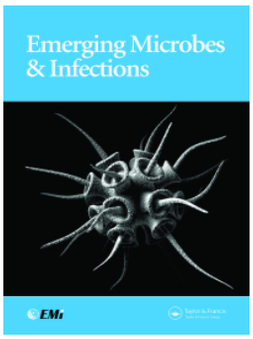

Emerging Microbes & Infections

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/temi20

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To cite this article: Julian W. Bakker, Helen J. Esser, Hein Sprong, Gert-Jan Godeke, Tabitha E. Hoornweg, Willem F. de Boer, Gorben P. Pijlman & Constantianus J. M. Koenraadt (2024) Differential susceptibility of geographically distinct *Ixodes ricinus* populations to tick-borne encephalitis virus and louping ill virus, Emerging Microbes & Infections, 13:1, 2321992, DOI: 10.1080/22221751.2024.2321992

To link to this article: https://doi.org/10.1080/22221751.2024.2321992

Emerging Microbes & Infections
2024, VOL. 13, 2321992 (13 pages)
https://doi.org/10.1080/22221751.2024.2321992

Differential susceptibility of geographically distinct *Ixodes ricinus* populations to tick-borne encephalitis virus and louping ill virus

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ABSTRACT

Tick-borne encephalitis virus (TBEV) is an emerging pathogen in the Netherlands. Multiple divergent viral strains are circulating and the focal distribution of TBEV remains poorly understood. This may, however, be explained by differences in the susceptibility of tick populations for specific viruses and viral strains, and by viral strains having higher infection success in their local tick population. We investigated this hypothesis by exposing Dutch *Ixodes ricinus* ticks to two different TBEV strains: TBEV-NL from the Netherlands and TBEV-Neudoerfl from Austria. In addition, we exposed ticks to louping Ill virus (LIV), which is endemic to large parts of the United Kingdom and Ireland, but has not been reported in the Netherlands. Ticks were collected from two locations in the Netherlands: one location without evidence of TBEV circulation and one location endemic for the TBEV-NL strain. Ticks were infected in a biosafety level 3 laboratory using an artificial membrane feeding system. Ticks collected from the region without evidence of TBEV circulation had lower infection rates for TBEV-NL as compared to TBEV-Neudoerfl. *Vice versa*, ticks collected from the TBEV-NL endemic region had higher infection rates for TBEV-NL compared to TBEV-Neudoerfl. In addition, LIV infection rates were much lower in Dutch ticks compared to TBEV, which may explain why LIV is not present in the Netherlands. Our findings show that ticks from two distinct geographical populations differ in their susceptibility to TBEV strains, which could be the result of differences in the genetic background of the tick populations.

KEYWORDS Ticks; vector-competence; artificial membrane feeding

Introduction

Tick-borne encephalitis virus (TBEV) is one of the most important arthropod-borne viruses in Europe and endemic in large parts of Eurasia. Each year, between 4,000 and 9,000 cases of tick-borne encephalitis (TBE) are reported in humans, with most cases occurring in Central- and Eastern-Europe and Russia [1]. TBEV is transmitted by hard ticks of the family Ixodidae, predominantly by *Ixodes ricinus* in West- and Central-Europe and *I. persulcatus* in Eastern-Europe [2]. An east to west spread of TBEV has been observed and the first autochthonous human cases of TBE were detected in the Netherlands in 2016, in the United Kingdom in 2019 and in Belgium in 2020 [3–6]. The distribution of TBEV was thought to be restricted by the need to have synchronous activity of TBEV-infected nymphs and uninfected larvae co-feeding on rodents, triggered by a rapid spring warming [7,8]. However, conventional TBEV risk maps based on rapid spring warming did not predict that the Netherlands and the United Kingdom are at risk for TBEV [9]. Besides climatic factors, other factors such as the vector competence of arthropods may play a role in the spread of pathogens, as observed in the spread of chikungunya virus in mosquitoes [10]. However, the vector competence of ticks for different tick-borne viruses is an underexplored topic, so studying vector competence of ticks may shed light on the distribution and spread of tick-borne viruses in Europe.

Besides TBEV, other tick-borne flaviviruses circulate in Europe, such as louping ill virus (LIV) in the United Kingdom and Ireland (Figure 1). While LIV is predominantly found on the British Isles, it has also been found in Norway [11], Denmark [12] and Russia [13]. Though closely related to TBEV, the transmission cycle of LIV has important differences in terms of vertebrate hosts, pathogenicity for humans, and habitats associated with LIV circulation. The LIV transmission cycle is dominated by sheep, red grouse

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/22221751.2024.2321992.

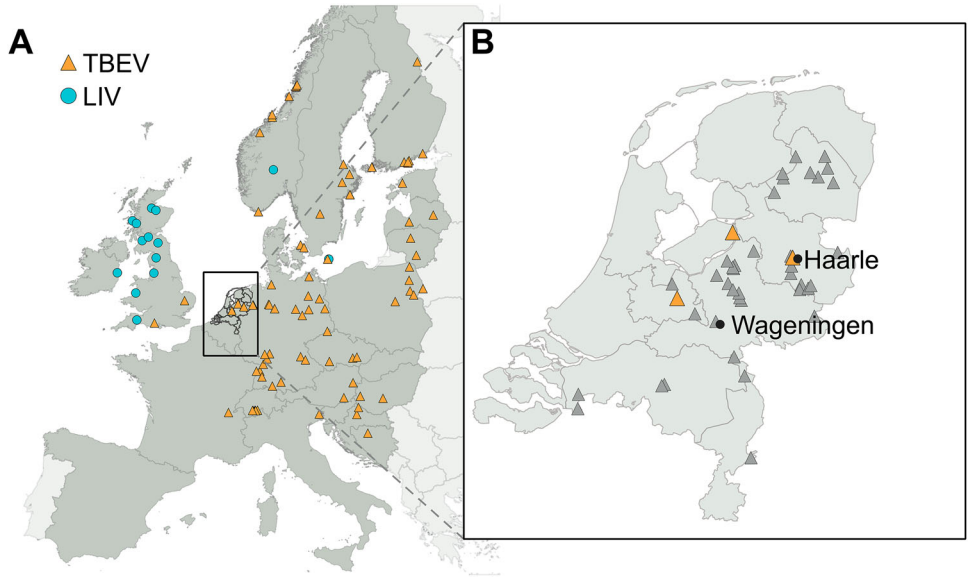

Figure 1. Tick-borne encephalitis virus (TBEV) European subtype and louping ill virus (LIV)in western Europe (A) and the Netherlands (B). (A) Blue circles indicate the presence of LIV, yellow triangles indicate the presence of TBEV European subtype. (B) Grey triangles indicate TBEV negative pools of *Ixodes ricinus*, yellow triangles indicate *I. ricinus* tick pools positive for TBEV. The tick sampling locations for this study are shown as Haarle and Wageningen. Data for tick surveillance in the Netherlands were reproduced from [27]. TBEV/LIV locations can be found in Supplementary Table S2.

and mountain hare as reservoir hosts and *I. ricinus* ticks as vector. Human LIV cases are very rare compared to TBEV [14–16]. The spread of LIV to the European mainland may be hindered by the availability of reservoir hosts[15], and, potentially, the vector competence of local *I. ricinus* populations, but this has so far not been tested.

