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Properties of the tick-borne encephalitis virus population during persistent infection of ixodid ticks and tick cell lines

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Abstract

Tick-borne encephalitis virus (TBEV) is the causative agent of tick-borne encephalitis (TBE), a vector-borne zoonotic neuroinfection. For successful circulation in natural foci the virus has to survive in the vector for a long period of time. Information about the effect of long-term infection of ticks on properties of the viral population is of great importance. In recent years, changes in the eco-epidemiology of TBEV due to changes in distribution of ixodid ticks have been observed. These changes in TBEV-endemic areas could result in a shift of the main tick vector species, which in turn may lead to changes in properties of the virus.

In the present study we evaluated the selective pressure on the TBEV population during persistent infection of various species of ticks and tick cell lines. TBEV effectively replicated and formed persistent infection in ticks and tick cell lines of the vector species (*Ixodes* spp.), potential vectors (*Dermacentor* spp.) and non-vector ticks (*Hyalomma* spp.). During TBEV persistence in *Ixodes* and *Dermacentor* ticks, properties of the viral population remained virtually unchanged. In contrast, persistent TBEV infection of tick cell lines from both vector and non-vector ticks favoured selection of viral variants with low neuroinvasiveness for laboratory mice and substitutions in the E protein that increased local positive charge of the virion. Thus, selective pressure on viral population may differ in ticks and tick cell lines during persistent infection. Nevertheless, virus variants with properties of the original strain adapted to mouse CNS were not eliminated from the viral population during long-term persistence of TBEV in ticks and tick cell lines.

Keywords: TBEV, ixodid ticks, tick cell lines, persistent infection, neuroinvasiveness, E protein

Introduction

Tick-borne encephalitis virus (TBEV) is a causative agent of neuroinfection with severe consequences in humans, tick-borne encephalitis (TBE), and is endemic in a large part of

Eurasia. TBEV has been detected in many ixodid tick species (Labuda and Nuttall, 2004), but *Ixodes ricinus* and *Ixodes persulcatus* are the main vectors. In some TBEV-endemic areas, where *I. ricinus* and *I. persulcatus* ticks are absent or few in number, other ixodid tick species play the main role as TBEV vectors: *Haemaphysalis* spp. in the Far East of the Russian Federation and in Korea (Hoogstraal, 1966; Ko et al., 2010), and *Ixodes ovatus* in Japan (Takashima, 1998).

TBEV is an enveloped, unsegmented positive-stranded RNA virus. Its virion consists of a nucleocapsid with viral RNA in complex with the C protein and a lipid envelope with viral proteins M and E on its surface. E protein plays a major role in infection by mediating cell receptor binding and membrane fusion, and is also the main target for neutralising antibodies. The viral RNA is nearly 11 kb in length and has a single open reading frame, encoding a polyprotein which is processed into three structural (C, (pr)M, E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins. This open reading frame is flanked by 5` and 3` non-translated regions (NTR) (Lindenbach et al., 2013).

There are three TBEV subtypes: European, Siberian and Far-Eastern (King et al., 2012). The main vector of the European subtype is *I. ricinus*, and that of the other subtypes is *I. persulcatus*. In central and northwestern parts of Russia and in eastern Estonia and Latvia, which are known to be areas sympatric for the above-mentioned tick species, the Siberian subtype can be isolated from *I. ricinus* ticks and the European subtype from *I. persulcatus* (Demina et al., 2010; Katargina et al., 2013). Currently, changes in habitat and climate have led to a change in the distribution of ticks (Bugmyrin et al., 2013; Jaenson et al., 2016; Tokarevich et al., 2011) and as a consequence to the emergence of new TBE foci and to switching of the vector. Thus, in Finland new TBE foci were described where unusual combinations of *I. ricinus/I. persulcatus* ticks and European and Siberian TBEV subtypes were present with no sign of sympatry of ticks or virus subtypes (Jääskeläinen et al., 2006, 2016).

Due to the changing TBE epidemiology, the question of the properties that determine the competence of ticks as TBEV vectors becomes relevant. The role of *Dermacentor* ticks in

maintaining virus circulation in natural foci has been actively discussed (Wójcik-Fatla et al., 2011; Belova et al., 2013a,b; Kahl and Dautel, 2013; Karbowiak et al., 2015; Földvári et al., 2016). There is evidence indicating the ability of *Dermacentor* ticks to harbour virus for a long time (Nosek and Kožuch, 1985), and to transmit it through co-feeding (Jones et al., 1987; Alekseev and Chunikhin, 1991), transstadially (Karbowiak et al., 2016) and transovarially (Danielová et al. 2002; Zhmaeva and Pchelkina, 1967). Nevertheless, an independent TBEV vector must not only be capable of harbouring and transmitting virus both horizontally and vertically, but must also be able to maintain TBEV circulation in nature independently. Therefore, until confirmatory evidence becomes available, *Dermacentor* ticks can only be considered as potential TBEV vectors.

