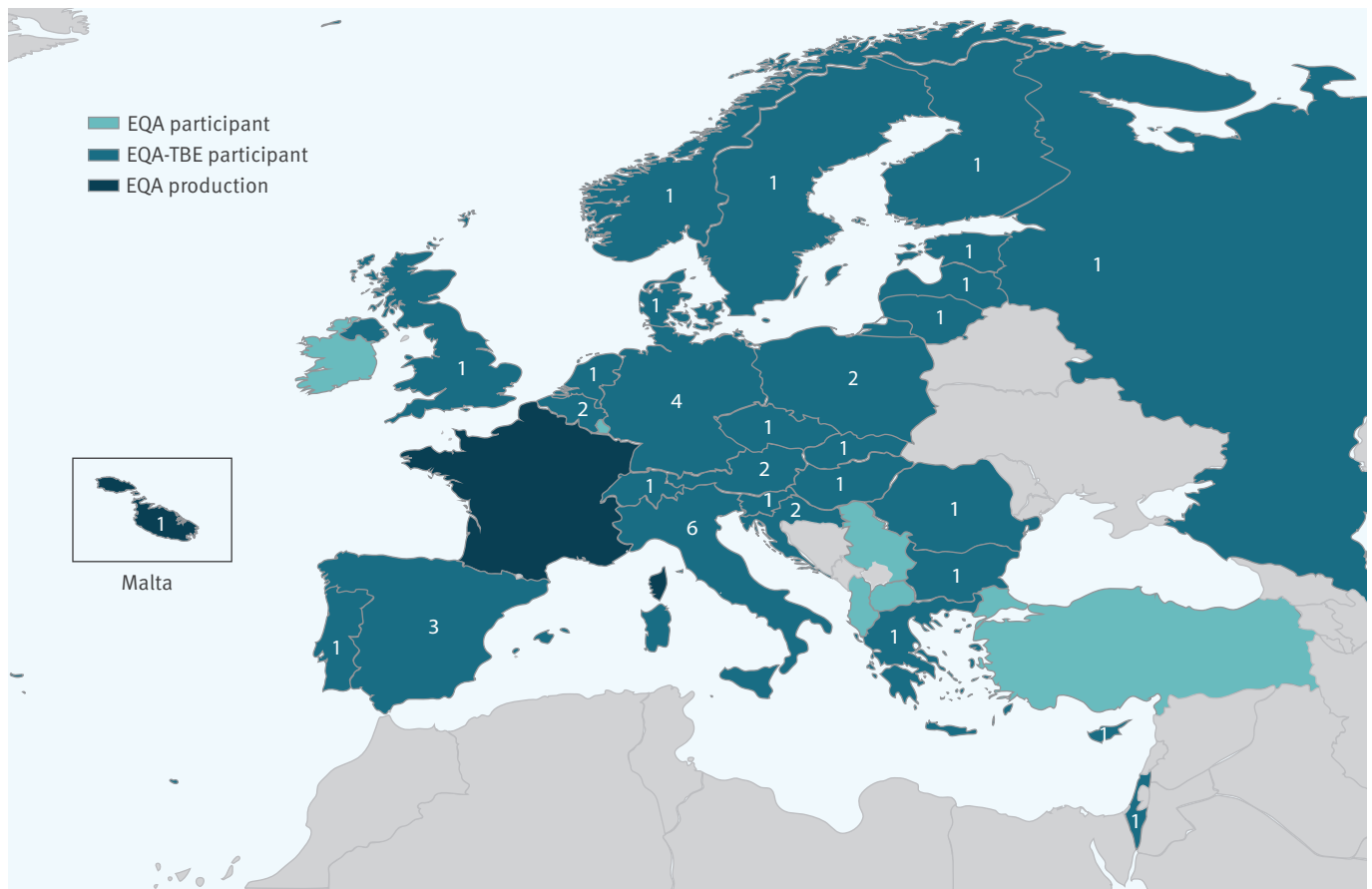
EEA countries that had never reported an autochthonous WNV case, while WNV lineage 2 was missed by two laboratories in two EU/EEA countries, one of which is endemic for WNV lineage 2. This was the third EQA of molecular detection of WNV within EVD-LabNet and its predecessor ENIVD [22,23]. The long history of WNV capability assessments and surveillance in Europe is likely to explain the good scores observed with WNV.

In this panel, USUV was the most recent emerging virus with still accumulating evidence of its relevance for public health and an increasing geographical distribution [13]. This might explain why the testing capability for USUV had the smallest geographical coverage (n = 18 countries) and number of laboratories (n = 28 laboratories). This was the first EQA that included USUV and there is no literature on clinically relevant viral loads in plasma. The concentration in this panel (1.6×10^4 copies/mL) was in the range of detected viral loads for the closely related WNV in plasma [34-36]. Looking at the currently known geographical distribution of USUV in Europe, all countries with USUV circulation except Switzerland participated with USUV testing. The USUV-positive sample was missed by seven laboratories in four EU/EEA countries. To gain better insight in the robustness of USUV detection in Europe, a dedicated EQA including a concentration range of USUV genome copies in different matrices (whole blood, plasma and urine) is to be planned.

Although the geographical distribution of TBEV in Europe is broader than that of WNV and the total number of tick-borne encephalitis cases is higher, the number

FIGURE 4

Number of laboratories per country that provided results for tick-borne encephalitis virus, external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 42 laboratories)



EQA: external quality assessment; TBEV: tick-borne encephalitis virus.

Blue: countries participating with TBEV RNA testing; dark blue: France (the Unite des Virus Emergents (National Reference Centre for arboviruses) at Aix Marseille University prepared and anonymised the panel); light blue: countries that submitted results for viruses other than TBEV.

of laboratories participating with TBEV testing (n = 42) and their country coverage (n = 28) was smaller than for WNV. The TBEV sample was missed by four laboratories in three countries, of which two display endemic presence of TBEV. This was the second EQA including molecular detection of TBEV within EVD-LabNet and its predecessor ENIVD [21]. However, overall results could not be compared as our EQA only assessed TBEV testing based on one single RNA viral load.

Based on our results, we cannot give advice on what methods to use for the molecular detection of the four viruses. This requires assessment of the whole routine procedure from sample receipt to generation of a result. The performance in the EQA is a combination of the extraction method and the RT-PCR method used, as would routinely be the case when processing real-life diagnostic samples. The set-up of the current EQA cannot assess the influence of the extraction method or RT-PCR system on the final outcome per sample. The background data provided by the participants

indicated an important diversity of the methods used for nucleic acid extraction (19 methods). It was impossible to link the extraction method to the quality of the results. To assess solely the quality of the RT-PCR, EQA panels consisting of extracted or synthetic RNA should be provided. Although our study was not designed to address the efficacy of the extraction technique per se, there are many arguments that favour automated extraction protocols over manual protocols. Automated extraction reduces the risk of cross-contamination, the turnaround and hands-on times, provide equivalent amounts of viral RNA and guarantee a better reproducibility compared with manual extraction [37–42]. EQA is an efficient tool to evaluate diagnostic procedures and to alert highlight where improvements are needed. Therefore, we recommend repeating the EQA for laboratories with unsatisfactory results, focusing at least on TOSV and USUV and investigating whether the required improvements are achieved. For these two viruses, we recommend real-time assays rather than classic or nested PCR protocols.

TABLE 6

RT-PCR methods used for tick-borne encephalitis virus RNA detection, external quality assessment for molecular detection of emerging neurotropic viruses, Europe (n = 42 laboratories)

Target	Method	Number of laboratories	False-negative
Tick-borne encephalitis virus			
TBEV-3'-UTR	Schwaiger et al., 2003 [63] ^a	17	None
TBEV-NS ₁	Achazi et al., 2011 [64] ^a	5	None
TBEV-NS ₅	Puchhammer-Stöckl et al., 1995 [65]	2	None
TBEV-NS ₄	Bago et al., 2002 [66] ^b	1	None
TBEV-5'-UTR	Schrader et al., 1999 [67]	1	None
TBEV-E	Briggs et al., 2011 [68]	1	None
TBEV-E	Gäumann et al., 2010 [69] ^a	1	None
TBEV-5'-UTR	Klaus et al., 2010 [70] ^a	1	None
TBEV-3'-UTR	Brinkley et al., 2008 [71] ^b	1	None
TBEV-E	Andreassen et al., 2012 [72] ^a	1	None
TBEV-E	Skarpaas et al., 2006 [73]	1	None
TBEV-various	Own design ^b	2	None
TBEV Unknown	Genesig (commercial) ^a	1	1
TBEV Unknown	Amplisense (commercial) ^a	2	1
TBEV Unknown	Viasure (commercial) ^a	1	None
Pan-flavivirus			
Pan-flavi NS ₅	Scaramozzino et al., 2001 [51]	7	None
Pan-flavi NS ₅	Sanchez-Seco et al., 2005 [52]	3	2
Pan-flavi NS ₅	Moureau et al., 2007 [53]	1	None
Pan-flavi NS ₅	Patel et al., 2013 [54]	2	None
Pan-flavi NS ₅	Briese et al., 1999 [55]	1	None
Pan-flavi NS ₅	Vina-Rodriguez et al., 2017 [56]	1	None
Pan-flavi NS ₅	Vazques et al., 2012 [57]	1	None
Pan-flavi unknown	Own design	2	None
Pan-flavi unknown	Genekam (commercial)	1	None
Pan-flavi unknown	TibMolBiol (commercial)	1	None

TBEV: tick-borne encephalitis virus.

^a Included in statistical analysis as classified as virus-specific real-time RT-PCR.

^b Excluded from statistical analysis since cannot be classified as real-time RT-PCR or conventional RT-PCR.

Conclusion

Early detection of neurotropic arboviruses allows for timely risk assessment and risk management measures. We observed wide variation in both extraction methods and RT-PCR tests, showing a profound absence of standardisation across European laboratories. Overall, the results were not satisfactory and indicated a need for improvement of capacity and capability. Testing for WNV and TBEV, for which EQAs had been organised previously, showed better results than testing for USUV and TOSV for which this EQA was the first. This trend is important to consider and suggests that EQA exercises for TOSV and USUV should be repeated in order to assess whether successful improvements have been made.

*Note

This designation is without prejudice to positions on status, and is in line with UNSCR 1244 and the ICJ Opinion on the Kosovo Declaration of Independence

Acknowledgements

We thank all EQA participants: Institute of Virology, University of Veterinary Medicine Vienna, Vienna, Austria; Center for Virology, Medical University of Vienna, Vienna, Austria; Institute of Tropical Medicine; Central Laboratory for Clinical Biology, Antwerp, Belgium; Viral diseases, Scientific Public Health Institute, Brussels, Belgium; Microbiology department, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria; Research unit, University Hospital for Infectious Diseases "Dr. Fran Mihaljevic", Zagreb; Microbiology, CNPHI, Zagreb, Croatia; Department of Molecular Virology, Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus; Dept. of molecular biology, Institute of Public Health, Ostrava, the Czech Republic; Virus and Microbiological Special Diagnostics, Statens Serum Institut, Copenhagen, Denmark; Virology Department, National Institute for Health Development, Tallinn, Lithuania;

University of Helsinki and Helsinki University Hospital, Department of Virology and Immunology, Helsinki, Finland; Centre for Biological Threats and Special Pathogens, ZBS1 Highly Pathogenic Viruses, Robert Koch Institute, Berlin, Germany; Charité – Universitätsmedizin Berlin, Institute of Virology, Berlin, Germany; Bundeswehr Institute of Microbiology; Central Diagnostic Unit, Munich, Germany; Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany; Public health Laboratories, Hellenic Pasteur Institute, Athens, Greece; National Public Health Institute, Budapest, Hungary; National Virus Reference Laboratory, University College Dublin, Belfield, Dublin, Ireland; Az. Ospedaliero-Universitaria di Bologna, Policlinico S. Orsola-Malpighi, Bologna, Italy; Microbiology and Virology Unit, Padova University Hospital, Padova, Italy; Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy; Infectious Diseases, Amedeo di Savoia Hospital, Torino, Italy; Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; Laboratory of Virology, National Institute for Infectious Diseases Lazzaro Spallanzani IRCCS, Rome, Italy; Riga East University hospital Latvian Centre of Infectious Diseases, Riga, Latvia; Clinical testing department, National public health surveillance laboratory, Vilnius, Estonia; Virology-Serology, LNS, Dudelange, Luxembourg, Luxembourg; Molecular Diagnostics Mater Dei Hospital, Msida, Malta; Department of Virology, Norwegian Institute of Public Health, Oslo, Norway; Department of Virology, National Institute of Public Health-National Institute of Hygiene, Warsaw, Poland; Virology Laboratory, BTTIC MIHE, Pulawy, Poland; Center for Vectors and Infectious Diseases Research, National Institut of Health. Águas de Moura, Portugal; Laboratory for vector-borne diseases, National Institute of Research Cantacuzino, Bucharest, Romania; Institute of Virology, Biomedical Research Center, Bratislava, Slovakia; Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; Exotic disease, CReSA-IRTA, Barcelona, Spain; Servicio de Microbiología; Hospital Virgen de las Nieves, Granada, Spain; Laboratory of Arboviruses and Viral imported Diseases. National Center of Microbiology. Institute of Health “Carlos III”, Madrid, Spain; Hospital Clinic de Barcelona, Barcelona, Spain; Public Health Agency Sweden, Microbiology, Stockholm Sweden; RIPL, NIS Public Health England Porton Down, UK. Control of Infectious Diseases Department, Institute of Public Health, Tirana, Albania; Laboratory for virology and molecular diagnostics, Institute of Public Health of the Republic of North Macedonia, Skopje, North Macedonia; Department for Laboratory Diagnostic, NRL for ARBO viruses and haemorrhagic fever, Institute of Virology, Vaccines and Sera “Torlak”, Belgrade, Serbia; Public Health Institution of Turkey, Ankara, Turkey. National Center for Zoonotic Viruses, Central Virology Laboratory Public Health Services, Israel Ministry of Health Chaim Sheba Medical Center Tel – Hashomer, Israel; Federal Budget Institution of Science “Central Research Institute of Epidemiology”, Moscow, Russia; Federal Office for Civil Protection, Spiez Laboratory, Spiez, Switzerland.