In contrast to LIV, the vertebrate hosts for TBEV are small rodents, which are assumed to be viraemic for only 2–4 days, so that systemic transmission plays a minor role in virus maintenance [17]. Conversely, ticks can remain infected with TBEV for over 120 days and therefore play an important role as reservoir for this pathogen [18]. Moreover, experimental studies have demonstrated that co-feeding of infected nymphs and uninfected larvae on rodent hosts is a highly efficient TBEV-transmission pathway that can even occur when ticks feed on TBEV-immune hosts [19,20]. Co-feeding transmission is therefore regarded as critical for TBEV maintenance [17,21,22].

TBEV is recognized to have a focal distribution [23]. Typically, genetic variation of TBEV strains within TBEV foci is low, whereas genetic variation between TBEV foci is high [24,25]. On the other hand, closely related TBEV strains can be found in geographically distant foci hundreds of kilometres apart [24]. In the Netherlands for example, three genetically divergent TBEV strains are known to circulate, including two classical European subtypes of TBEV as well as a more unique strain, here termed TBEV-NL [26]. The first European subtype of TBEV was found in the Utrechtse Heuvelrug region (TBEV NL/UH) and is closely related to a Swedish strain (strain 1993/783, Figure 2). The second European subtype of TBEV was found near Dronten (TBEV NL-RMB2) and is closely related to a German strain (Rauher Busch) [27]. The third forms an outgroup of the known European subtypes and has only been found in the Sallandse Heuvelrug region of the Netherlands (strain TBEV-NL) and in the United Kingdom [28]. Migratory birds most likely play a role in long-distance spread of TBEV, but it remains unclear why local spread of TBEV strains does not occur more frequently.

A possible explanation for the limited local spread could be differences in vector competence in tick populations for specific viral strains. The establishment of tick-borne viruses in Dutch *I. ricinus* ticks, such as LIV and TBEV, could therefore be restricted by local differences in vector competence. We therefore quantified to what extent different strains of TBEV and LIV affect infection success, as proxy for vector competence, and aimed to determine the infection success of TBEV and LIV *in vitro* in tick and human cell lines and *in vivo* in field-collected *I. ricinus* ticks, by the use of an artificial feeding system. Furthermore, we tested whether the studied viruses produced similar infection patterns in ticks from geographically different populations.

Materials and methods

Cells and viruses

Human lung carcinoma A549 (ATCC CCL-185) and African green monkey kidney Vero E6 (ATCC CRL-1586) cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin (Gibco) and

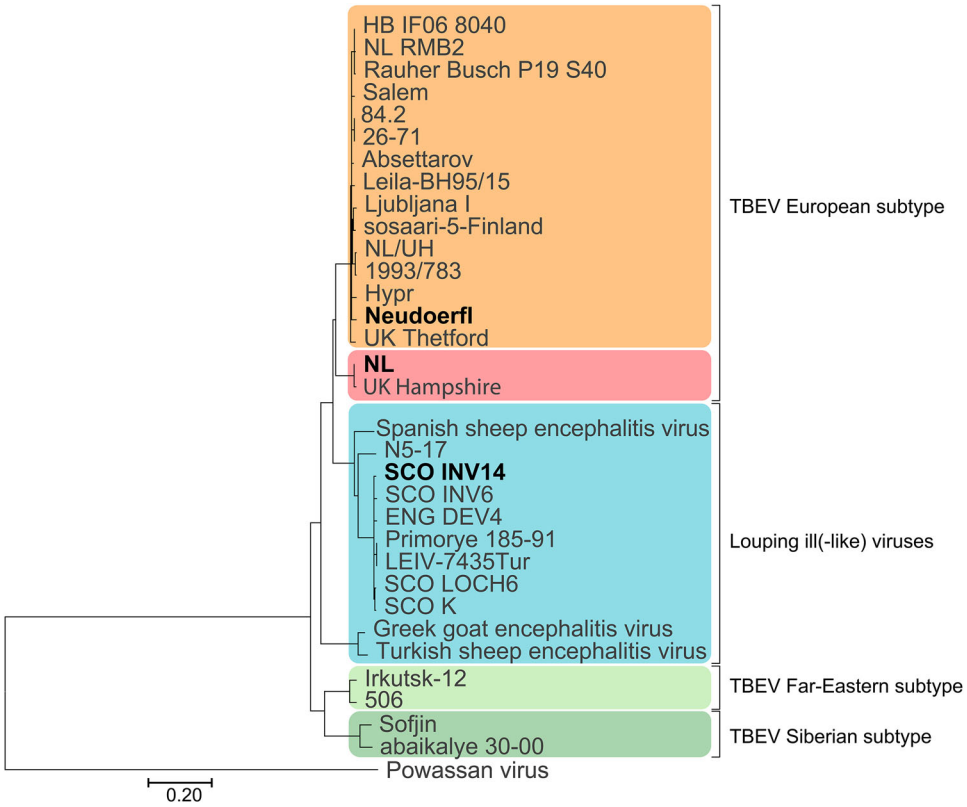

Figure 2. Maximum likelihood phylogenetic tree of the polyprotein of selected tick-borne flaviviruses. GenBank accession numbers for the tick-borne flaviviruses included are shown in Supplementary Table S1. Powassan virus was used as outgroup. Strains used in this study are highlighted in bold. The scale bar represents the number of substitutions per site.