Besides biological and ecological characteristics of the vector, the ability of TBEV to adapt to and replicate in both the vertebrate host and the tick is an important factor for successful virus circulation in the natural focus. The level of virus replication in ticks can be one of the main factors that determine vector competence. It has been shown that the European subtype of TBEV replicates more intensively and reaches 10-100 times higher titres in tick cell lines from the main virus vector (*I. ricinus*), than in cell lines from non-vector ticks (*Hyalomma anatolicum, Rhipicephalus [Boophilus] microplus, Rhipicephalus appendiculatus* and *Ornithodoros moubata*) (Ruzek et al., 2008b).

Change of the main vector may cause changes in virus population properties. TBEV variant M, adapted to *Hyalomma marginatum* ticks, reached higher titres in *Hyalomma turanicum* ticks than the parental strain EK-328, and maintained its properties during 5 successive passages through mouse brains (Dzhivanian et al., 1988; Romanova et al., 2007). Variant M also differed from the parental strain in displaying low neuroinvasiveness (ability of the virus to enter the central nervous system (CNS) and cause disease) for laboratory mice, and small-plaque phenotype in PEK cells. Variant M differed from the parental strain EK-328 genome in 15 nucleotide and 6 amino acid substitutions (Romanova et al., 2007). Moreover, the

amino acid substitution in the E protein of Glu_{122}→Gly, increasing the local positive charge (lpc) of the virion, was shown to be responsible for the respective phenotypes of the viral populations.

Most likely, TBEV variants with low neuroinvasiveness for mice and lpc-substitutions in the E protein have an advantage during virus replication in *I. ricinus* ticks (Labuda et al., 1994; Khasnatinov et al., 2009). It is likely that substitutions in the other genome regions could also be connected with neuroinvasiveness of the virus (Ruzek et al., 2008a).

In active natural TBE foci, virus is continually transferred from the vector to the host and vice versa. In situations with a small number of hosts and unfavourable climatic conditions, virus may be able to survive for a long time in the natural focus due to persistent infection of the vector or the vertebrate host (Bakhvalova et al., 2006).

In the present study, using usual and unusual vector ticks and cell lines derived from unusual and non-vector tick species, we analysed the effect of persistent infection with the Siberian subtype of TBEV on genetic and phenotypic properties of the viral population.

Materials and methods

Cells

A pig embryo kidney (PEK) cell line (Institute collection, originally obtained from Mechnikov Moscow Research Institute of vaccines and sera, 1959–1965) was maintained at 37° C in Medium 199 (PIPVE, Russia), supplemented with 5% foetal bovine serum (FBS, Gibco) (Romanova et al., 2007).

In the present investigation we used cell lines derived from embryos of the ticks *Hyalomma anatolicum* – HAE/CTVM8 (Bell-Sakyi, 1991) and *Ixodes ricinus* – IRE/CTVM19 (Bell-Sakyi et al., 2007) provided by the Tick Cell Biobank, then at The Pirbright Institute. The tick cell lines were maintained at, respectively, 32° C and 28° C as described earlier (Bell-Sakyi, 1991; Weisheit et al., 2015).

Ticks

In all experiments we used first generation laboratory tick colonies (adult ticks derived from eggs laid under laboratory conditions by female ticks collected from the field) of the following species:

- *I. ricinus* (parental females from Kaluga region, Russia);
- *I. persulcatus* (parental females from the Republic of Karelia, Russia);
- *Dermacentor reticulatus* (parental females from Kaluga region, Russia)

All parental females were analysed after oviposition for contamination with TBEV, *Borrelia burgdorferi* s.l., *Anaplasma phagocytophilum, Ehrlichia chaffeensis/E. muris* using the commercial kit AmpliSens® "TBEV, B. burgdorferi s.l., A. phagocytophilum, E. chaffeensis / E. muris-FRT" (FBIS Central Research Institute of Epidemiology, Russia) and found to be negative. Live ticks were kept at room temperature in humidified glass tubes (Belova et al., 2012).