Funding: This work was supported by the European Centre for Disease Prevention and Control (ECDC) under specific contract no.3-ECD.8456 for implementation of the framework contract ECDC/2016/002. Several virus strains were provided by the European Virus Archive goes Global (EVAg) project that has received funding from the European Union’s Horizon 2020 research and innovation program under grant agreement No 653316.

Conflict of interest

None declared.

Authors’ contributions

Chantal Reusken: design, organisation, analysis, pretested the panel, MS corrections. Cecile Baronti: preparation of the material of EQA. Ramona Mögling: logistics, analysis of the results, MS corrections. Anna Papa: design, pretested the panel, MS corrections. Katrin Leitmeyer: design, analysis of the results, MS corrections. Remi N. Charrel: design, organisation, supervised material preparation, results analysis, MS writing.

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Predicting and mapping human risk of exposure to *Ixodes ricinus* nymphs using climatic and environmental data, Denmark, Norway and Sweden, 2016

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Citation style for this article:

Kjær Lene Jung, Soleng Arnulf, Edgar Kristin Skarsfjord, Lindstedt Heidi Elisabeth H, Paulsen Katrine Mørk, Andreassen Åshild Kristine, Korslund Lars, Kjelland Vivian, Slettan Audun, Stuen Snorre, Kjellander Petter, Christensson Madeleine, Teräväinen Malin, Baum Andreas, Klitgaard Kirstine, Bødker René. Predicting and mapping human risk of exposure to *Ixodes ricinus* nymphs using climatic and environmental data, Denmark, Norway and Sweden, 2016. Euro Surveill. 2019;24(9):pii=1800101. <https://doi.org/10.2807/1560-7917.ES.2019.24.9.1800101>

Article submitted on 05 Mar 2018 / accepted on 01 Aug 2018 / published on 28 Feb 2019

Background: Tick-borne diseases have become increasingly common in recent decades and present a health problem in many parts of Europe. Control and prevention of these diseases require a better understanding of vector distribution. **Aim:** Our aim was to create a model able to predict the distribution of *Ixodes ricinus* nymphs in southern Scandinavia and to assess how this relates to risk of human exposure. **Methods:** We measured the presence of *I. ricinus* tick nymphs at 159 stratified random lowland forest and meadow sites in Denmark, Norway and Sweden by dragging 400m transects from August to September 2016, representing a total distance of 63.6 km. Using climate and remote sensing environmental data and boosted regression tree modelling, we predicted the overall spatial distribution of *I. ricinus* nymphs in Scandinavia. To assess the potential public health impact, we combined the predicted tick distribution with human density maps to determine the proportion of people at risk. **Results:** Our model predicted the spatial distribution of *I. ricinus* nymphs with a sensitivity of 91% and a specificity of 60%. Temperature was one of the main drivers in the model followed by vegetation cover. Nymphs were restricted to only 17.5% of the modelled area but, respectively, 73.5%, 67.1% and 78.8% of the human populations lived within 5 km of these areas in Denmark, Norway and Sweden. **Conclusion:** The model suggests that increasing temperatures in the future may expand tick distribution geographically in

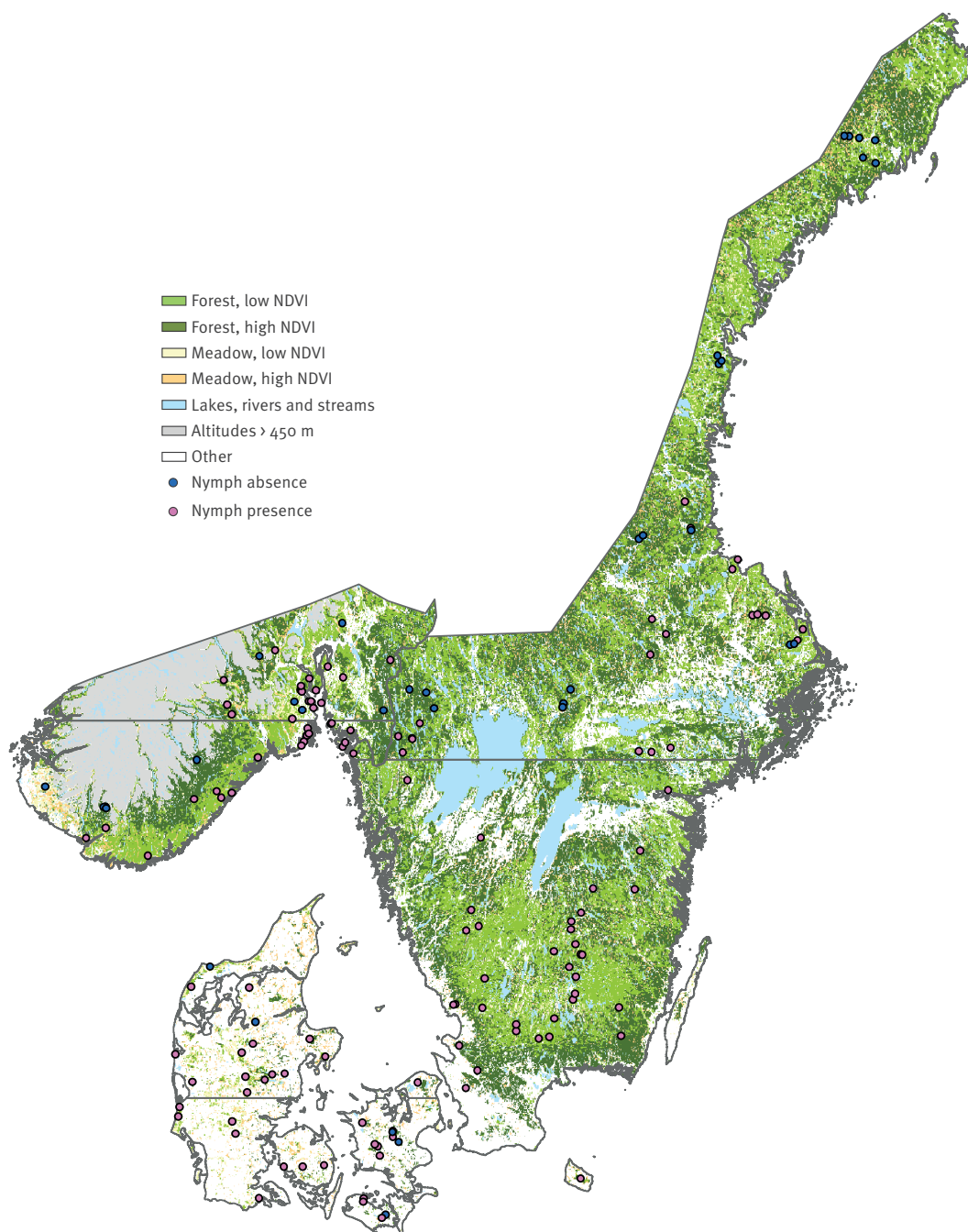
northern Europe, but this may only affect a small additional proportion of the human population.

Introduction

Ticks are one of the most important vectors for pathogens, impacting a wide range of vertebrates, and transmit more pathogens than any other arthropod [1,2]. In Europe, the main vector for tick-borne pathogens is *Ixodes ricinus* [3,4], which is also the most common tick species in Scandinavia [3-5]. Over the last decades, the incidence and geographical range of tick-borne diseases have increased [3,6,7] and pose a risk to both human and animal health. Scandinavia constitutes the edge of the northern distributional range of *I. ricinus* [4]. The incidence of Lyme borreliosis (LB) and tick-borne encephalitis (TBE) is increasing in both Norway and Sweden [5,8-10]. In Norway, LB and TBE have mostly been reported along the coastline in the southern parts of the country [5,8]. However, tick-borne encephalitis virus (TBEV) has been found in *I. ricinus* nymphs as far north as ca 115 km from the Arctic Circle [11,12]. In Sweden, LB is widespread in the southern and eastern regions [4,13,14], whereas TBE is concentrated in the south-central and coastal regions, with the annual TBE incidence around Stockholm exceeding 4 per 100,000 inhabitants [9,10,15,16]. In Denmark, LB seems endemic and widespread [3], whereas TBEV-infected ticks have only been confirmed

FIGURE 1

Stratification of the study area, showing 159 sample sites and presence/absence of *Ixodes ricinus* nymphs, Denmark, Norway and Sweden, 15 August–30 September 2016



NDVI: normalised difference vegetation index.

Forest includes the cover types: broad-leaved forest, coniferous forest and mixed forest. Meadow includes: land principally occupied by agriculture with significant areas of natural vegetation, natural grasslands, moors and heathland, and transitional woodland-shrub. The lines divide each country into equally sized northern and southern strata. Only parts of Norway and Sweden were included in the field study for logistic reasons.

TABLE 1

Environmental predictors used in the boosted regression tree models to predict probability of the presence of *Ixodes ricinus* nymphs in the modelled Scandinavian region, Denmark, Norway and Sweden, 15 August–30 September 2016

Source	Variables
Modis (Fourier transformed), 2001–12 ^a [44]	Middle infra-red
	Daytime land surface temperature
	Night-time land surface temperature
	Normalised difference vegetation index (NDVI)
	Enhanced vegetation index (EVI)
WorldClim 1.4, 1960–90 [49]	Altitude
BioClim (WorldClim), 1960–90 [49]	BIO1: Annual mean temperature
	BIO2: Mean diurnal range (mean of monthly (max–min temperature))
	BIO3: Isothermality (BIO2/BIO7) × 100
	BIO4: Temperature seasonality (standard deviation × 100)
	BIO5: Max temperature of warmest month
	BIO6: Min temperature of coldest month
	BIO7: Temperature annual range (BIO5–BIO6)
	BIO8: Mean temperature of wettest quarter
	BIO9: Mean temperature of driest quarter
	BIO10: Mean temperature of warmest quarter
	BIO11: Mean temperature of coldest quarter
	BIO12: Annual precipitation
	BIO13: Precipitation of wettest month
	BIO14: Precipitation of driest month
	BIO15: Precipitation seasonality (coefficient of variation)
	BIO16: Precipitation of wettest quarter
	BIO17: Precipitation of driest quarter
	BIO18: Precipitation of warmest quarter
	BIO19: Precipitation of coldest quarter
Harmonized World Soil Database v 1.2 (FOA, IIASA), 2009 [50]	Soil types, depicted by Soil Mapping Unit Code of major soil group (FAO-90 soil classification system)
Gridded Population of the World Dataset (SEDAC), 2015 [47]	Population counts per 1 km ²

^a For each variable, the Fourier processing output includes mean, minimum, maximum, variance in raw data, combined variance in annual, bi-annual, and tri-annual cycles as well as amplitude, phase and variance of annual, bi-annual and tri-annual cycle.

All predictors come as raster files with a resolution of 1 km².

on the island of Bornholm and at one emerging site in northern Zealand with two human cases [17,18].