100 µg/mL streptomycin (Sigma-Aldrich, Zwijndrecht, the Netherlands) and maintained as monolayers in T25 flasks at 37 °C with 5% CO_2. For virus growth kinetics, infectivity assays and end-point dilution assays with ticks, Vero E6 cells were cultured in HEPES-buffered DMEM medium (DMEM + GlutaMax™, Gibco) supplemented with gentamicin (50 µg/ml; Gibco) and fungizone (2.5 µg/ml of amphotericin B and 2.1 µg/ml of sodium deoxycholate; Gibco). *Ixodes ricinus* IRE/CTVM19 cells [29] were cultured in sealed flat-sided culture tubes (Nunc™) in Leibovitz L-15 medium (Gibco) supplemented with 20% heat-inactivated FBS, 10% Tryptose phosphate broth (Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin at 28 °C in a total volume of 2.2 mL in flat-sided cell culture tubes (Nunc™).

A passage 3 (P3) stock of LIV-INV14 [30] (GenBank accession no. MK007541), a P4 stock of TBEV-Neudoerfl [31] (GenBank accession no. U27495), and a P3 stock of TBEV-NL [32] (GenBank accession no. ON502378) were used for infection experiments. All virus stocks were grown on A549 cells and viral titres were determined using end point dilution assays (EPDA) on Vero E6 cells as described below.

Virus growth kinetics

A 6-well plate with 70-80% confluent monolayer of Vero E6 cells was incubated with the indicated virus at a multiplicity of infection (MOI) of 0.01 for 2 h and washed 3 times with 1x PBS (Gibco). Two mL of cell culture medium was added, and cells were incubated at 37 °C with 5% CO_2. IRE/CTVM19 cells were seeded to a density of 5×10^5 cells/mL in a total volume of 2.2 mL in flat-sided cell culture tubes and incubated with the indicated virus. Two hours after incubation, cells were washed three times with PBS by centrifugation at 1,000 *g* for 5 min. Cells were resuspended in cell-culture medium. At the indicated timepoints, 30 µl samples of Vero E6 or IRE/CTVM19 cell culture medium were removed and directly frozen at -80°C until further analysis. Virus samples were titrated on Vero E6 cells using EPDAs.

Ticks

Ixodes ricinus nymphs were collected by blanket dragging between September 2020 and April 2021 in Wageningen (Dorschkamp, 51°58'38.5″N 5°41'58.4″E), where TBEV is absent, and between July 2021 and September 2021 in Haarle (Hellendoornse berg, 52°22'18.1″N 6°25'45.3″E), in the Sallandse Heuvelrug region where the TBEV-NL strain was previously isolated [26]. Ticks were stored in batches of 25 nymphs in 15 mL tubes (Falcon) with pierced lids. Tubes were stored in a box with a water layer to create a humid environment and placed in an incubator at 18 °C and 16:8 light:dark cycle. Nymphs were stored for a

maximum of 21 days before use in subsequent experiments.

Artificial membrane feeding system

An artificial membrane blood-feeding system was adopted from Krull et al. [33] and Oliver et al. [34]. The feeding unit consisted of a polycarbonate tube (50 × 30 × 2 mm, Flexinplex kunststoffen, the Netherlands) glued to a silicon membrane and closed with a *Drosophila* cultivation plug (ceaprene stopper, 36 mm, Greiner bio-one). A hole was cut in the centre of the lid of a 125 ml polypropylene container (sample tub type 2118, Carl Roth, Germany), the feeding unit was glued into the lid using Elastosil E4 silicone glue (Wacker, Munich, Germany) and the lid was screwed onto the tub containing the blood meal (Figure 3). The silicon membrane was made by using lens-cleaning paper (120 × 70 mm, Tiffen Lens Cleaning Tissue) and a 10 mL mixture of components A and B of Ecoflex 00-10 soft rubber (Smooth-On, Inc.) and supplemented with 2 mL n-hexane (Sigma Aldrich). The lens-cleaning paper was fixed to a transparent acetate A4 sheet on a flat surface using tape and the silicon mixture was spread onto the cleaning paper using a thin putty knife, after which excess silicon rubber was scraped off. Membranes were allowed to dry for a minimum of 12 h after which the membrane thickness was checked using a digital micrometer. Only 50-70 µm thick membranes were used. The polycarbonate tubes were glued on the membranes using ELASTOSIL E4 silicone glue. The silicone glue was dried overnight and membrane integrity was tested by adding 5-10 mL of 70% ethanol to the assembled feeding units for 15 min after which the membranes were checked for leakage. In contrast with previous studies using artificial membrane blood-feeding units, no tick frass or physical stimuli were used [33–35]. For blood-feeding experiments, 100–125 nymphs were added to each feeding unit.

Infectious blood meal

Nymphs were fed on sterile, heparinized bovine blood which was supplemented with 4 mg/mL glucose monohydrate (Sigma-Aldrich) to stabilize blood cells. We used blood up to 6 days after collection. Bovine blood was obtained from the Carus animal research facility (Wageningen University & Research, the Netherlands) under animal ethics protocol no.