Viruses

In our work we used

- strain EK-328, Siberian subtype of TBEV, isolated from a pool of *I. persulcatus* ticks in 1972 in Estonia; differs from the GenBank sequence (GenBank ID DQ486861.1; Romanova et al., 2007) by 2 mutations in nucleotide positions 483 and 9888, and by 2 positions (2965 and 5241) with minor heterogeneity. All substitutions were synonymous and did not affect strain properties. Passage history: 10 passages through mice brain, 1 passage in PEK cells.
- Variant M obtained after strain EK-328 adaptation to *H. marginatum* ticks (Chunikhin and Dzhivanyan, 1979; Romanova et al., 2007). Passage history: 3 passages through mice brain of the strain EK-328 (original isolate from the pool of *I. persulcatus* ticks), 13 parenteral passages in *H. marginatum* ticks, 1 cloning in PEK cells, 4 parenteral passages in *H. marginatum* ticks, 5 passages through mice brain, 1 passage in PEK cells. Differs from the sequence (Romanova et al., 2007) by 2 synonymous mutations in positions 19 and 10104, and

2 nonsynonymous mutations in positions 2249 (E protein Ile_{426}→Ile/Thr) and 3300 (protein NS1 Glu_{280}→Asp), which did not affect variant properties.

Viruses were used as culture supernate from infected PEK cells.

Ethics statement

Animals were maintained according to international guidelines for animal husbandry and Chumakov IPVE ethical guidelines, including the recommendations of CIOMS (1985).

Tick feeding

Adult *D. reticulatus* ticks were fed on rabbits (breed "Soviet Chinchilla", FSBI "Scientific Center of Biomedical Technologies" of Russian Academy of Sciences, "Elektrogorskiy" branch). First a plastic Elizabethan-style collar was put on the rabbit to prevent it from grooming the area where the ticks were to be applied. Then a 25 cm^2 area of hair in the region of the rabbit's shoulder blades was shaved and one end of a cloth tube was glued (BF-6, Russia) to the skin of the rabbit. After drying of the glue (12 h), ticks (10 females and 5 males) were put into the cloth tube for feeding, and the open end of the tube was folded over and sealed with rubber bands. The tick feeding process was observed daily by opening the end of the cloth tube, and individuals were removed when engorged.

Plaque assay in PEK cells

The plaque assay for determining virus titre was carried out as described previously (Belova et al., 2012). Briefly, PEK cells were seeded in 6-well plates. After 3 days, cells were infected with 10-fold dilutions of virus, prepared in Medium 199. Virus was adsorbed for 1 h at 37° C, then each well was overlaid with 5 ml of 1% agar (Difco) in Earle's balanced salt solution (PIPVE, Russia) containing 7.5% FBS, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), Fungizone (1 μg/ml, Gibco,) and 0.015% neutral red, and incubated at 37° C up to 14 days. The size of plaques (diameter) was measured on the 7th day post infection (d.p.i.). Plaques were counted on the 14th day p.i. and virus titres were calculated as the decimal logarithm of plaque-forming units per ml (log_{10}PFU/ml).

Preparation of tick suspensions

Individual ticks were washed in 70% ethanol and twice in saline solution (0.9% NaCl) with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), then ground with a pestle in separate mortars and diluted with 600 µl of Medium 199 with Earle's salts (PIPVE, Russia) with antibiotics as above. Thus, we obtained suspensions from unfed *Ixodes* and *D. reticulatus* ticks comprising 0.14% and 0.58% tick material, respectively, given that the weights of adult ticks were about 0.85 mg (*Ixodes*) and 3.50 mg (*D. reticulatus*).

Persistent infection of ticks with TBEV

Unfed ticks were infected by the percoxal route (Belova et al., 2012). In total, 30 females and 30 males each of *I. ricinus* and *I. persulcatus* and 90 female and 50 male *D. reticulatus* were inoculated with doses of TBEV strain EK-328 at equivalent multiplicity of infection (MOI) based on the size of the ticks: for *Ixodes* females this was 4.7 $\log_{10}$PFU/1 µl, for males 4.4 $\log_{10}$PFU/0.5 µl, and for *D. reticulatus* ticks 5.0 $\log_{10}$PFU/2 µl. Infected ticks were kept in humidified glass tubes at room temperature (23–25° C) for up to 5 months. At different time points we collected between 2 and 6 ticks and evaluated TBEV titre in individual ticks by plaque assay.