The increase in incidence and geographical range of pathogens and their tick vector is likely to be a combination of several factors, e.g. climate and availability of host species [6,7], which all affect the ticks' life cycle and therefore their distribution and the possibility of tick-borne diseases being present in specific regions [7,19]. Many hard ticks, as *I. ricinus*, are sensitive to climate and weather [1,6], and are restricted to live in areas with high rainfall and vegetation that keeps a humidity of at least 80%, to prevent desiccation when the ticks are off-host [1,7]. Knowing the distribution of ticks may help pinpoint potential risk areas for disease transmission and guide health authorities in determining where to focus surveillance efforts, where to use preventive measures, or where to put emphasis on informing people.

Determining tick distribution can be a difficult task depending on the size of the area of interest. Throughout Scandinavia, there have been several field studies on ticks and their associated pathogens [3,8,12,20–24], but in order to predict tick presence in unsampled regions in the present but potentially also for the future, we need repeatable survey methods and to find factors associated with tick abundance that can aid us in developing models with high predictive power. In Norway, Jore et al. [2] used sheep serum antibody-positive for tick-borne *Anaplasma phagocytophilum* as a proxy for tick presence, finding effects of temperature, abundance of large cervids and farm animals as well as land cover on tick distribution. Studies in Sweden found significant effects of climate, vegetation parameters and length of vegetation period on tick abundance and distribution [13,14]. In Denmark, Jensen [23] found that *I. ricinus* nymph abundance was significantly affected by the interaction between soil water capacity and the number of hunted roe deer.

TABLE 2

Number of sites surveyed and data on presence/absence of *Ixodes ricinus* nymphs, Denmark, Norway and Sweden, 15 August–30 September 2016

Country	Total number of sites surveyed	Number of sites with presence of <i>Ixodes ricinus</i> nymphs	Number of sites with absence of <i>Ixodes ricinus</i> nymphs
Denmark	37	32	5
Norway	47	38	9
Sweden	75	55	20

Several other studies from Europe and North America have also found a link between environmental factors and tick distribution, such as temperature, vegetation indexes and vapour pressure [25-27].

Although climate, land cover and host abundance may all play a role in tick distribution, it can often be difficult to obtain extensive data on host species, whereas environmental, weather and climate data are more readily available from satellite images and weather models. Machine learning techniques are increasingly used in developing models for vector predictions as they are flexible, can account for nonlinearity and interactions and can handle different types of predictor variables, such as satellite images of environmental data [28,29]. Machine learning techniques combined with environmental predictors have been used in modelling biting midges (*Culicoides* sp.) [30-33], and mosquitoes [28,34,35], and studies on ticks include modelling tick distribution or abundance [36-38] as well as the distribution of tick-borne human diseases [15,39].

The risk of human exposure to ticks, and potentially tick-borne diseases, depends on tick and host dynamics as well as human behaviour [40]. Several studies have reported that living in areas in close proximity to forest increases the risk of LB or TBE [41-43] as *I. ricinus* is more abundant in forest habitats [21,40].

We here present a novel map of nymphal *I. ricinus* distribution for Scandinavia using machine learning algorithms applied to field data, collected in a strict standardised design in the period from 15 August to 30 September 2016. Furthermore, we relate our modelling results of tick distribution to public data on human population density and to the distance to the predicted suitable tick habitats, in order to assess the potential public health impact.

Methods

Stratification of study region and site selection

This study was part of a larger study, where additional objectives were to measure tick abundance and collect nymphs for pathogen detection in Denmark, Norway and Sweden. The field collection region for *I. ricinus* nymphs was for logistical reasons limited to 274,660 km² including all of Denmark, southern

Norway and southern Sweden as well as the Swedish eastern coastal zone (Figure 1). Within this area, we excluded all altitudes of 450 m above sea level and higher (19,926 km²), where ticks are rare or absent [5]; these altitudes were also excluded from the final prediction map.

We stratified the remaining land area (234,191 km², excluding lakes and waterways) using Fourier processed satellite imagery of the normalised difference vegetation index (NDVI) [44] and Corine land cover data (1 km² resolution) [45] to define forest and meadow habitats. Other land cover categories were not sampled for ticks and were left out of the prediction map. For details about the stratification and Fourier-processed satellite imagery, see the Supplement.

We randomly selected 30 first-priority sample sites (80% forest and 20% meadow, Supplementary Table S2) in each of the three countries (R 3.4.2 [46] and sampleStratified in the raster package). This number was logistically the maximum number of sites feasible to visit within a reasonable timeframe. We decided to collect 80% of the samples from forested areas, as forest areas are the most important tick habitat [21,40]. Furthermore, 10 alternative sites for each first-priority site were randomly selected in the same stratum and ordered in priority after shortest distance to original site. These alternative sites were created in case of problems with access to the priority area or difficulties collecting nymphs (for pathogen detection). If a priority area could not be sampled, we would move on to the first alternative site and so forth, keeping the abundance data from the original site if available. For each meadow site, we additionally created 10 alternative forest sites, to be sampled should it prove impossible to collect ticks in meadows.

Because we were interested in investigating tick abundance along the Oslo Fjord in detail, we chose a further 20 random sites along the fjord (maximum distance of 800 m from the coast), with 10 alternatives for each of the 20 sites (same setup as above, Supplementary Table S3).

Field study

For logistical reasons, we conducted the field study between 15 August and 30 September 2016. We

measured tick abundance during the day between 11:00 and 16:00, using a 100 m north- and a 100 m east-facing transect, meeting at a 90° angle at one end. We sampled for questing *I. ricinus* ticks by dragging a white flannel cloth (1.05 × 1.15 m, containing lead weights at one end) 100 m along each transect, turning and dragging it 100 m back; we removed and counted larvae, nymphs, adult male and adult female ticks every 50 m. As some sites had very low abundance of nymphs or none, an alternative site with lower priority was chosen for nymph collection, while keeping abundance data from the original sites, thus resulting in a different number of sites with abundance measures per country. If one or more nymphs were found on the two transects, the site was classified as ‘nymph presence’ else it was classified as ‘nymph absence’.

Presence/absence modelling

We developed a boosted regression tree (BRT) prediction model on the presence/absence data for nymphs, using 92 environmental predictors (Table 1). BRT is a machine learning technique based on two algorithms: regression trees and gradient boosting [29]. This technique allows predictions of a response variable, in our case presence/absence. The estimated probability of presence (PP) can then be plotted as a risk map with a resolution of 1 km². For additional details regarding the environmental predictors, the BRT method used, balancing of the data and cross validation of the model, see the Supplement.

The MODIS-derived data (Table 1) stem from time series data (12 years), whereas our field sampling only occurred in the year 2016. However, at any given time, the abundance and presence of *I. ricinus* instars are influenced by environmental conditions in previous years (adult females surviving to lay eggs, survival of eggs during winter, prolonged diapause of nymphs and larvae) and are not just dependent on the environmental conditions in the collection year. Time series data provide us with data on seasonality and the potential range of the environmental variables, allowing us to make more general predictions on *I. ricinus* distribution in southern Scandinavia.

Human risk of tick exposure

After identifying a final prediction map, we used the Gridded Population of the World dataset (raster with 1 km² resolution [47], Table 1), to identify the number of people living in areas within various distances to forest and meadows where the PP was higher or equal to 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%. We chose distances from 1 km to 5 km to depict people living in close proximity to potential tick habitats. Details can be found in the Supplement.

Results

Field study

We measured tick abundance at 37 sites in Denmark, 75 sites in Sweden and 47 sites in Norway. The 159

sites constitute 63.6 km of dragged transects (Table 2, Figure 1).

Presence/absence modelling

The final BRT model had an accuracy of 0.85, a sensitivity of 91% and a specificity of 60% (given a fixed cut-off of 50% PP). The area under the curve for the receiver operating characteristic was 0.86 (Supplementary Figure S2) [29]. As specificity was only 60% (with the default PP cut-off of 50%), we plotted the prediction errors (observed data – mean predicted probability of presence over the folds and the repeats) in order to visualise a potential spatial pattern (Supplementary Figure S3). From the spatial map, we concluded that the low specificity was mainly due to sites in Denmark and Norway (close to the Swedish border). The final prediction map encompassed 100%, 68.4% and 85.8% of Denmark, Norway and Sweden’s total land area, respectively (Figure 2). We only made predictions for forest and meadow habitats that corresponded to our sampling sites. Habitats with at least 50% PP of tick nymph presence (17.5% of the total modelled area) constituted 15.7% of Denmark’s, 7.4% of Norway’s and 23.9% of Sweden’s land area within the modelled region. Assuming that tick presence in the areas of northern Norway and Sweden not included in the modelled region was below 50% PP, the percentage of a predicted tick risk of at least 50% was 5.1% and 20.5% of the total land area of Norway and Sweden, respectively.

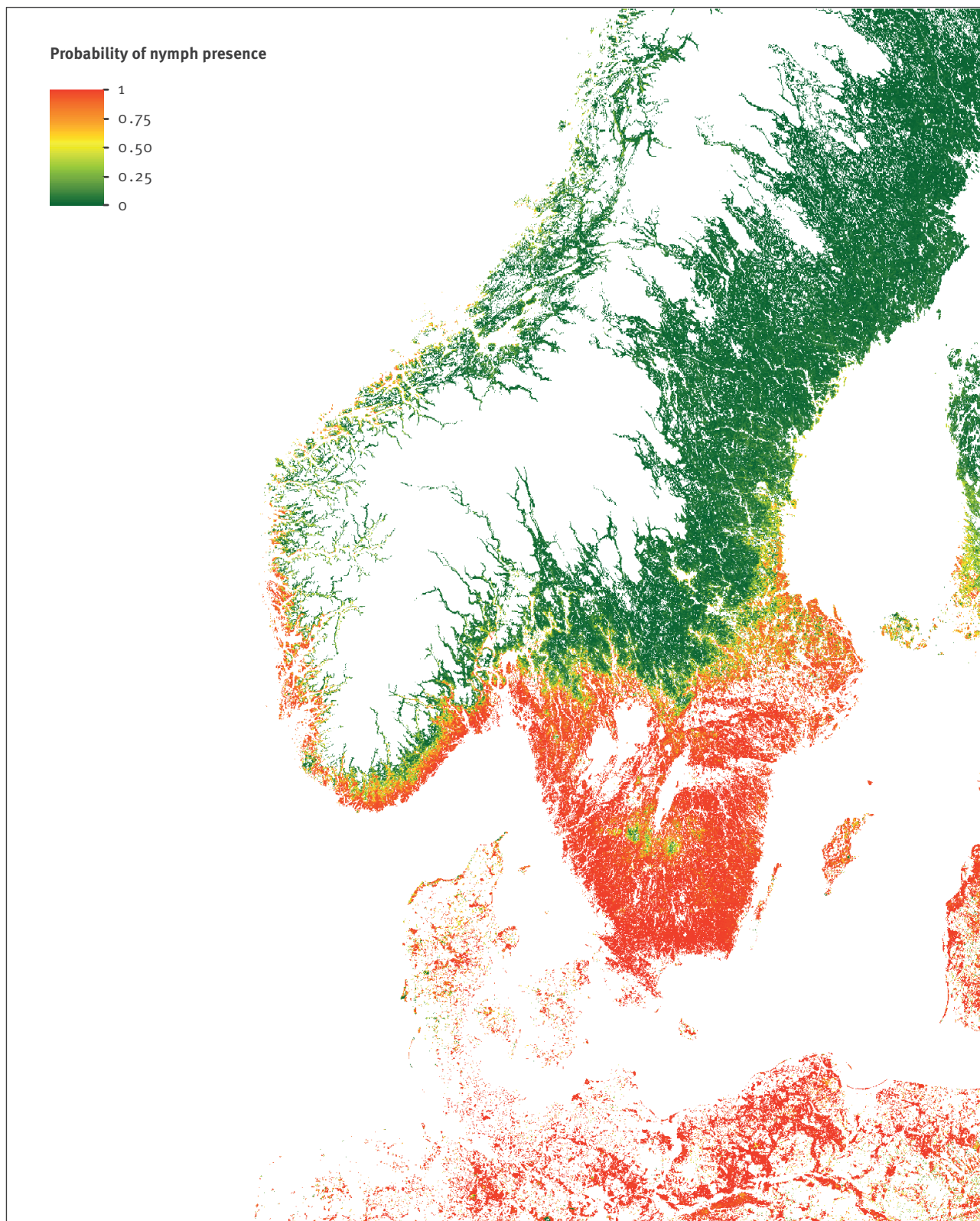
The most important predictors in the final model were day- and night-time land surface temperatures and other parameters related to temperature, land cover (lower PP in transitional woodland-shrub compared with the other cover types), the middle infrared index and related parameters, and parameters related to the vegetation indices enhanced vegetation index (EVI) and NDVI (see plots of the top 5 predictors, Supplementary Figure S4).