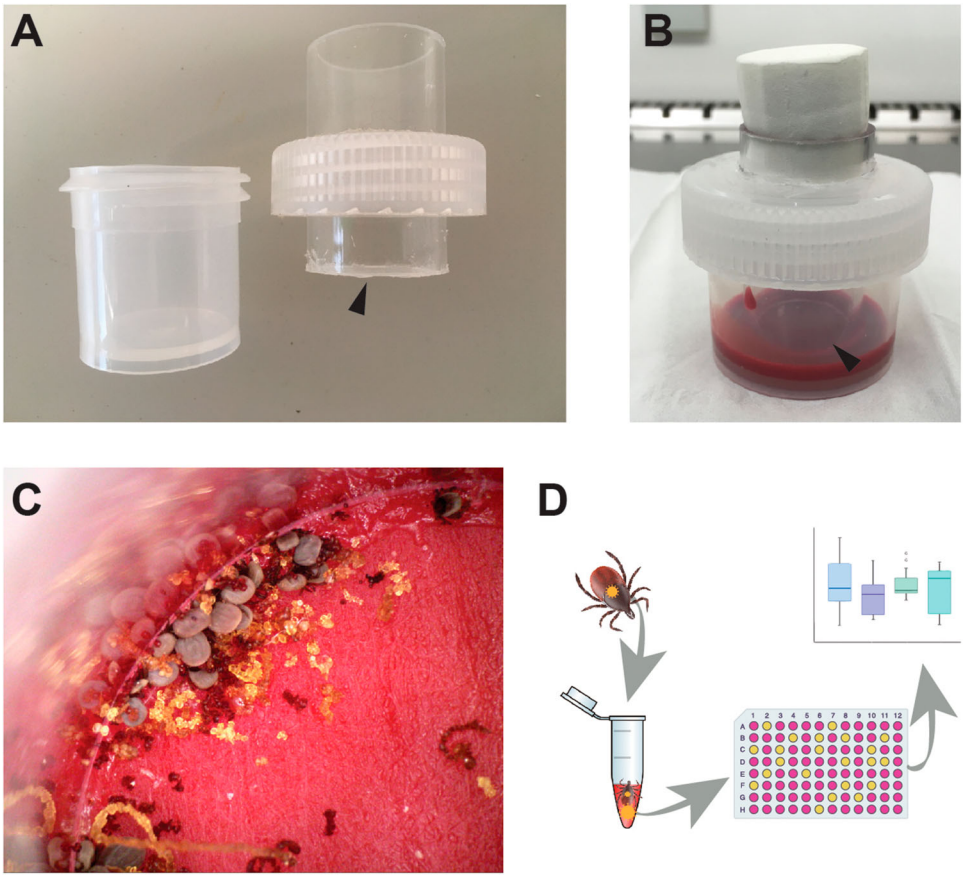

Figure 3. Blood-feeding unit for artificial membrane feeding of ticks. (A) Feeding unit with container and screw cap. The arrow head indicates the position of the membrane. (B) Feeding unit fully assembled with the arrow head indicating the position of the membrane. (C) Feeding *Ixodes ricinus* nymphs. Aggregation is observed as nymphs feeding closely to each other. (D) Methodological overview of virus infectivity assay. Ticks are homogenized and the homogenate is added to mammalian cells. The presence of virus is determined based on CPE.

AVD1040020173624. We did not supplement the blood with antibiotics or antimycotics to avoid disruption of the tick microbiome [36]. A P3 stock of LIV-INV14 and TBEV-NL and a P4 stock of TBEV-Neudoerfl was diluted in 500 µL DMEM medium to a titre of 4×10^7 $TCID_{50}$/mL (50% tissue culture infectious dose /mL) and added to 3.5 mL blood (final titre of 5×10^6 $TCID_{50}$ /mL blood). Virus-spiked blood was replaced every 24 h and membranes were rinsed with 0.9% NaCl solution during each blood change. After 24 h at 37°C, 8×10^5 $TCID_{50}$/mL of infectious virus remained (Supplementary Figure S1). Within each experiment (either the experiment with ticks from Wageningen or Haarle), the different viral treatments were replicated with ticks collected on different sampling days. The batches of ticks collected on different sampling days are henceforth termed "replicate". Four replicates for ticks from Wageningen and three replicates for ticks from Haarle were conducted, respectively. Multiple feeders (2-4) per virus per replicate were used. For every replicate, a control group of ticks, which were fed only on blood mixed with 500 µL DMEM medium, was included to control for the presence of other pathogens, which could cause CPE in the infectivity assay. Ticks were allowed to feed for up to a maximum of nine days in an incubator set at 37°C without light. Feeders were placed in a box containing a layer of water to supply a humidity gradient. Detached engorged ticks were removed using blunt-end tweezers and placed individually in 1.5 mL microcentrifuge tubes with pierced lids. The tubes were stored in a container with 80% to 90% RH created by a saturated potassium chloride solution, 21°C and 16:8 light:dark cycle for 21 or 60 days post engorgement (dpe). Ticks were considered dead when they did not respond with leg or palp movement after touching with blunt-ended tweezers. Dead ticks were removed from the experiment. Ticks were stored at -80°C after their incubation period.

Infectivity assay

Frozen ticks were homogenized in 1.5 mL microcentrifuge tubes using a combination of zirconium oxide beads (2.0 mm) and stainless steel beads (0.9–2.0 mm) in a Bullet blender (Next Advance, USA). Briefly, samples were homogenized for 2 min (speed 12) and spun down at 12,000 *g* for 30 seconds in an Eppendorf 4125 centrifuge. Next, 100 µL of DMEM-HEPES cell culture medium was added and samples were again homogenized for 2 min at max speed and spun down for 1.5 min at 12,000 *g*. Thirty µL of each tick homogenate was added to a Vero E6 monolayer of 70-80% confluency in a 96-well plate. After 2 h, the medium was removed, the cells were washed once with 1×PBS and 100 µL HEPES-buffered DMEM cell culture medium was added. Cytopathic effect (CPE) was scored at 6 days post infection (dpi).

Virus titration

$TCID_{50}$ was determined using 10 µL of supernatant, tick homogenate or blood meal in an end-point dilution assay (EPDA). Briefly, Vero E6 cells were detached using trypsin (Gibco) and diluted to 5×10^5 cells/mL in DMEM-HEPES cell culture medium. Virus samples were tenfold serial-diluted (10^{-1} up to 10^{-10}) in DMEM-HEPES. The Vero E6 cells were added in a 1:1 ratio to the virus dilutions and 10 µL of each virus suspension was added to each of 6 wells in a 60-well Micro-Well Plate (Nunc, Roskilde, Denmark). The plate was incubated in a humidified box. CPE was scored at 6 dpi and viral titres were expressed as the $TCID_{50}$/mL calculated according to the Reed and Muench method [37].

Phylogenetic analysis

Phylogenetic analysis of the tick-borne flavivirus sequences (Supplementary Table S1) was performed on the complete polyprotein coding region. Sequences were aligned using MUSCLE [38] within the program suite Geneious (version 2019.0.4). Powassan virus (Genbank accession number MZ576219) was used as outgroup. Phylogenetic analysis was inferred by using the Maximum Likelihood method and General Time Reversible (GTR) model with invariant sites and a gamma-distribution model (GTR + I + G) was found to suit the data set best [39], as selected by jModeltest [40] (version 2.1.7), followed by bootstrap analysis of 1000 replicates. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log-likelihood value. Evolutionary analyses were conducted in MEGA11 [41].

Statistical methods

Generalized linear models (GLMs) with a binomial distribution and log-link function were used to test the effect of virus (TBEV-Neudoerfl vs. TBEV-NL vs. LIV) and incubation time on infection rate. We built separate models for the data from Wageningen and Haarle, as ticks from these locations were collected at different times. Thus, we treated these datasets as two separate experiments. There was no interaction between virus and incubation time for the data from either Wageningen or Haarle and we therefore did not include an interaction term in the model. Replicate was included as fixed factor (to account for potential seasonal differences in tick

populations within a location) because the number of levels (n = 4 for Wageningen and n = 3 for Haarle) was too low to include it as random factor. Generalized linear mixed models (GLMMs) with a truncated negative binomial distribution and log-link function were used to test the effect of virus and incubation time on virus titres. Model diagnostics were performed using the "DHARMa" package [42]. Significant overdispersion of viral titres was alleviated by including individual ticks as random factor. GLMs/GLMMs were constructed using the R package "glmmTMB" [43]. Estimated marginal mean infection rates and viral titres were calculated using the package "emmeans" [44]. Pairwise contrasts of significant effects were performed with a Tukey HSD. A Kruskal–Wallis test was used to test for differences in back-titrated bloodmeal viral titres. All statistical analyses were carried out with the statistical software package R version 3.6.3 [45] using RStudio [46].