Two control groups were used to analyse the mortality of infected ticks after inoculation. Ticks from one group were inoculated percoxally with an equal volume of supernate from uninfected PEK cells, and ticks from another group remained intact. Control groups were kept in the same conditions as test ticks and their survival was monitored. *I. persulcatus* ticks from both infected and control groups were observed for 54 days due to their high mortality in laboratory conditions.

Some of the infected *D. reticulatus* ticks after long-term persistence (128 days) were fed on a rabbit to study the kinetics of TBEV replication during feeding. After 30 h, 104 h and 7 days of feeding, 2-3 females were removed and assayed for virus titre.

Infection of tick cell lines with TBEV

The kinetics of TBEV reproduction were studied in two tick cell lines, IRE/CTVM19 and HAE/CTVM8. Cells in flat-sided culture tubes (Nunc) were infected with strain EK-328 or variant M at MOI 1 PFU/cell and incubated at 28° C (IRE/CTVM19) or 32° C (HAE/CTVM8). Uninfected cells were kept as a negative control. Medium was changed approximately weekly by removal and replacement of half of the volume of culture medium (1.1 ml). Every 3-4 weeks the tick cells were subcultured 1:1 (Bell-Sakyi, 1991). After the first subculture, all subsequent subcultures were performed only with one tube from the pair, while culture supernate and cells from the other tube were harvested separately for determination of virus titre.

Virulence in mice

In the present work virus neuroinvasiveness for laboratory mice was defined as the number of plaque-forming units required to cause death of 50% of animals after intraperitoneal (i/p) inoculation of the virus. In experiments, 8-week-old BALB/c mice (FSBI "Scientific Center of Biomedical Technologies" of Russian Academy of Sciences, "Stolbovaya" branch) in groups of five were injected with 10-fold dilutions of virus (300 μl) and observed for clinical symptoms and mortality for 21 days. The lethal dose of virus resulting in 50% mortality ($log_{10}LD_{50}$) was calculated according to the Kerber method (Lorenz and Bogel, 1973).

Obtaining viral clones

Virus cloning was carried out as modified plaque assay in PEK cells. At 6-10 d.p.i., agar columns directly above and around separate plaques were removed and dissolved in 500 μl Medium 199 with 50% FBS. For further study, the clones after freeze-thaw procedure were multiplied in the cell line in which the parental strain had been cultured (PEK, IRE/CTVM19 or HAE/CTVM8).

Sequencing

Viral RNA was isolated from tick suspensions, supernate from infected cells or cell pellets with TRI Reagent LS (Sigma-Aldrich) according to the manufacturer's protocol. M-MLV transcriptase (Promega, USA) reverse transcription was carried out with specific primers

according to the manufacturer's protocol. cDNA was amplified by PCR using overlapping sets of TBEV-specific primers (all primer sequences available upon request). Sequencing was carried out in both directions directly from PCR products on an ABI PRISM 3730 (Applied Biosystems) sequencer using ABI PRISM® BigDye™ Terminator v. 3.1. The sequences were aligned with Clustal-X 2.0.11 (Larkin et al., 2007).

Results

TBEV reproduction during adaptation to persistent infection of ixodid ticks

I. ricinus, I. persulcatus and *D. reticulatus* ticks were percoxally infected with TBEV strain EK-328 and kept alive for up to 5 months to obtain TBEV variants adapted to long-term persistence in the tick.

TBEV strain EK-328 successfully replicated and formed persistent infection in ticks of all three species (Fig. 1). The highest virus titres were observed in *D. reticulatus* ticks throughout the observation period. TBEV reproduction in *I. ricinus* and *I. persulcatus* ticks was generally similar, with greater fluctuation than seen in *D. reticulatus*, but from the 14th d.p.i. virus reproduction in *I. persulcatus* was lower than in *I. ricinus*.

According to our previous reports, TBEV replicates much faster and more intensely in feeding than in unfed *I. ricinus* ticks (Belova et al., 2012). Twelve TBEV-infected *D. reticulatus* ticks at the 128th d.p.i. were allowed to feed on rabbits. Strain EK-328 showed active reproduction in feeding ticks. During the first 104 hours of feeding the TBEV titre increased from 2.90±0.05 to 5.40±0.03 $\log_{10}$PFU/tick and on the 7th day the virus titre reached 6.30±0.03 $\log_{10}$PFU/tick.