Human risk of tick exposure

The modelled region incorporating all altitudes included 19.4 million people, with 5.5 million (28.4%), 4.5 million (23.2%) and 9.4 million (48.5%) in Denmark, Norway and Sweden, respectively, which corresponded to 100% of the total Danish population, 91% of the total Norwegian population and 97% of the total Swedish population (based on the population density raster file). The proportion of people living within 1 km of forest and meadow was consistently lower for Denmark (ranging from 11% to 7% with increasing PP) than for Norway (ranging from 37% to 13% with increasing PP) and Sweden (ranging from 37% to 26% with increasing PP) for all PP values (Figure 3). This number increased consistently as distance to forest and meadow reached 5 km with 76–61%, 88–44% and 85–73% of the regional population living within 5 km of forest or meadow with PP values ranging from 0.1 to 0.9 for Denmark, Norway and Sweden, respectively (Figure 3). Figure 4 depicts

FIGURE 2

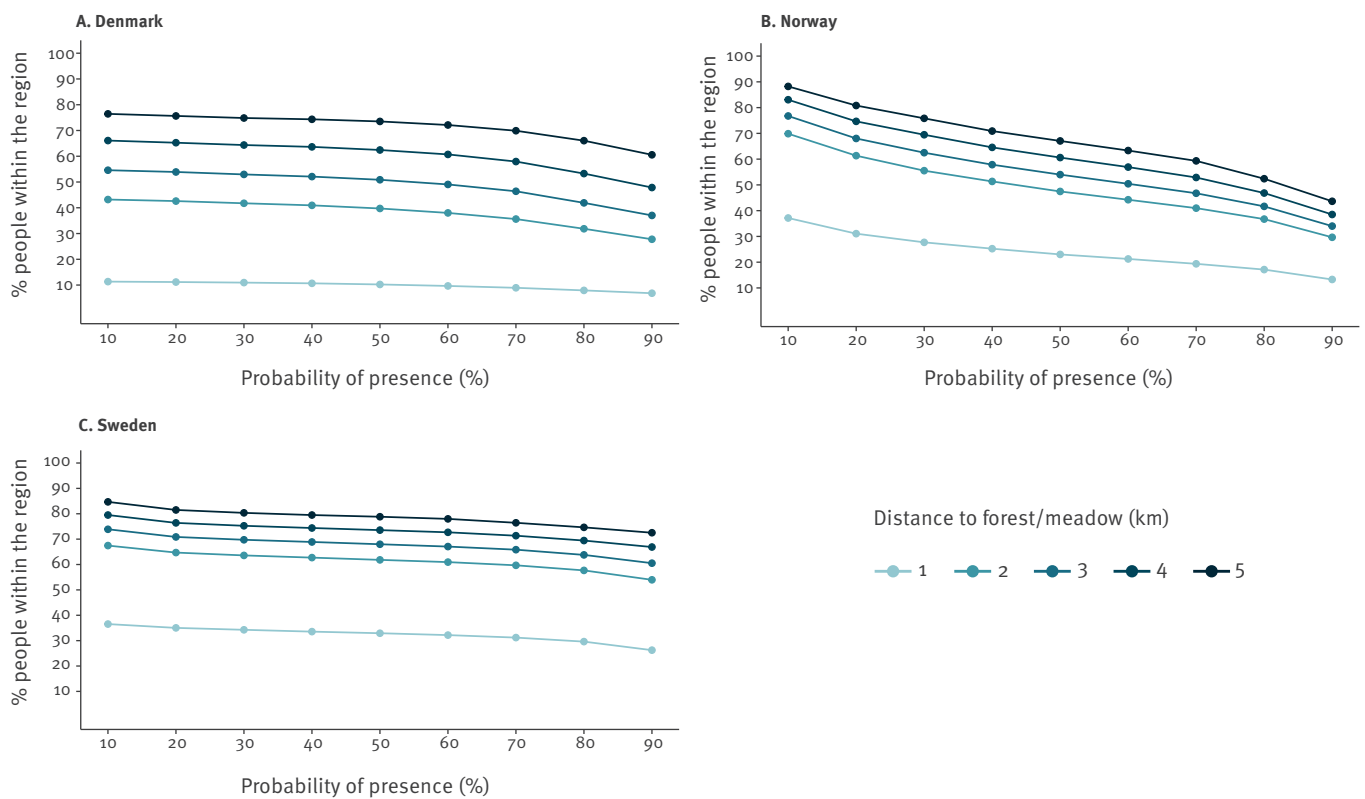
Predicted probability of presence of nymphal *Ixodes ricinus*, produced by the final boosted regression tree model, Denmark, Norway and Sweden, 15 August–30 September 2016



This map depicts the predicted region (100%, 68.4% and 85.8% of Denmark's, Norway's and Sweden's total land area). White areas within Denmark, Norway and Sweden are altitudes above 450 m or lakes, rivers and streams, or habitats other than our sampled forest and meadow habitats (not predicted).

FIGURE 3

Percentage of people in the predicted region living within 1, 2, 3, 4 and 5 km of forest and meadow with different cut-offs for probability of presence of nymphal *Ixodes ricinus*, Denmark, Norway and Sweden, 15 August–30 September 2016



areas where people live within 1, 3 and 5 km of forest or meadow for a fixed PP value of 50%.

Discussion

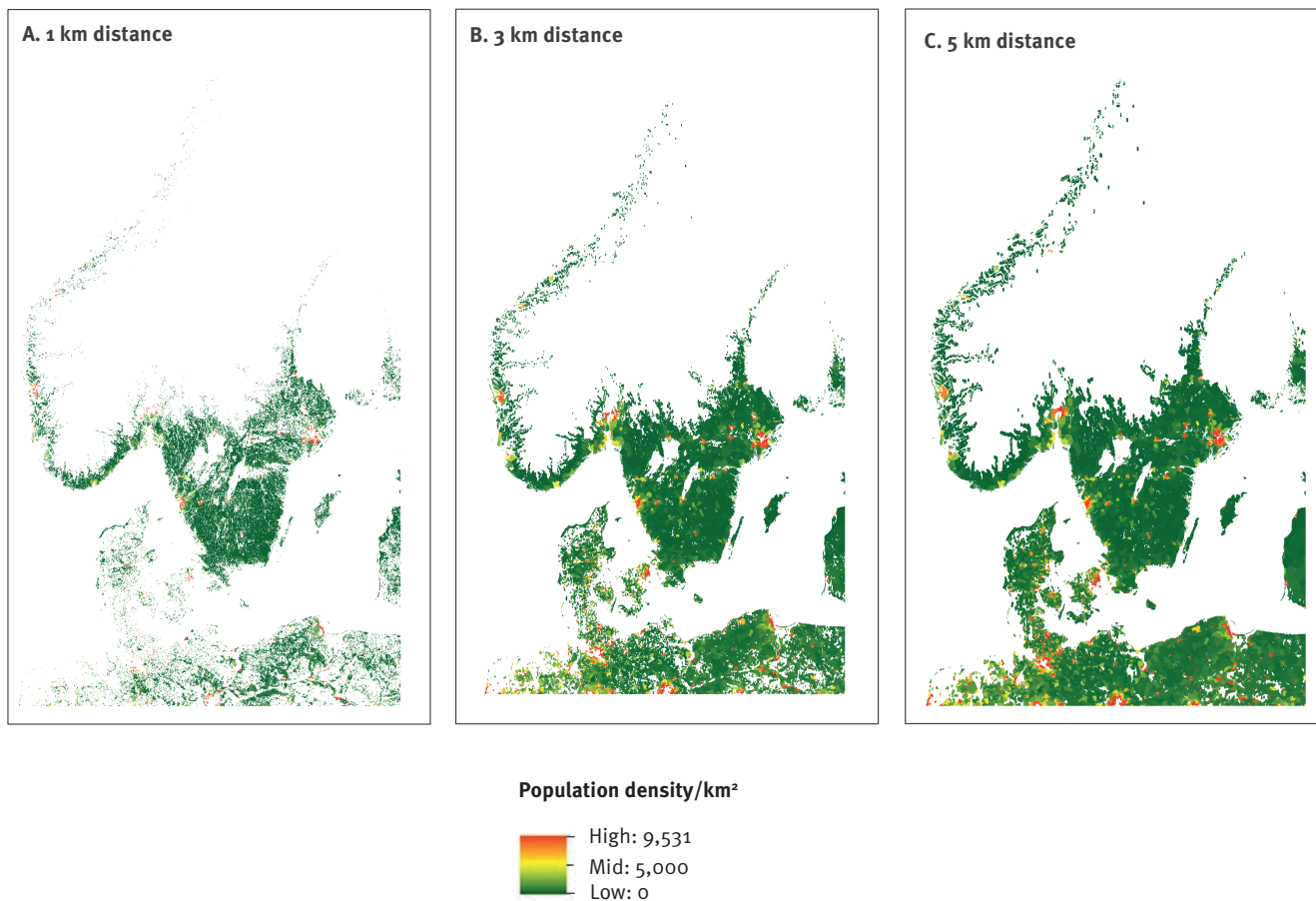
Using the machine learning technique Boosted Regression Trees, we were able to create maps of the probability of nymphal *I. ricinus* presence in Scandinavia with high predictive power based on a standardised repeatable procedure. The predicted distribution corresponded well with what is generally believed about tick distribution in Scandinavia, assuming that a PP lower than 50% is a true absence. The higher probabilities of presence around the southern Norwegian coast line is in agreement with the distribution maps known for Norway [5,24]. In Sweden, we found higher PPs in the southern parts, with a boundary north of the large lakes, above which PP was low. This border coincides well with the biogeographical and climatic boundary called Limes Norrlandicus (LN) that separates the species-rich boreo-nemoral zone with shorter and milder winters in the south, from the boreal zone in the northern parts of Sweden [48]. Before the 1980s, LN used to be the range limit for *I. ricinus* in Sweden [4], but since then, the range of *I. ricinus* has expanded beyond this biogeographical border albeit at low abundances [4]. Our model reflected this pattern, showing higher PP below LN and a quick drop in PP

above LN, but with a low PP throughout this northern region. The distinct patches of low PP below the great lakes in Sweden follow observed lower temperatures at these two elevated areas (Supplementary Figure S5). The PP was high throughout Denmark, except for the dry heathlands and sandy habitats of central and western Jutland. This pattern corresponds well with what we know about tick biology and the need for a high relative humidity to sustain ticks in a given habitat [7].

Our model had low specificity compared with the sensitivity. Since the main priority of this study was prediction of true presences, we refrained from increasing the specificity, which could have been obtained by choosing a higher cut-off value than the fixed 50%. In general, certainty of true absence can be hard to obtain, as presence/absence is always dependent on the sampling effort. Our recorded absences may not have been true absences and our model may still have predicted presence based on the environmental variables for that specific site. Conversely, high local abundance of deer hosts may facilitate establishment of ticks in areas for which the model predicted absence. In our data, we had a low proportion of absences (21%) and for Denmark alone, this number was 15.6%. Even though we used balancing methods to account for this disproportion, it is possible that our empirically collected sample could

FIGURE 4

Areas with people living at different distances to forest/meadow that have a probability of presence of nymphal *Ixodes ricinus* of at least 50%, Denmark, Norway and Sweden, 15 August–30 September 2016



not feed the model with enough absence data to learn how to accurately predict absences, thus resulting in low specificity.

We were able to create a model with high predictive power based on environmental predictors. We found that land surface temperatures as well as measures of high vegetation cover (middle infrared light is absorbed by leaves and vegetation, thus densely vegetated areas reflect less middle infrared light) positively influenced the probability of nymph presence. However, the resulting modelled distribution may be due to other environmental factors correlated with these predictors, such as the climatic impact on vegetation and host species. Although ticks can be directly affected by temperatures and humidity [1,4,6,7], they are also dependent on their host species for survival and dispersal [4,7,9]. Abundance of host species may in turn be directly and indirectly affected by climate and weather [4,7,13], thus making it hard to separate factors into causal and confounding. Despite lacking fine resolution data on host abundance, we were able to use environmental

predictors to create a biologically plausible model for *I. ricinus* presence/absence in Scandinavia.