Results

Growth kinetics of tick-borne flaviviruses in A549 and IRE/CTVM19 cells

To test whether there is a difference in kinetics of the TBEV-variants in mammalian and tick cells, we studied the growth kinetics of LIV, TBEV-NL and TBEV-Neudoerfl in human A549 cells and *I. ricinus* IRE/CTVM19 cells. Both LIV and TBEV-NL replicated faster and to higher titres in A549 cells compared to TBEV-Neudoerfl (Figure 4A). Mean peak titres of 3.8×10^8 and 2.2×10^8 $TCID_{50}$/ml for LIV and TBEV-NL, respectively, were reached after 48 hpi, compared to 1.6×10^8 $TCID_{50}$/mL for TBEV-Neudoerfl after 72 hpi. LIV replicated slower in IRE/CTVM19 cells compared to TBEV-NL and TBEV-Neudoerfl (Figure 4B). Nevertheless, similar mean titres for LIV and TBEV-Neudoerfl of 2.0×10^8 $TCID_{50}$/mL and 1.6×10^8 $TCID_{50}$/mL, respectively, were reached after 7 dpi. TBEV-NL reached a mean peak titre of 4.2×10^7 $TCID_{50}$/mL after 5 dpi.

Susceptibility of Ixodes ricinus for tick-borne flaviviruses

We tested the susceptibility of ticks collected from the forest plot near Wageningen for TBEV-NL, TBEV-Neudoerfl and LIV. In this region, no TBEV transmission has been detected thus far [27]. This experiment was conducted over two seasons (autumn 2020 and spring 2021) and included a total of 2,825 *I. ricinus* nymphs, of which 1,260 (45%) fed to repletion. After 60 days of incubation, 39% (n = 355) of the nymphs moulted into females and 43% (n = 388) into males, whereas 18% (n = 159) of the ticks did not moult. We tested ticks at 0 dpe to determine if tick infection was successful. For TBEV-NL and TBEV-Neudoerfl, 100% of the tested ticks contained virus (n = 43 and 42, respectively), whereas for LIV 93% (n = 43) of the ticks contained virus (Supplementary Figure S2A). Viral titres of engorged nymphs at 0 dpe were significantly different among viruses (Supplementary Figure S2B, Likelihood Ratio Tests, LRT, $\chi^2 = 8.69$, df = 2, $p < 0.05$). Back-titrated blood meals showed that bloodmeals contained similar viral titres at the start of feeding (Kruskal–Wallis, H = 1.66, df = 2, $p = 0.44$, 3.2×10^6 $TCID_{50}$/mL for TBEV-NL, 4.9×10^6 $TCID_{50}$/mL for TBEV-Neudoerfl and 4.1×10^6 $TCID_{50}$/mL for LIV, Supplementary Figure S3A), indicating that the differences observed in viral titres of the ticks at 0 dpe were not caused by differences in initial virus spiked bloodmeals. No CPE was observed for the control group where ticks only fed on blood mixed with medium.

Infection rates of *I. ricinus* ticks were also determined after 21 and 60 days of incubation. Likelihood ratio tests showed that the probability of infection did not depend on incubation time (LRT, $\chi^2 = 0.38$, df = 1, $p = 0.53$), but

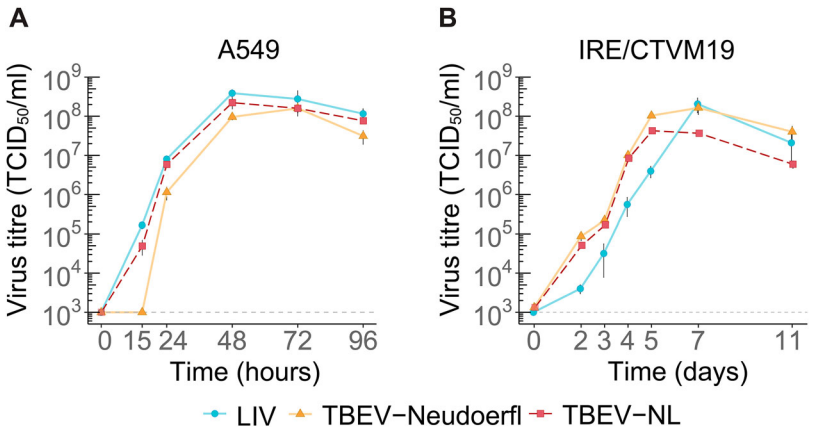

Figure 4. Growth kinetics of tick-borne flaviviruses in human A549 (A) and *Ixodes ricinus* IRE/CTVM19 (B) cells over time. Cells were infected with TBEV-NL, TBEV-Neudoerfl and LIV at an MOI of 0.01. The mean titres of three replicates ± the standard error of the mean are shown. Dashed line indicates the detection limit of the end-point dilution assay at 1×10^3 $TCID_{50}$/mL.

was affected by virus strain (LRT, $\chi^2 = 104{,}5$, df = 2, $p < 0.001$) and replicate (LRT, df = 3, $\chi^2 = 40.74$, $p < 0.001$). Estimated marginal mean infection rates for TBEV-Neudoerfl (64.0%, 95% CI: 58.5–69.2, Figure 5A) were significantly higher than those of TBEV-NL (42.0%, 95% CI: 36.7–47.5) and LIV (25.8%, 95% CI: 21.2–30.9). Specifically, the odds of infection with TBEV-Neudoerfl were 2.5-fold higher compared to TBEV-NL (95% CI: 1.71–3.52, $p < 0.001$) and 5.1-fold higher compared to LIV (95% CI: 3.51 - 7.52, $p < 0.001$). The odds of infection with TBEV-NL were 2-fold higher compared to LIV (95% CI, 1.43 - 3.02, $p < 0.001$).