Thus, TBEV successfully replicated, formed persistent infection in unfed *I. ricinus*, *I. persulcatus* ticks and *D. reticulatus* ticks, and showed rapid increase in virus titre in feeding *D. reticulatus* ticks..

Phenotypic characteristics of TBEV during adaptation to persistent infection of ixodid ticks

On the basis of data on changes in the viral population properties after adaptation to ticks (Labuda et al., 1994; Romanova et al., 2007), in our experiments with persistent TBEV infection of ticks we expected appearance of the lpc-mutants, i.e. virus variants with increased lpc of the E protein. We chose size of plaques in PEK cells as a marker of the lpc-mutants (Kozlovskaya et al., 2010). Analysis of strain EK-328 plaque phenotype during its adaptation to persistent infection in ticks showed the development of heterogeneity in the viral population, but only during the later time periods at 90 d.p.i. (data not shown) and later (Table 1). In PEK cells, variants Dr128d and Dr128d104hF derived from, respectively, unfed and feeding *D. reticulatus* ticks at 128 d.p.i., formed equal proportions of 7-8 mm plaques, standard for strain EK-328, and smaller plaques of 1 mm and 2.5 mm (Table 1). In the earlier time periods (14-15 d.p.i.), TBEV variants derived from *D. reticulatus* and *I. ricinus* ticks maintained their original phenotypic characteristics and formed only large plaques. Interestingly, heterogeneity of the viral population was observed after 18 days of persistence in *I. persulcatus* ticks (variant Ip18d): the variant formed pale <1 mm plaques in addition to large plaques in the approximate ratio of 1:25 (Table 1). However, the TBEV variant that persisted for 48 days in ticks of the same species (variant Ip48d) demonstrated parental large-plaque phenotype. A similar large plaque variant Dr128d7dF was obtained after 128 days' persistence followed by 7 days' feeding in *D. reticulatus* ticks. It is possible that small plaques were present but masked by the large plaques due to interference phenomena, or the rate of selection of such variants depends on individual variation between ticks.

The data show that long-term persistence of the virus in ticks increases virus population heterogeneity.

Neuroinvasiveness for mice of TBEV variants obtained during persistent infection of ixodid ticks

We evaluated neuroinvasiveness for laboratory mice by i/p inoculation of several EK-328 variants obtained by persistence in *I. ricinus, I. persulcatus* and *D. reticulatus* ticks. Strain EK-328 after additional passage in PEK cells that was used for infection of ticks showed lower neuroinvasiveness (1.9 $\log_{10}$PFU) than the original strain (0.9 $\log_{10}$PFU). All the studied variants (Ir15d, Ip18d, Dr14d, Dr128d and Dr128d104hF) obtained by persistent infection of ticks showed increased neuroinvasiveness (0.3, 0.8, 0.7, 0.8 and 1.2 $\log_{10}$PFU, respectively), similar to that of the strain EK-328 obtained by replication in mouse brain (Table 1). Thus, the high neuroinvasiveness shown by the parental virus adapted to the CNS of mice is preserved during long-term persistence of the virus in ticks.

The nucleotide sequences of the genome fragments encoding protein E of TBEV variants adapted to persistent infection of ixodid ticks

We analysed the nucleotide sequence of the genome fragment encoding E protein from the variants Dr40d (40 days of persistence in unfed *D. reticulatus*) and Dr128d7dF (persistence for 128 days in unfed and 7 days in feeding *D. reticulatus*). The studied variants showed large-plaque phenotype similar to the parental strain EK-328 (Table 1). Nucleotide substitutions were not found. However, variants Dr128d and Dr128d104hF, which had persisted in ticks for a similar period as the analysed variant Dr128d7dF, showed heterogeneity of plaques in PEK cells that indicated a change in the composition of the viral population.

TBEV replication during persistent infection in I. ricinus and H. anatolicum tick cell lines

Tick cell lines are a model system for studying the kinetics of TBEV replication in ticks. We analysed the kinetics of Siberian subtype TBEV replication in tick cell lines derived from *I. ricinus* (IRE/CTVM19) and *H. anatolicum* (HAE/CTVM8) maintained at 28° C and 32° C respectively. We used strain EK-328 and variant M, previously selected during adaptation of the strain EK-328 to *H. marginatum* ticks (Romanova et al., 2007). As mentioned above, variant M

reached higher titres in *H. turanicum* ticks, but had lower titres in PEK cell supernate, than the parental strain EK-328 (Romanova et al., 2007). Therefore, we expected EK-328 and variant M to differ in virus titre and, possibly, in growth kinetics in these tick cell lines.