Overlaying our distribution maps for tick nymphs with human population density maps revealed the proportion of people potentially at risk for tick exposure. Based on studies estimating the risk of LB or TBE in relation to landscape characteristics around residential homes [41-43], we set the maximum distance from forest or meadow to 5 km. In general, we found that a large percentage of the population in the region live within 5 km of forest and meadows with a risk of tick presence, even if we set the cut-off for PP to be higher than the default 50%. Particularly for Norway, our model predicted high probability of nymph presence only for a very small area around the coast line; with a 50% PP cut-off, this area amounted to just 5.1% of Norway's total land area. Whereas this small area seems negligible, human population densities in Norway are relatively higher in these areas, exposing more people to tick habitats than we would expect by looking at the area alone, as 67% of the Norwegian population live within 5 km of forest and meadow

with PP \geq 50%. That changing the PP cut-off value had a larger effect on the percentage of people at risk in Norway compared with Denmark and Sweden is probably due to a steep temperature gradient as we move away from the coast, caused by elevation-dependent temperatures (Supplementary Figure S5).

In the United States, Glass et al. [42] found that the odds of contracting LB increased within ca 1 km of living close to forested habitats. The proportion of people living within 1 km of forest or meadow is particularly low for Denmark no matter the PP cut-off (11–7%). This may however be a gross underestimation of exposure risk as Denmark has many fragmented small forest patches interspersed with agricultural fields and urban areas and these small patches may not show up in our coarse resolution of 1 km². However, little is known about how likely these non-sampled areas are as tick habitats. In Norway and Sweden, a higher proportion of the population (between 37% and 13% at different PP cut-off values) are living within 1 km of forest or meadow.

This study showed that given the current distribution of ticks in Scandinavia, a high percentage of inhabitants are already exposed to the risk of tick bites (within a distance of 5 km to forest or meadow with a 50% PP, respectively 73.5%, 67.1% and 78.8% of the Danish, Norwegian and Swedish population may be at risk). The northward expansion of ticks and tick-borne pathogens in Norway and Sweden is a considerable public health concern [9]. However, human population densities in northern Norway and Sweden are low compared with the southern regions, and a tick range expanding north will therefore affect a smaller proportion of the human population. Our results therefore suggest that it may be desirable to target our surveillance and preventive measures in areas with high human population density and where ticks are well established, i.e. the whole of Denmark, the southern coastal parts of Norway, southern Sweden and Sweden's densely populated eastern coast along the Bothnian Bay.

Machine learning techniques allowed us to produce models and maps with high accuracy and predictive sensitivity for the whole region without having to sample every habitat. These models have highlighted areas at high risk of tick exposure and thus potentially of vector-borne diseases, and can help in targeting these areas for costly surveillance and preventive measures. It is important to note that our model reflects a moment in time, and does not show annual variation in tick distribution or how a future potential increase in temperatures may affect tick distribution and thus the potential for human exposure. Still, the study design is consistent between sites and repeatable, ensuring reliable future comparisons of tick distribution, and the produced maps allow for easy external validation. The resolution used to create our models may be too coarsely grained to catch small hotspots of tick presence/absence and the potential for human

exposure. This is particularly evident for Denmark, which, throughout the country, has numerous small forest fragments smaller than 1 km².

Acknowledgements

We thank Laura Mark Jensen, Simon Friis-Wandall, Mette Frimodt Hansen, Caroline Greisen, Ana Carolina Cuellar, Najmul Haider, Leif Kristian Sortedal, Philip Thomassen Neset, Preben Ottesen, Alaka Lamsal, Ruchika Shakya, Leif Kristian Sortedal, Martin Strnad, Hanne Quarsten, Sølvi Noraas, Åslaug Rudjord Lorentzen, Chiara Bertacco, Kevin Hohwald, Catharina Schmidt, Coco de Koning, and Wenche Okstad for assistance in the field. We would also like to thank the Danish Ministry of the Environment, The Forest and Nature Agency as well as many private landowners for allowing us access to their properties to conduct our sampling. This study was funded by the Interreg V Program (the ScandTick Innovation project, grant number 20200422).

Conflict of interest

None declared.

Authors' contributions

LJK planned and managed the field work set-up, performed field work in Denmark and contributed to field work in Sweden, analysed the data and drafted the manuscript. RB planned the original study, contributed to analysis and drafting the manuscript. AS, KSE, HEHL, KMP, AKA, LK, VK, AS, and SS contributed to field work in Norway and drafting the manuscript. PK, MC, and MT contributed to field work in Sweden and drafting the manuscript. AB contributed to analysis and drafting the manuscript. KK contributed to drafting the manuscript. All authors read and approved the final version of the manuscript.

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Tick-borne encephalitis in Europe, 2012 to 2016

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Citation style for this article:

Beauté Julien, Spiteri Gianfranco, Warns-Petit Eva, Zeller Hervé. Tick-borne encephalitis in Europe, 2012 to 2016. *Euro Surveill.* 2018;23(45):pii=1800201. <https://doi.org/10.2807/1560-7917.ES.2018.23.45.1800201>

Article submitted on 18 Apr 2018 / accepted on 16 Aug 2018 / published on 08 Nov 2018

Since 2012, tick-borne encephalitis (TBE) is a notifiable in the European Union. The European Centre for Disease Prevention and Control annually collects data from 28 countries plus Iceland and Norway, based on the EU case definition. Between 2012 and 2016, 23 countries reported 12,500 TBE cases (Ireland and Spain reported none), of which 11,623 (93.0%) were confirmed cases and 878 (7.0%) probable cases. Two countries (Czech Republic and Lithuania) accounted for 38.6% of all reported cases, although their combined population represented only 2.7% of the population under surveillance. The annual notification rate fluctuated between 0.41 cases per 100,000 population in 2015 and 0.65 in 2013 with no significant trend over the period. Lithuania, Latvia and Estonia had the highest notification rates with 15.6, 9.5 and 8.7 cases per 100,000 population, respectively. At the subnational level, six regions had mean annual notification rates above 15 cases per 100,000 population, of which five were in the Baltic countries. Approximately 95% of cases were hospitalised and the overall case fatality ratio was 0.5%. Of the 11,663 cases reported with information on importation status, 156 (1.3%) were reported as imported. Less than 2% of cases had received two or more doses of TBE vaccine.

Background

Tick-borne encephalitis (TBE) is an infectious disease of the central nervous system caused by a flavivirus and usually transmitted by the bite of infected *Ixodes* spp. These ticks can be found from western Europe to Japan [1]. Less frequently, humans can be infected by drinking contaminated milk. Many vertebrate species can be infected by the TBE virus but ticks are the main reservoir for the virus. There are three subtypes of the TBE virus: the European subtype (TBEV-Eu) is mainly transmitted by *I. ricinus* while both the Far-eastern (TBEV-FE) and Siberian (TBEV-Sib) subtypes are mainly transmitted by *I. persulcatus*. Recent findings from Finland suggest that *I. ricinus* can also transmit TBEV-Sib [2]. In Europe,

most cases are infected by TBEV-Eu but cases infected with TBEV-FE were reported in Estonia and Latvia [1] and with TBEV-Sib in Estonia [3] and Finland [4].

The typical course of the disease is biphasic. After a median incubation period of 8 days, the first stage consists of a few days of non-specific symptoms such as fever, fatigue and body pain. After a symptom-free week, approximately one-third of infected persons can develop neurological conditions [5], ranging from mild meningitis to severe encephalitis [1]; increasing age is a known risk factor for severe TBE. Infection with TBEV-FE is associated with more severe disease with case fatality as high as 20–40% compared with 1–2% with TBEV-Eu [6].

There is no curative treatment for TBE but a vaccine is available. This vaccine is highly immunogenic [7] and the impact of mass vaccination in Austria is suggestive of good effectiveness [8]. Vaccine schedules for the two vaccines licensed in Europe based on TBEV-Eu strains require three doses followed by boosters [1]. In a position paper on TBE vaccination published in 2011, the World Health Organization (WHO) recommended that TBE vaccination should be offered to all age groups in highly endemic areas (i.e. areas with TBE incidence above 5 cases per 100,000 population) [9].

In Europe, most cases occur during June–September [10]. *Ixodes* spp. are found in large parts of Europe but areas at risk for TBE are mainly located in central and eastern Europe and the Baltic and Nordic countries [11]. Between 2000–2010, the annual number of TBE cases reported in the European Union and European Economic Area (EU/EEA) fluctuated between 2,000–3,500 cases [11,12]. Spikes in cases of TBE have occurred in some years, e.g. 2006, but this was likely a result of changes in human behaviour based on suitable weather conditions (e.g. increased outdoor recreational activities) [13]. More recently, some countries, e.g. Belgium and

Box

European Union case definition for tick-borne encephalitis

A confirmed case is defined as any person meeting the clinical criteria i.e. symptoms of inflammation of the central nervous system

AND

- Has laboratory-confirmation i.e. at least one of the following five:
- Tick-borne encephalitis (TBE) specific IgM and IgG antibodies in blood.
- TBE specific IgM antibodies in cerebrospinal fluid.
- Sero-conversion or fourfold increase of TBE-specific antibodies in paired serum samples.
- Detection of TBE viral nucleic acid in a clinical specimen.
- Isolation of TBE virus from clinical specimen.

A probable case is defined as any person meeting the clinical criteria and the laboratory criteria for a probable case i.e. detection of TBE-specific IgM-antibodies in a unique serum sample

OR

Any person meeting the clinical criteria with exposure to a common source (unpasteurised dairy products).

the Netherlands, reported possible new endemic foci having found antibodies to the TBE virus in roe deer and cattle [14,15] and in 2016, the Netherlands reported their first locally-acquired human case [16]. The mapping of endemic foci is essential to make recommendations for vaccination programme and travel advice [17]. In 2011, the first attempt to collect TBE surveillance data at the EU/EEA level underlined the need for an agreed case definition and systematic data collection [11]. Therefore, in 2012, the European Commission included TBE in the list of notifiable diseases in the EU/EEA [17].

Here, we describe TBE cases reported in the EU/EEA between 2012 and 2016.

Methods

Since 2012, the European Centre for Disease Prevention and Control (ECDC) requires all 28 EU Member States, plus Iceland and Norway, to annually report their TBE data to the European Surveillance System (TESSy) database using the EU case definition (Box) [18]. More detailed information on surveillance systems is available elsewhere [10]. We included all cases reported during the years 2012–2016 meeting the EU case definition in the analysis.

TBE Information received included age, sex, date of disease onset, probable place of infection, place of residence, importation status, hospitalisation status, vaccination status, and clinical outcome. Coded values

for variables with geographical information (probable place of infection and place of residence) followed the nomenclature of territorial units for statistics (NUTS) of the EU [19].

We used population denominator data provided by the Statistical Office of the EU (Eurostat) for calculating rates (data extracted on 22 September 2017). We compared continuous variables by the Mann–Whitney U test and categorical variables using the chi-squared test. We estimated annual rates of change and their 95% confidence intervals (CI) using a log-linear regression of notification rates over the period 2012–2016. We assessed goodness of fit of linear regressions using F statistics. We used Stata software release 14 (StataCorp. LP, United States) for all data management and statistical analyses.

Results

Case classification and notification rate

Over the 2012–2016 period, 23 countries reported 12,500 TBE cases (Ireland and Spain reported no cases), of which 11,622 (93.0%) were confirmed cases and 878 (7.0%) probable cases (Table 1). We excluded 31 cases with unknown classification (11 cases for Austria, 15 cases for Lithuania, four cases for Poland and one case for Slovenia). Cyprus, Iceland, Malta, and Portugal had no TBE surveillance and Denmark did not report any data.

Most countries (18/23) reported over 90% of cases as confirmed. Slovakia (552/638; 86.5%), France (36/44; 81.8%), Hungary (131/171; 76.6%), Latvia (683/953; 71.7%), and Poland (712/1,040; 68.5%), classified the lowest proportions of their cases as confirmed. The mean annual notification rate was 0.54 cases per 100,000 population.

Importation

Of the 11,664/12,500 cases reported with information on importation status, 156 (1.3%) were reported as imported (Table 1). Importation status was missing for cases reported by Bulgaria, Croatia, and Finland. All cases reported in Belgium, Luxembourg, and the United Kingdom (UK) were imported. Information on the probable country of infection was available for 152 of these imported cases (97.4%). Top destinations for travel-associated TBE were Austria (32 cases, 21.1% of all imported cases), Sweden (19 cases, 12.5%) and Finland (18 cases, 11.8%). Four countries (the Czech Republic, Germany, Lithuania, and Sweden) reported 102/156 (65.3%) of all imported cases. Imported cases were slightly younger than locally-acquired cases (median age for imported cases: 46 years; locally-acquired cases: 48 years; $p = 0.03$) and more likely to be male (imported cases: 71% males; locally-acquired cases: 59% males; $p < 0.01$).

Geographical distribution

Two countries (Czech Republic and Lithuania) accounted for 4,825/12,500 (38.6%) of all reported cases (Table 1).