Besides the infection rates, we tested for the effect of different viruses on the viral titres in blood-fed ticks at 21 and 60 dpe. There was a significant interaction between virus and incubation time (LRT, $\chi^2 = 7.54$, df = 2, $p < 0.022$). At 21 dpe, estimated marginal mean viral titres were not significantly different between ticks infected with LIV (1.1×10^5 $TCID_{50}$/mL, Figure 5B, Supplementary Table S3), TBEV-Neudoerfl (4.3×10^5 $TCID_{50}$/mL) or TBEV-NL (2.9×10^5 $TCID_{50}$/mL). At 60 dpe, LIV had significantly lower estimated mean viral titres (5.6×10^4 $TCID_{50}$/mL) compared to TBEV-NL (1.6×10^6 $TCID_{50}$/mL) and TBEV-Neudoerfl (8.6×10^5 $TCID_{50}$/mL, Figure 5B, Table S3). The viral titres of TBEV-NL and TBEV-Neudoerfl did not differ significantly at 60 dpe.

Susceptibility of ticks from a TBEV-endemic region

Ticks collected from forest plots at Haarle were infected with TBEV-NL, TBEV-Neudoerfl and LIV to investigate whether the susceptibility of ticks from a TBEV-NL endemic region showed similar patterns as the ticks collected from forests near Wageningen, a non-endemic region. A total of 2,125 *I. ricinus* nymphs were used for the experiment of which 818 (39%) fed to repletion. After 60 days of incubation, 46% (n = 242) of the nymphs moulted into females, and 36% (n = 189) into males, whereas 17% (n = 91) of the ticks did not moult. Mean infection rates of ticks directly removed after engorgement (0 dpe) were 100% for TBEV-NL (n = 14) and TBEV-Neudoerfl (n = 14) and 83% for LIV (n = 6, Supplementary Figure S4A). At 0 dpe, viral titres in ticks from Haarle were significantly different between the viruses (LRT, $\chi^2 = 12.05$, df = 2, $p < 0.01$, Supplementary Figure S4B). Back titrated blood meals showed similar viral titres for all viruses (Kruskal–Wallis, H = 0.81, df = 2, $p = 0.67$, Supplementary Figure S3B) indicating that the differences observed in viral titres of the ticks at 0dpe were not caused by differences in initial virus spiked bloodmeals. No CPE was observed for the control group where ticks only fed on blood mixed with medium.

Infection rates of *I. ricinus* ticks were determined after 21 and 60 days of incubation. Infection rates did not differ between incubation time (LRT, $\chi^2 = 3.71$, df = 1, $p = 0.054$). While controlling for a significant replicate effect (LRT, $\chi^2 = 31.29$, df = 2, $p < 0.001$), we detected a strong effect of virus on the likelihood of infection (LRT, $\chi^2 = 280.24$, df = 2, $p < 0.001$, Figure 6A). Estimated marginal mean infection rates were 15% (95% CI: 10.5–20.8) for LIV, 64.5% (95% CI: 58.3–70.3) for TBEV-Neudoerfl and 89.7% (95% CI:

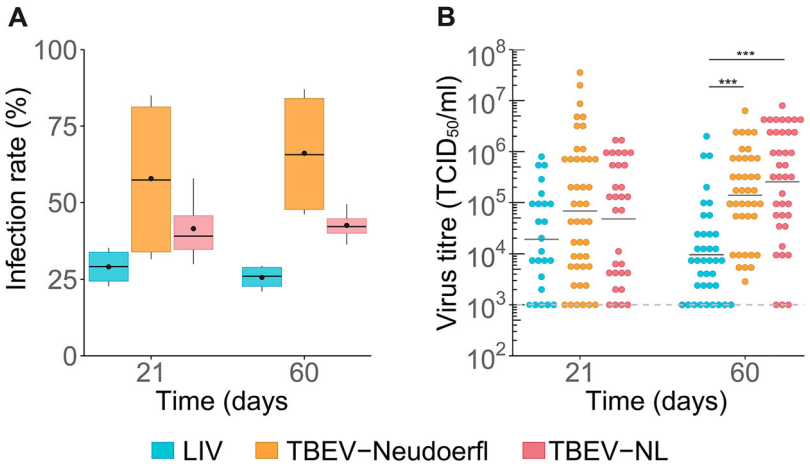

Figure 5. Infection rates with, and virus titres of, different tick-borne flaviviruses in *Ixodes ricinus* nymphs collected from forest plots near Wageningen. *Ixodes ricinus* nymphs were blood-fed on virus-spiked blood and incubated for 21 or 60 days post engorgement (dpe). Data are shown for four individual replicates making totals of 83, 82 and 85 ticks for LIV, TBEV-Neudoerfl and TBEV-NL at 21 dpe, respectively with totals of 288, 285 and 309 ticks for LIV, TBEV-Neudoerfl and TBEV-NL at 60 dpe, respectively. (A) Infection rates were determined by infectivity assays on Vero cells and shown as the median infection rate of four replicates. Incubation time did not affect infection rate (LRT, $p > 0.05$). Virus infection rates were significantly different (Tukey's HSD, $p < 0.001$) for all combinations. The mean infection rate is shown as black dot. (B) Virus titres are shown as the $TCID_{50}$/mL determined by EPDA. The horizontal bar represents the median viral titre per treatment. Tukey's HSD test results are indicated when significant (*** = $p < 0.001$). Dashed line indicates the detection limit of the end-point dilution assay at 1×10^3 $TCID_{50}$/mL