TABLE 1

Number of reported cases^a of tick-borne encephalitis, percentage of imported cases, notification rate per 100,000 population and trend, in 25 countries, European Union and European Economic Area, 2012–2016 (n = 12,500)

Country	2012		2013		2014		2015		2016		Total cases			Trend	
	Number of cases	Notification rate	Number of cases	Notification rate	Number of cases	Notification rate	Number of cases	Notification rate	Number of cases	Notification rate	Number of cases	Imported cases n (%) ^b	Notification rate	Annual variation (%)	95% CI
Austria	38	0.45	100	1.18	81	0.95	79	0.92	95	1.09	393	6 (1.7)	0.92	15.2	- 19.6 to 49.9
Belgium	2	0.02	1	0.01	0	0.00	1	0.01	1	0.01	5	3 (100)	0.01	NA	NA
Bulgaria					0	0.00	2	0.03	0	0.00	2	Unknown	< 0.01	NA	NA
Croatia	45	1.05	44	1.03	23	0.54	26	0.62	6	0.14	144	Unknown	0.68	- 45.1	- 90.5 to 0.3
Czech Republic	573	5.45	625	5.94	410	3.90	349	3.31	565	5.35	2,522	16 (0.6)	4.79	- 6.2	- 33.0 to 20.6
Estonia	178	13.43	114	8.64	83	6.31	116	8.82	81	6.16	572	2 (0.3)	8.68	- 15.4	- 39.0 to 8.2
Finland	39	0.72	38	0.70	47	0.86	68	1.24	61	1.11	253	Unknown	0.93	14.4	0.7 to 28.1
France	1	< 0.01	2	< 0.01	9	0.01	10	0.02	22	0.03	44	9 (21.4)	0.01	77.3	42.0 to 112.7
Germany	195	0.24	419	0.52	264	0.33	219	0.27	348	0.42	1,445	47 (3.5)	0.36	4.6	- 31.1 to 40.2
Greece	0	0.00	0	0.00	1	0.01	1	0.01	0	0.00	2	0	< 0.01	NA	NA
Hungary	44	0.44	53	0.53	31	0.31	24	0.24	19	0.19	171	2 (1.2)	0.35	- 24.5	- 42.9 to - 6.1
Ireland	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0	0	NA	NA
Italy			0	0.00	0	0.00	5	0.01	48	0.08	53	3 (6.3)	0.02	NA	NA
Latvia	229	11.20	230	11.36	149	7.44	141	7.10	204	10.36	953	0	9.51	- 6.3	- 30.2 to 17.7
Lithuania	494	16.45	487	16.39	353	11.99	336	11.50	633	21.91	2,303	18 (0.9)	15.64	2.2	- 28.3 to 32.7
Luxembourg	NA	NA	NA	NA	0	0.00	1	0.18	0	0.00	1	1 (100)	0.06	NA	NA
The Netherlands	NA	NA	NA	NA	NA	NA	NA	NA	4	0.02	4	2 (50.0)	0.02	NA	NA
Norway	7	0.14	6	0.12	13	0.25	9	0.17	12	0.23	47	7 (15.9)	0.18	13.7	- 13.9 to 41.3
Poland	187	0.49	226	0.59	195	0.51	149	0.39	283	0.75	1,040	8 (0.8)	0.55	4.2	- 22.4 to 30.7
Romania	3	0.01	3	0.01	1	0.01	0	0.00	0	0.00	7	0	< 0.01	NA	NA
Slovakia	102	1.89	163	3.01	116	2.14	84	1.55	173	3.19	638	5 (0.8)	2.36	3.8	- 31.2 to 38.9
Slovenia	164	7.98	307	14.91	100	4.85	62	3.01	83	4.02	716	1 (0.2)	6.95	- 29.7	- 78.6 to 19.2
Spain	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0	0	NA	NA
Sweden	287	3.03	209	2.19	178	1.85	268	2.75	238	2.42	1,180	21 (1.8)	2.44	- 2.2	- 24.3 to 19.9
UK	3	< 0.01	0	0.00	2	< 0.01	0	0.00	0	0.00	5	5 (100)	< 0.01	NA	NA
EU/EEA	2,591	0.64	3,027	0.65	2,056	0.43	1,950	0.41	2,876	0.58	12,500	156 (1.3)	0.54	- 6.6	- 29.1 to 16.0

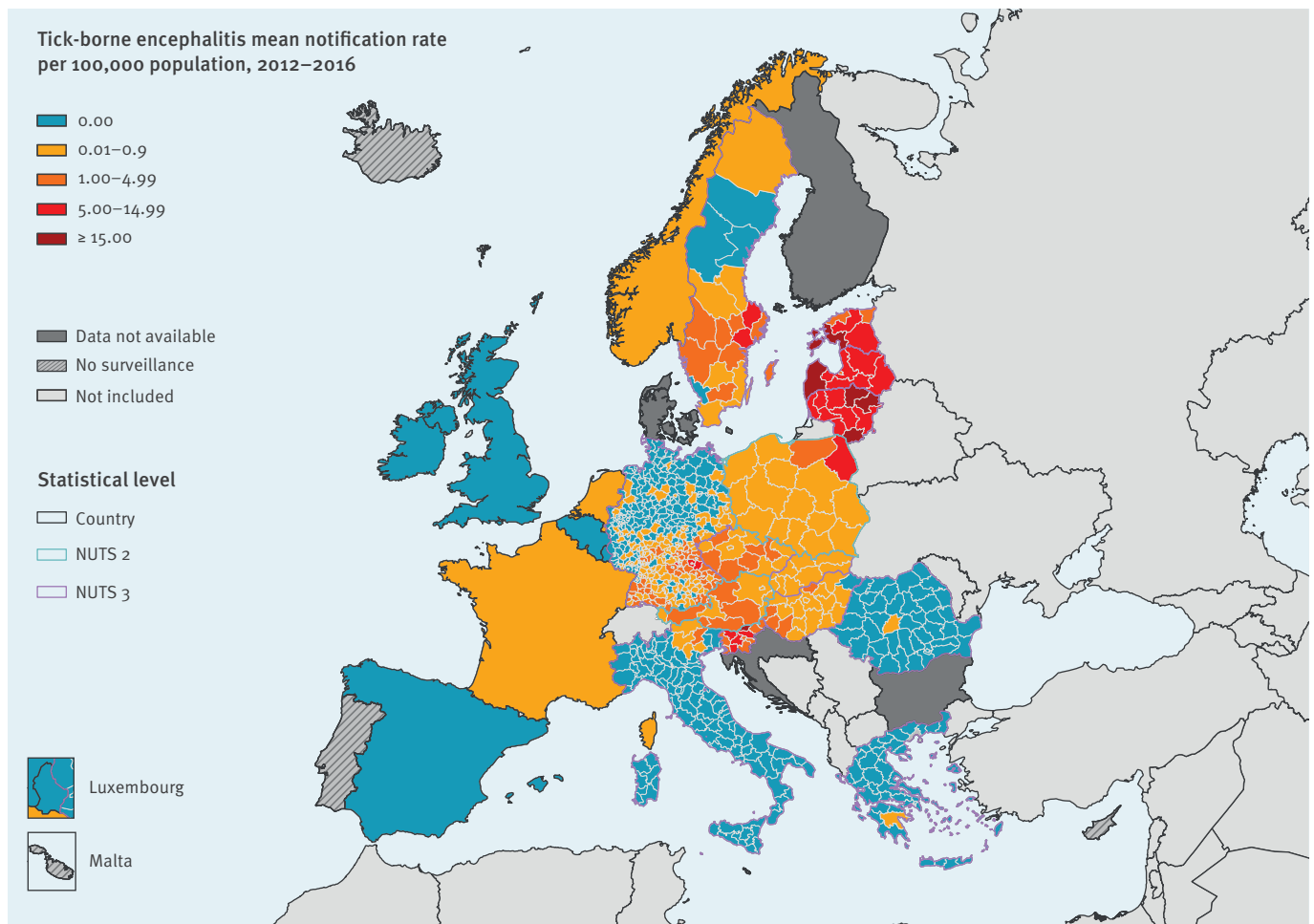
CI: confidence interval; EU/EEA: European Union and European Economic Area; NA: not available; UK: United Kingdom.

^a Number of cases includes both confirmed and probable cases.

^b cases with known importation status.

FIGURE 1

Rate of locally acquired tick-borne encephalitis per 100,000 population, by place of infection, European Union and European Economic Area countries, 2012–2016



ECDC. Map produced on: 19 July 2018

Of the 23 countries that reported cases, 16 had mean notification rates below one case per 100,000 population. Over the 2012–2016 period Lithuania, Latvia and Estonia had the highest notification rates with 15.6, 9.5 and 8.7 cases per 100,000 population, respectively (Table 1 and Figure 1). Among the 23 countries that reported cases, 17 had locally-acquired cases. Of these, 12 provided geographical information at NUTS3 level, two at NUTS2 (Austria and Poland), and three did not have information at subnational level (France, the Netherlands, and Norway) (Figure 1). At the subnational level, six regions had mean annual notification rates above 15 cases per 100,000 population: Utena county, Lithuania (44.5), Lääne-Eesti, Estonia (27.7), Kurzeme, Latvia (23.8), Alytus county, Lithuania (22.1), Panevėžys county, Lithuania (19.1) and Carinthia, Slovenia (15.1) (Figure 1). Twenty-nine regions in seven countries (Estonia, Germany, Latvia, Lithuania, Poland, Slovenia and Sweden) had notification rates above five cases per 100,000 population. In Lithuania, Telšiai County was the region with the lowest mean annual notification rate (5.6).

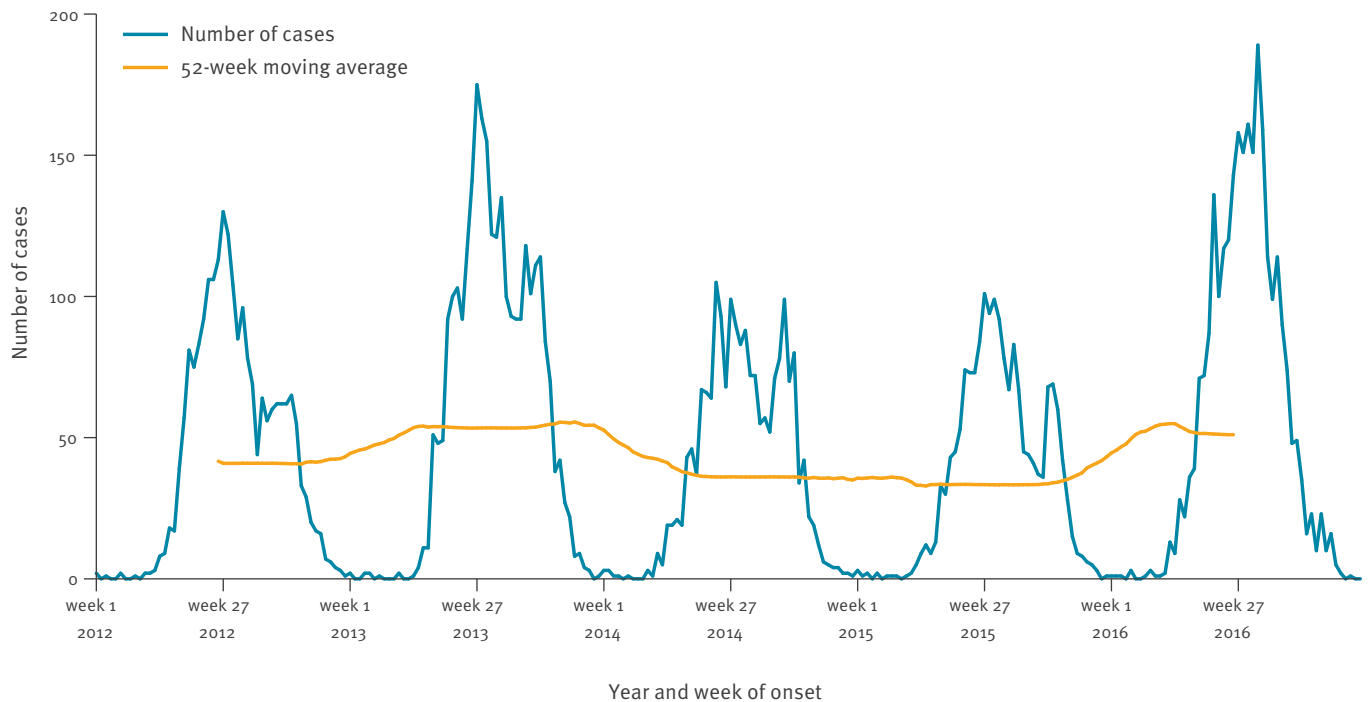
Trend and Seasonality

The overall annual notification rate fluctuated between a minimum of 0.41 cases per 100,000 population in 2015 and a maximum of 0.65 in 2013 with no significant trend over the period (annual variation of - 6.6% (95% CI: - 29.1 to 16; $p = 0.4$) (Table 1). We observed significant trends for three countries: the TBE notification rate increased at an annual rate of 14.4% (95% CI: 0.7 to 28.1) in Finland and 77.3% (95% CI: 42.0 to 112.7) in France and decreased at an annual rate of 24.5% (95% CI: 6.1 to 42.9) in Hungary.

Of the 11,397 cases reported with onset date, 10,632 (93.3%) had an onset month May–October and 135 (1.2%) had an onset month December–March (off-season) (Figure 2). We observed a comparable seasonality in the 12 countries reporting at least 100 cases over the period with onset month (Figure 3). There were peaks in 2012 (Estonia and Sweden), 2013 (Germany, Hungary, Slovakia, Czech Republic and Slovenia), 2014 (Austria), 2015 (Austria, Estonia, Finland, Sweden), and

FIGURE 2

Number of reported tick-borne encephalitis cases by month of onset, and 12-month moving average, 19 European Union and European Economic Area countries, 2012–2016



Includes data from: Austria, Czech Republic, Estonia, France, Germany, Greece, Hungary, Italy, Latvia, Lithuania, Luxembourg, Netherlands, Norway, Poland, Romania, Slovakia, Slovenia and Sweden.

2016 (Czech Republic, Germany, Lithuania, Poland and Slovakia).

Demographics

Of the 12,470 cases reported with information on age, 6,782 (54.4%) were in the 40–69 years old group (Table 2). TBE was more common in males with a male-to-female rate ratio of 1.5:1. Notification rates increased with age in both sexes, peaking at 0.89 cases per 100,000 population in males aged 60–69 years, and then decreased in older age groups (Figure 4). At date of disease onset, females (median 51 years, interquartile ratio (IQR): 35–62) were older than males (median 47 years, IQR: 31–61) ($p < 0.01$).

Outcome

Of the 8,081 cases reported with hospitalisation status, 7,672 (94.9%) were admitted to hospital (Table 2). Of the 9,889 cases reported with known outcome, 48 (0.5%) died and 247 (2.5%) had neurological sequelae. The case fatality ratio did not differ significantly by sex (0.5% in males vs 0.4% in females, $p = 0.30$). The case fatality ratio was higher in older age groups (3.1% in cases aged 80 years or older, 2.0% in cases aged 70–79 years and $< 0.5%$ in cases aged below 70 years).

Vaccination

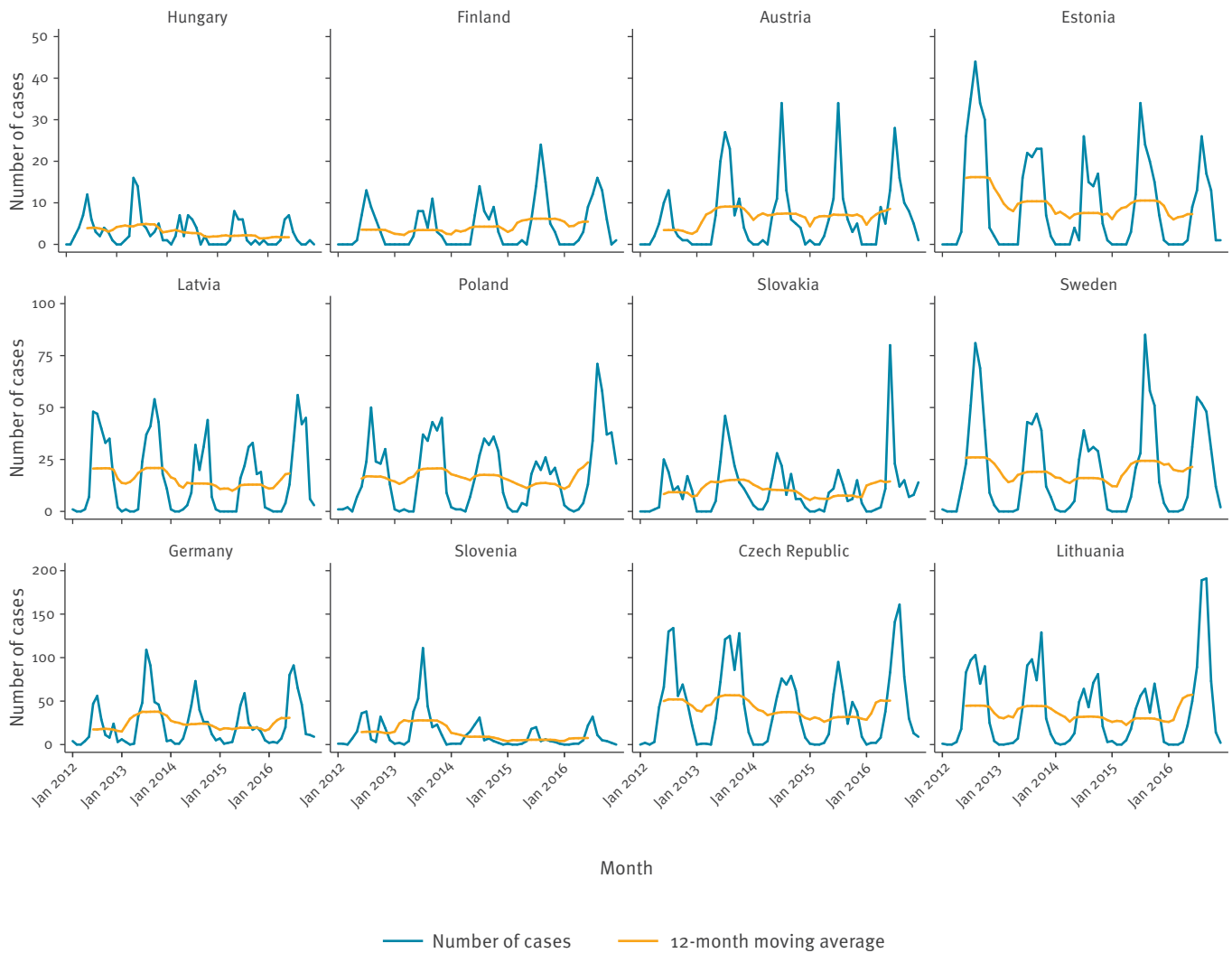
Of the 5,205 cases with known vaccination status, 5,066 (97.3%) were not vaccinated, 60 had received one or two doses (1.2%), 60 (1.2%) three doses or more and 19 (0.4%) an unknown number of doses (Table 2). Of the 20 cases with fatal outcome and known vaccination status, 19 were not vaccinated and one had received one dose of the vaccine. The proportion of cases that received two doses or more of vaccine was higher in the extreme age groups compared with the other groups (2.5% in both cases aged 20 years or younger and 70 years or older). No imported cases had received more than one vaccine dose.

Discussion

The European TBE surveillance data suggest a stable trend over the years 2012–2016 with no reported changes in national surveillance systems; continuing the long-term trend observed in Europe since the mid-1990s [12]. The number of TBE cases reported in Europe, excluding Russia, increased over the years 1990–1994, probably reflecting the start of surveillance in many countries [12]. Over the following 15 years (1995–2009), the trend was stable with an annual number of TBE cases fluctuating between 2,000 and 4,000 cases. Peaks occurred when a set of countries reported unusually high numbers of TBE cases, e.g. 2006 and 2009 [12]. In 2013, several European countries experienced

FIGURE 3

Number of reported tick-borne encephalitis cases by week of onset and 52-week moving average, 12 European Union and European Economic Area countries, 2012–2016



a peak in TBE cases, which resulted in the highest number of TBE cases (> 3,000) observed in Europe that year. An analysis carried out in eight European countries suggested that human behaviour in response to good weather conditions, e.g. increased outdoor recreational activities, was the main explanation for the 2006 spike rather than tick abundance [13].

The overall stable trend observed in TBE surveillance data is mainly driven by a few countries reporting the majority of cases, potentially masking important disparities both between and within countries. For example, two countries (Czech Republic and Lithuania) accounted for 38.6% of all reported TBE cases, although their combined population represented only 2.7% of the population of the 25 countries included in this analysis. All countries with average annual notification rate above one TBE case per 100,000 population had a stable trend over the period. We only observed an increase in Finland and France. In France,

the notification rate almost tripled in 2016 compared with previous years in the Alsace region where most cases occurred [20]; some newly identified foci such as the Alpine region could also have contributed to the upsurge in cases. However, the reasons behind this increase are yet to be determined. In Finland, the emergence of new foci reported during 1995–2013 could partly explain the increase [21]. A decrease in TBE cases was observed in Hungary over the years 2012–2016, to our knowledge there is no explanation as to why. Trends at country level, such as these, may mask changes at local level as TBE endemicity is very focal and countries do not have a uniform risk across all territories/regions/counties etc. In Lithuania, which had the highest average annual notification rate, there was an eightfold difference between counties with highest and counties lowest TBE incidence. An analysis of epidemiological patterns of TBE in Lithuania suggested different trends across counties with more pronounced increases in eastern and northern parts

TABLE 2

Main characteristics of reported cases of tick-borne encephalitis, European Union and European Economic Area countries, 2012–2016 (n = 12,500)

Characteristics	Number of cases	Percent	Notification rate per 100,000 persons
Total	12,500	100	0.54
Age group (years)			
< 20	1,402	11.2	0.29
20–29	1,257	10.1	0.44
30–39	1,575	12.6	0.50
40–49	2,200	17.6	0.65
50–59	2,474	19.8	0.77
60–69	2,108	16.9	0.80
70–79	1,183	9.5	0.63
≥ 80	271	2.2	0.23
Unknown	30	NA	NA
Sex			
Female	5,118	40.9	0.43
Male	7,381	59.1	0.65
Unknown	1	NA	NA
Importation status			
Imported	156	1.3	NA
Locally-acquired	11,507	98.7	NA
Unknown	837	NA	NA
Hospitalisation			
Yes	7,672	94.9	NA
No	409	5.1	NA
Unknown	4,419	NA	NA
Outcome			
Alive	9,594	97.0	NA
Dead	48	0.5	NA
Neurological complications	247	2.5	NA
Unknown	2,611	NA	NA
Vaccination status			
Four doses	24	0.5	NA
Three doses	36	0.7	NA
Two doses	27	0.5	NA
One dose	33	0.6	NA
Vaccinated unknown doses	19	0.4	NA
Not vaccinated	5,066	97.3	NA
Unknown	7,295	NA	NA

NA: not available.

of the country [22]. Similarly, diverging trends across regions were reported in Austria [23]. Decreasing trend were observed in north-east of Lower Austria whilst the alpine regions in the west of Austria became highly endemic.

Independently of what happens in animal reservoirs, we can classify factors driving TBE incidence in three

groups: (i) tick abundance, (ii) population at risk, (iii) surveillance characteristics. Factors related to tick abundance are multiple (e.g. land, weather, reservoirs etc.) and can be very focal. The impact of climate change is debated with possibly different effects in different settings. A study carried out in Sweden suggested that milder winters were associated with increased TBE incidence in the mid-1980s [24]. Yet, a general circulation model predicted that TBE transmission could be disrupted by climate change with a contraction of TBE areas to higher altitudes in central Europe and northern latitudes in Scandinavia [25]. This would result in a decreased incidence in the coming decades but such change would probably not be captured over a 5-year period. Changes in human behaviour (e.g. increase of at-risk outdoor recreational activities) can put people at greater risk of exposure to ticks and thus TBE. However, with increased vaccine coverage such risk could be improved. Finally, better clinical awareness, testing and reporting would improve the ability of the surveillance system to detect cases.

The geographical granularity of our data (at best NUTS3) does not allow fine monitoring of TBE foci, which countries are best placed to perform. However, during the first effort to collect TBE data at the EU/EEA level most of the recommendations were followed [11]. We implemented standard EU case definition for TBE and initiated routine collection of surveillance data from EU/EEA countries, to at least NUTS-3 geographical level for most of the countries. ECDC encourages all countries to report their cases at subnational level.