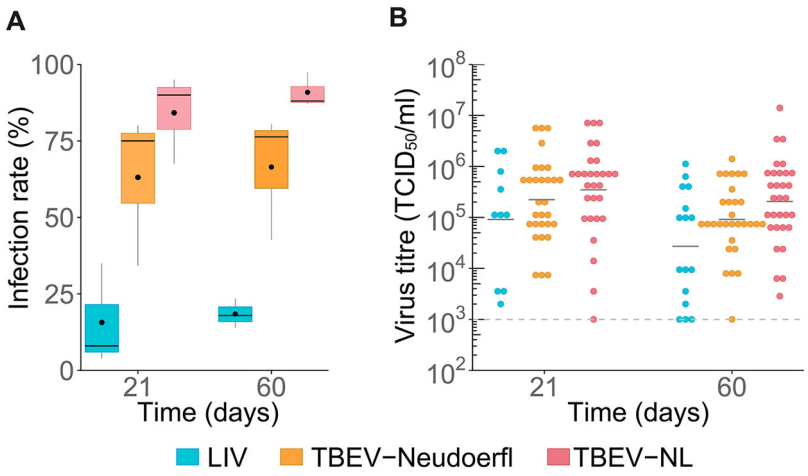

Figure 6. Infection rates with, and virus titres of, different tick-borne flaviviruses in *Ixodes ricinus* nymphs collected from forest plots near Haarle. *Ixodes ricinus* nymphs were blood-fed on virus-spiked blood and incubated for 21 or 60 days post engorgement (dpe). Data are shown for three individual replicates making totals of 70, 95 and 97 ticks for LIV, TBEV-Neudoerfl and TBEV-NL at 21 dpe, respectively and with totals of 116, 198 and 208 ticks for LIV, TBEV-Neudoerfl and TBEV-NL at 60 dpe, respectively. (A) Infection rates were determined by infectivity assays on Vero cells and shown as the median infection rate of three replicates. Incubation time did not affect infection rate (LRT, $p > 0.05$). Differences between virus infection rates were significantly different for all combinations (Tukey's HSD, $p < 0.001$). The mean infection rate is shown as black dot. (B) Virus titres are shown as the TCID50/mL determined by EPDA. The horizontal bar represents the median viral titre per treatment. Viral titres decreased significantly over time (LRT, $p < 0.05$). Estimated marginal mean viral titres for both timepoints differed only significantly between TBEV-NL and LIV, with TBEV-NL having significantly higher viral titres (Tukey's HSD, $p < 0.01$). Dashed line indicates the detection limit of the end-point dilution assay at 1×10^3 $TCID_{50}$/mL.

85.6–92.7) for TBEV-NL. The odds of infection were 49.4-fold higher for TBEV-NL compared to LIV (95% CI: 25.4–96.5, $p < 0.001$) and 4.8-fold higher compared to TBEV-Neudoerfl (95% CI: 6.9 - 8.2, $p < 0.001$). In addition, the odds of infection for TBEV-Neudoerfl were 10.3-fold higher (95% CI: 4.8–18.3, $p < 0.001$) compared to LIV.

Next to infection rates, the viral titres of TBEV-NL, TBEV-Neudoerfl and LIV infected ticks were determined after 21 and 60 days of incubation. A significant two-fold decrease in viral titre was observed after 60 dpe (LRT, $\chi^2 = 5.36$, df = 1, $p < 0.05$) compared to 21 dpe. Median viral titres at 21 days of incubation were 1.1×10^5 $TCID_{50}$/mL for LIV, 2.0×10^5 $TCID_{50}$/mL for TBEV-NL and 6.3×10^5 $TCID_{50}$/mL for TBEV-Neudoerfl (Figure 6B). After 60 days of incubation, median viral titres for LIV were 4.9×10^4 $TCID_{50}$/mL, for TBEV-NL 2.7×10^5 $TCID_{50}$/mL and for TBEV-Neudoerfl 8.3×10^4 $TCID_{50}$/mL. Estimated marginal mean viral titres for both timepoints differed only significantly between TBEV-NL and LIV, with TBEV-NL having significantly higher viral titres (Supplementary Table S4).

Discussion

The vector competence of ticks for a given virus is key to virus maintenance as it determines to what extent the virus can be acquired and subsequently transmitted to a new host. The current study investigated whether ticks from two distinct locations differed in their susceptibility to TBEV-Neudoerfl, TBEV-NL and LIV, and how these differences might explain the difference in emergence of tick-borne viruses in the Netherlands.

We used wild *I. ricinus* tick populations from two regions in the Netherlands: one tick population from the surroundings of Wageningen without active TBEV circulation, and one tick population from a TBEV-endemic region at Haarle [27]. Ticks from both regions were susceptible to LIV, TBEV-NL and TBEV-Neudorfl. However, the infection rates for LIV were significantly lower compared to the two TBEV strains. We observed variation in virus titres and infection rates in engorged ticks directly after they had fed on virus-spiked blood. This observation was more marked for LIV than for the TBEV strains. Nevertheless, the back titrated blood meals did not show differences among the viruses indicating that the observed differences in virus titres and infection rates in the engorged ticks was most likely caused by differences in virus susceptibility of the ticks. Furthermore, although we only tested viral susceptibility and replication in the ticks, our results suggest that Dutch *I. ricinus* are less competent in transmitting LIV than TBEV. Additional studies on TBEV and LIV transmission from ticks to their hosts are needed to test this hypothesis.

Although the primary vectors of TBEV are known [47–50], variation in vector competence of local tick populations has rarely been studied [51]. The two TBEV strains used in this study had different infection

rates in the two tick populations tested. TBEV-NL infected a larger proportion of ticks from a TBEV-NL-endemic region than TBEV-Neudoerfl. In contrast, ticks from a TBEV-free region had lower infection rates for TBEV-NL compared to TBEV-Neudoerfl. In agreement with our study, Liebig et al. [52] showed that ticks infected with a locally circulating TBEV strain had higher infection rates compared to infection with a geographically different TBEV strain. Variations in vector-competence of geographically different arthropod populations have previously been observed for a variety of tick-borne pathogens, including *Borrelia burgdorferi* and *Ehrlichia canis* [53–55], and may be the result of differences in genetic background of the vector population [54,56]. Previous genetic studies showed geographically-structured dispersal patterns of *I. ricinus* as a result of geographical barriers against mammal migration [57]. Ticks from Great Britain were genetically different compared to ticks from mainland Europe [57]. A similar pattern was also observed in ticks from Great Britain and Latvia [58]. Whether these genetic differences within *I. ricinus* species underlie the observed differences in vector-competence of ticks for TBEV or other tick-borne pathogens remains unclear.

Infection rates of TBEV in our experiments ranged from a median of 41% to 91%. Previous studies also found high variation in infection rates between ticks from TBEV-endemic and TBEV non-endemic regions [51,52]. A study using an artificial membrane feeding system found infection rates around 75% in nymphs moulted from larvae [59]. However, comparing infection rates in ticks among studies is difficult as little standardization is used in the artificial membrane feeding systems [60]. Moreover, artificial membrane feeding systems to study tick-borne viruses have only been used in a small number of studies [51,52,59]. In our study, the ticks from Haarle had higher overall infection rates for the two TBEV strains compared to ticks from Wageningen. It should be stressed that the two tick populations were not collected from the field simultaneously, and thus not infected simultaneously, and that the different times of the year in which the experiments were conducted could have contributed to the differences in infection rates, as observed in a recent study [51]. We did indeed find a significant difference between infection rates of the replicates conducted either in autumn or in spring in ticks from Wageningen. Replicates with ticks collected in autumn had a higher infection rate compared to ticks collected in spring and this indicates that other (unknown) biotic or abiotic factors including differences in tick age or nutritional status may influence infection success of viruses during artificial membrane feeding in *I. ricinus* [61].

Another explanation for differences in infection rates observed between the different tick populations could be the presence or absence of specific microorganisms in the ticks [53,62,63]. Ticks harbour a plethora of symbiotic microorganisms, from facultative symbionts, tick-specific viruses to human pathogens, and co-infections of these microorganisms are frequently observed [64,65]. Studies on the effect of co-infections on tick-borne flavivirus replication are scarce. A previous study found that a preceding infection with *Borrelia* decreased the likelihood of a TBEV infection in artificially blood-fed ticks [51], though this result was only observed for ticks from one out of two geographical regions tested. In the present study, the presence of *Borrelia* most likely did not influence TBEV infection in ticks, as we used bovine blood in our experiment, in which complement-mediated *Borrelia* killing has been observed [66] and is suggested to eliminate *Borrelia* from artificially fed ticks [67].

Besides *Borrelia*, other microorganisms present in ticks may alter the viral replication kinetics of TBEV and subsequent transmission to the vertebrate host. We previously collected ticks from the two regions used in this study and we observed differences in the presence of microorganisms (Supplementary Table S5). *Anaplasma phagocytophilum* prevalence was higher in ticks from Haarle compared to ticks from Wageningen (18.69% versus 4.20%, respectively). In contrast, the prevalence of *Neoehrlichia mikurensis* in ticks from Haarle was low compared to ticks from Wageningen (0.75% versus 12.30%, respectively). We did not confirm the presence of microorganisms in the TBEV-infected ticks used in the current study. Co-infection studies in sheep infected with TBEV or LIV and *A. phagocytophilum* showed increased virus titres in *A. phagocytophilum*-infected animals [68,69]. Moreover, *A. phagocytophilum* infection was correlated with increased Langat virus titres in an *I. ricinus* cell line [70].

Recent studies discovered the presence of arthropod-specific viruses in ticks [63,71,72]. Although their role in pathogen transmission is unknown for ticks, some mosquito-specific viruses can interfere with the transmission of arthropod-borne viruses [73]. These examples show that co-infections of TBEV with other microorganisms are important to consider and further experimental studies are needed to test the potential of microorganisms to interfere with the transmission of TBEV.

During the artificial membrane blood-feeding experiments, we did not find any evidence for a negative effect of virus infection on tick survival, as mortality in the different groups of incubated ticks was negligible. Interestingly, virus infection rates in blood-fed ticks did not drop after their moult between day 21 and day 60 day of the experiment. Slovak and colleagues reported that transstadial transmission of TBEV was low in ticks that acquired

the virus via co-feeding [74]. However, besides using laboratory-reared ticks which may influence viral kinetics, the study of Slovak et al. used the TBEV-Hypr strain, which lacks a 263 bp region in the 3′ untranslated region (UTR). This could cause the observed difference in transstadial transmission, as the 3′UTR of flaviviruses influences replication and dissemination in ticks and mosquitoes [75–78]. A drop in TBEV viral titre after moulting has previously been observed in *I. ricinus* [79]. We observed a two-fold reduction in viral titres from 21 to 60 dpe, but this effect was only observed in ticks from Haarle. The high efficiency of transstadial transmission observed in the current study could be the result of a high initial viral load acquired by the feeding ticks or optimal incubation conditions such as high relative humidity.

At this point in time, the establishment and maintenance of TBEV foci at specific geographic locations is not well understood. In some regions in Europe, TBEV foci have persisted for decades, whereas others have disappeared [25,80]. The geographical spread of TBEV is characterized by a relatively high variation of virus strains in small geographical regions [27,81]. This type of pattern may be the consequence of the introduction of TBEV-infected ticks by birds into regions with ecological conditions that support the establishment of TBEV. The TBEV-NL strain is an unique strain which forms an outgroup to the known European subtypes of TBEV. A highly similar TBEV strain (99.5% similarity) has been found recently in the United Kingdom [28]. As the United Kingdom and the Netherlands are not connected by land, the two closely related TBEV strains found are most likely introduced by birds migrating between these two countries. Bird migration indeed occurs as for example the pied fly catcher (*Ficedula hypoleuca)* migrates between the United Kingdom and the Netherlands and has been found carrying TBEV infected ticks in Russia [82,83]. Indeed, arthropod-borne virus dispersal by birds between the United Kingdom and the Netherlands is probably occurring, as identical Usutu virus sequences have also been found in both countries [84]. This underlines the importance of birds in the spread of arboviruses.

With regard to the spread of LIV, this virus remains restricted to the British Isles but has been sporadically found in Norway, Denmark and Russia [11–13]. Next to the absence of suitable vertebrate hosts such as red grouse and mountain hare [15], the low infection rates of LIV in the experimentally-infected ticks from the Netherlands indicates that they may be less susceptible to infection with this virus, which may partly explain the restricted geographical distribution of LIV in mainland Europe.

Conclusion

Our findings show that ticks from two distinct populations were susceptible to different TBEV strains, which provides additional support for the existence of local amplification and transmission of TBEV in the Netherlands. We also showed that LIV, sporadically found on the European mainland, had much lower infection rates in Dutch ticks compared to TBEV. This could explain why LIV is not present in the Netherlands. Remarkably, we observed differences in infection success of two TBEV strains between two geographically distant tick populations. Specifically, ticks from an TBEV-endemic region had higher infection rates for a locally circulating TBEV-strain than ticks from a non-endemic region. Further studies are needed to assess to what extent these results can be extrapolated to other regions where tick-borne viruses circulate.

Acknowledgements

We thank Stephanie Becker and Katrin Liebig for their help and training with the artificial membrane feeding of ticks, Manoj Fonville for tick pathogen analysis, Alain Kohl and Colin McInnes for providing the LIV INV14 strain, the Tick Cell Biobank at the University of Liverpool for providing the IRE/CTVM19 cell line, Carus for providing bovine blood and Staatsbosbeheer for tick collection from the Sallandse Heuvelrug and the Dorschkamp. We also thank Marcel Dicke for providing comments on an earlier version of the manuscript.

Funding

This work was supported by the Graduate School for Production Ecology & Resource Conservation of Wageningen University & Research and by the Dutch Ministry of Health, Welfare, and Sports (VWS).

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