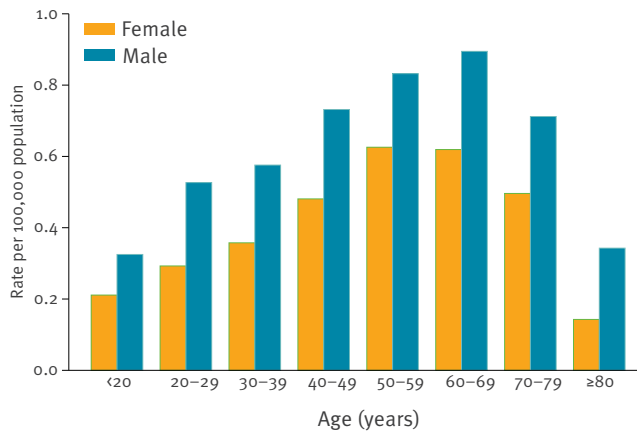
The reported TBE cases followed a pronounced seasonality with most cases occurring during the warmer months May–October, which is likely due to human habits with people spending a greater amount of time outdoors in areas e.g. forests where ticks populations are high [1]. Cases infected during colder months are possible, however, especially in central Europe.

Cases of TBE are more common in older age groups, with the highest number of cases occurring in those aged 40–69 years. The highest notification rate, in those aged 60–69 years, most likely reflects high exposure to tick populations at an age where individuals have increased time for outdoor recreational activity, but also fall into the known higher severity seen in older age groups [26].

Almost 95% (7,672/8,081) of reported TBE cases were admitted to hospital, which is not unexpected given that the clinical criteria used in the case definition selects severe cases. Even though the overall case fatality was relatively low, it was far from negligible in older age groups at ca 2–3% above 70 years of age. Previous reviews suggested that a third of patients could suffer long-lasting sequelae [1]. Our analysis found a much lower proportion but it is likely that our data could not capture long-term sequelae that would

FIGURE 4

Notification rates of tick-borne encephalitis per 100,000 population, by sex and age group and male-to-female rate ratio by age group, European Union and European Economic Area countries, 2012–2016



be diagnosed later in time from acute infection and thus not reported.

Vaccination remains the most effective protective measure against TBE [27]. However, studies have reported vaccine failures, especially in older age groups [28]. We found that 87/5,205 (1.7%) of cases were supposedly vaccinated (at least two doses of vaccine), mostly in extreme age groups. This would be in line with results from studies suggesting that age and number of vaccine doses were the most important factors determining the immunological response to vaccination [29]. The extended period between doses may mean that people are less likely to comply to the recommendations as shown in Germany where compliance after the first dose was low [30]. Another reason for not receiving or completing TBE vaccination is cost. TBE vaccination is not reimbursed in most EU/EEA countries and the willingness to pay for vaccination may not be sufficient to ensure uptake in residents or visitors frequenting areas considered high risk for tick populations and TBE [31]. A survey published in 2008, reported that Austria, Finland, Germany, Hungary, Latvia and Slovenia included TBE in their routine vaccination programme at least for some specific groups or areas [32].

In this study, we only found a few TBE cases in international travellers. Cases that are resident in countries with little or no risk of TBE are less likely to be vaccinated or diagnosed [33]. Increased awareness of TBE is required to improve vaccination coverage in travellers and promote the best practices to avoid tick bites. Currently, the WHO recommends vaccination of travellers who are at risk of TBE exposure during outdoor activities in rural endemic areas during the period of transmission [34].

Conclusion

The overall TBE notification rate remained stable during 2012–2016. Surveillance at EU/EEA level helped provide reliable and comparative data allowing better mapping of the disease risk both at the national and subnational level. Countries with regions where the disease is highly endemic should consider strengthening information campaigns on preventive measures against tick bites as well as introducing TBE vaccine recommendations if these are not already proposed. ECDC encourages countries to report better quality and more complete data on TBE diagnoses, particularly on the sub-national geographic distribution and on imported cases.

Acknowledgements

We would like to thank all people involved in the surveillance of TBE in the participating countries as well as the data managers at ECDC, without whom surveillance of TBE at EU/EEA level would not be possible. In addition, we would like to thank the following persons for their contribution to TBE surveillance in Europe: Heidemarie Holzmann and Karin Stiasny (Austria); Tinne Lernout, Vanessa Suin and Marjan Van Esbroeck, (Belgium); Sanja Kurecic Filipovic, Iva Pem Novosel and Goranka Petrovic (Croatia); Bohumír Kříž Marek Malý and Helena Šebestová (Czech Republic); Markku Kuusi, Jussi Sane and Pirjo Turtiainen (Finland); Isabelle Leparç-Goffart (France); Doris Altmann and Wiebke Hellenbrand (Germany); Theano Georgakopoulou, Kassiani Gkolfinopoulou, Anna Papa, and Danai Pervanidou (Greece); Jeff Connell and Sarah Jackson (Ireland); Giovanni Rezza, Caterina Rizzo and Giulietta Venturi (Italy); Antra Borman, Tatjana Klemjacionoka and Irina Lučenko (Latvia); Saulius Čaplinskas, Aidas Spiečius and Milda Žygutienė (Lithuania); Eelco Franz and Agnetha Hofhuis (The Netherlands); Solveig Jore and Heidi Lange (Norway); Cornelia Ceianu, Romana Rebreanu and Anca Sirbu (Romania); Edita Staroňová and Elena Tichá (Slovakia); Marta Grgic-Vitek, and Maja Socan (Slovenia). We are also grateful to Silviu Lucian Ionescu for his support in GIS.

Conflict of interest

None declared.

Authors' contributions

EWP and HZ initiated the work. GS and JB ran the analysis. All authors contributed to the interpretation of the findings. JB wrote the first draft of the manuscript. All authors revised the manuscript, providing substantial intellectual input.

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National bulletins

ALBANIA

Health bulletin
Institute of Public Health
Quarterly, online. In English.
<http://www.ishp.gov.al/wp-content/uploads/2014/04/Bulletin-20141-2.pdf>

AUSTRIA

Public Health Newsletter - Mitteilungen für das österreichische Gesundheitswesen
Bundesministerium für Gesundheit/ Ministry of Health, Vienna
Published monthly. Distribution only by email. In German.
Link to past editions: http://www.bmg.gv.at/home/Schwerpunkte/Krankheiten/Newsletter_Public_Health/
Link to registration: <http://bmg.gv.at/home/Service/Newsletter/>

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Vlaams Infectieziektebulletin
Department of Infectious Diseases Control, Flanders.
Bimonthly, online. In Dutch, summaries in English.
<http://www.infectieziektebulletin.be>
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Scientific Institute of Public Health, Brussels
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<https://epidemo.wiv-isp.be/ID/Pages/flashes.aspx?lcid=1036>
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Institute for Public Health of the Federation of Bosnia and Herzegovina
<http://www.zzjzfbih.ba/epidemioloski-bilteni/>
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Bulletin of the National Centre of Infectious and Parasitic Diseases, Sofia
Print version. In Bulgarian.
<http://www.ncipd.org/>

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Medical and Public Health Services, Ministry of Health, Nicosia
Biannual, print and online. In Greek.
<http://www.moh.gov.cy>

CZECH REPUBLIC

Zprávy CEM (The Bulletin of Centre for Epidemiology and Microbiology)
Státní zdravotní ústav (National Institute of Public Health), Prague
Monthly, print and online (6 month later after print version). In Czech, with abstracts in English.
<http://www.szu.cz/publications-and-products/zpravy-epidemiologie-a-mikrobiologie>
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Státní zdravotní ústav (National Institute of Public Health), Prague
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DENMARK

EPI-NEWS
Department of Infectious Disease Epidemiology and Prevention, Statens Serum Institut, Copenhagen.
Weekly, via e-mail subscription and online. In Danish and English (one week later).
<https://en.ssi.dk/news/epi-news>

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Monthly, online. In English
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National Institute for Health and Welfare (THL), Department of Health Security. In Finnish
<https://thl.fi/fi/web/infektiaudit>

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Bulletin épidémiologique hebdomadaire (BEH)
Santé publique France, Saint-Maurice
Bi-monthly, online. In French, with abstracts in English
Bi-monthly, online. In French, with abstracts in English
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Weekly, print and online. In German.
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Updates, online. In Greek.
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National Center For Epidemiology, Budapest
Weekly, online. In Hungarian.
<http://www.oek.hu/oek.web?to=839&nid=41&pid=7&lang=hun>

ICELAND

EPI-ICE
Landlknisembtti, Directorate Of Health, Seltjarnarnes
Monthly to quarterly, online. In Icelandic and English.
<https://www.landlaeknir.is/english/epi-ice/>

IRELAND

EPI-INSIGHT
Health Protection Surveillance Centre, Dublin
Monthly, online. In English.
<http://www.hpsc.ie/epi-insight/>

ITALY

Notiziario dell'Istituto Superiore di Sanita
Istituto Superiore di Sanita, Reparto di Malattie Infettive, Rome
Monthly, online. In Italian.
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Istituto Superiore di Sanita, Reparto di Malattie Infettive, Rome
Monthly, online. In Italian.
<http://www.epicentro.iss.it/ben>

LATVIA

Epidemiologijas Biletēni
Sabiedrības veselības agentūra

Public Health Agency, Riga
Online. In Latvian.
<http://www.sva.lv/epidemiologija/bileteni>

LITHUANIA

Epidemiologijos žinios
Užkrečiamųjų ligų profilaktikos ir kontrolės centras
Center for Communicable Disease Prevention and Control, Vilnius
Online. In Lithuanian.
<http://www.ulac.lt/index.php?pl=26>

MALTA

IDCU notifiable infectious disease tables
Infectious Disease Prevention and Control Unit, Department of Health
Promotion and Disease Prevention
Monthly and annually, online. In English.
https://ehealth.gov.mt/HealthPortal/public_health/idcu/library/library_menu.aspx

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Rijksinstituut voor Volksgezondheid en Milieu
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Monthly, online. In Dutch.
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Nytt om smittevern
Folkehelseinstituttet, Oslo.
Online. In Norwegian.
<http://www.fhi.no/tema/smittevern-og-overvaaking>

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Panstwowy Zakład Higieny
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Fortnightly, online. In Polish and English.
http://www.pzh.gov.pl/epimeld/index_p.html#o1

PORTUGAL

Portugal Saúde em Números / Health by Numbers Portugal
Ministério da Saúde,
Direcção-Geral da Saúde, Lisbon.
Digital only. In Portuguese and English.
<https://www.dgs.pt/publicacoes/revista-cientifica-da-dgs.aspx>

ROMANIA

Centrul pentru Prevenirea și Controlul Bolilor Transmisibile, National Centre
of Communicable Diseases Prevention and Control, Institute of Public Health,
Bucharest
Sporadic, print only. In Romanian.
<http://www.cnscbt.ro/>

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eNboz - Elektronske novice s področja nalezljivih bolezní in okoljskega
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Intitit za varovanje zdravja, Center za nalezljive bolezni
Institute of Public Health, Center for Infectious Diseases, Ljubljana
Monthly, online. In Slovene.
<http://www.nijz.si/sl/e-nboz-o/>

SPAIN

Boletín Epidemiológico Semanal
Centro Nacional de Epidemiología, Instituto de Salud Carlos III, Madrid
Fortnightly, print and online. In Spanish.

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Nyheter och press
Folkhälsomyndigheten, Stockholm.
Weekly, online. In Swedish.
<https://www.folkhalsomyndigheten.se/nyheter-och-press/>

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ENGLAND AND WALES

Health Protection Report
Public Health England, London
Weekly, online only. In English.
<https://www.gov.uk/government/collections/health-protection-report/latest-infection-reports>

NORTHERN IRELAND

Transmit: Health protection service bulletin
Public Health Agency, Belfast.
Monthly. In English.
<http://www.publichealth.hscni.net/search/node/transmit>

SCOTLAND

Health Protection Scotland Weekly Report
Health Protection Scotland, Glasgow.
Weekly, print and online. In English.
<http://www.hps.scot.nhs.uk/ewr/index.aspx>

EUROPEAN UNION

“Europa” is the official portal of the European Union. It provides up-to-date
coverage of main events and information on activities and institutions of the
European Union.
<http://europa.eu>

EUROPEAN COMMISSION - PUBLIC HEALTH

The website of European Commission Directorate General for Health and
Consumer Protection (DG SANCO).
<http://ec.europa.eu/health/>

HEALTH-EU PORTAL

The Health-EU Portal (the official public health portal of the European Union)
includes a wide range of information and data on health-related issues and
activities at both European and international level.
<http://ec.europa.eu/health-eu/>

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

European Centre for Disease Prevention and Control (ECDC)
The European Centre for Disease Prevention and Control (ECDC) was
established in 2005. It is an EU agency with aim to strengthen Europe’s
defences against infectious diseases. It is seated in Stockholm, Sweden.
<http://www.ecdc.europa.eu>

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Paper TQ-AD-19-003-EN-C ISSN 1025-496X

PDF TQ-AD-19-003-EN-N ISSN 1560-7917

Graphic design © ECDC, Stockholm



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