The kinetics of reproduction of the studied viruses in both tick cell lines were similar (Fig. 2, A, B). Virus titres in cell supernate reached 7-8 $\log_{10}$PFU/ml by 48 hours post infection (h.p.i.), decreased gradually, and remained at a relatively high level (3-4 $\log_{10}$PFU/ml) until the end of the 15-36 d.p.i. observation period and without signs of cytopathic effect. The kinetics of TBEV replication differed in tick cell lines by the time point at which virus titre increased. In the HAE/CTVM8 cell line TBEV titre began to increase after 12 h.p.i. (Fig. 2, A), and in the IRE/CTVM19 cell line by 24 h.p.i. (Fig. 2, B). Thus, TBEV strain EK-328 adapted to mouse brain and variant M adapted to *H. marginatum* ticks showed similar kinetics of virus replication in tick cell lines derived from vector and non-vector ticks.

Long-term persistent infection with strain EK-328 and variant M was examined in cells from *I. ricinus* ticks (IRE/CTVM19). The observation period was 120 days. The kinetics of strain EK-328 and variant M replication in the tick cell line were generally similar (Fig. 3). However, an intense increase in variant M titre was observed at 50 d.p.i., reaching 5.7±0.1 $\log_{10}$PFU/ml at 57 d.p.i. Such an increase in EK-328 titre was not observed. After 57 d.p.i. strain EK-328 replication was characterised by a gradual increase in viral titre to 5.3±0.6 $\log_{10}$PFU/ml at 85 d.p.i. From 66 d.p.i., strain EK-328 and variant M showed similar fluctuations of viral titre in the range 4.8-6.9 $\log_{10}$PFU/ml. Thus, both viruses were characterised by an increase of titre at 50 d.p.i., but for variant M this rise in titre began earlier than for the parental strain EK-328.

Phenotypic characteristics of TBEV during persistent infection of I. ricinus and H. anatolicum tick cell lines

We conducted two different experiments: 1) we characterised clones from the viral populations of EK-328 and variant M that persisted in IRE/CTVM19 and HAE/CTVM8 cell

lines for 84-93 days; 2) we analysed properties of EK-328 and variant M viral populations and clones after 120 days of persistence in IRE/CTVM19.

Characterisation of the EK-328 and variant M clones from population that persisted in IRE/CTVM19 and HAE/CTVM8 cell lines for 84-93 days

Four clones were obtained from the EK-328 population after 84 days of persistence in IRE/CTVM19 cells, two clones from the EK-328 population after 84 days of persistence in HAE/CTVM8 cells and one clone from the variant M population after 93 days of persistence in IRE/CTVM19 cells (Table 2).

Only clones with changed plaque phenotype were picked and then passaged in the same tick cell line in which the original persistent infection was established. In general, clones from EK-328 formed smaller plaques (1-3 mm) than the parental strain. The clone M93dIr from variant M population showed mixed but mostly large-plaque phenotype unlike the parental variant M (Table 2).

Neuroinvasiveness of the clones was studied in BALB/c mice. Clone M93dIr retained the low neuroinvasiveness of the parental variant M. Clones from the strain EK-328 population mainly showed high neuroinvasiveness similar to the parental strain, except for clone Ek84dHa-1 that was selected after 84 days of virus persistence in the HAE/CTVM8 cell line, whose neuroinvasiveness was very low, similar to variant M.

Sequencing of the E protein-coding genome region revealed some interesting substitutions that could be related to some changes in virus properties. Two out of four EK-328 clones from IRE/CTVM19 cell culture and both clones from HAE/CTVM8 cell culture had a Tre_{115}→Ala substitution (Table 2). This substitution, increasing hydrophobicity of the E protein, was described before and was presumed to be adaptive in *I. ricinus* ticks (Khasnatinov et al., 2009).

Clone Ek84dIr-1 had an additional substitution Lys_{296}→Met (Table 2), which was not described before. This substitution decreased lpc of the E protein. Clones Ek84dHa-1 and Ek84dHa-2, in addition to Tre_{115}→Ala, had the lpc-substitution Asp_{203}→His. A similar

substitution, Asp_{203}→Gly, was observed previously (Mandl et al., 2001) and led to virus phenotype changes: small plaque size in porcine kidney stable (PS) cells and low neuroinvasiveness for mice. It is possible that the substitution Asp_{203}→His was also responsible for decreased neuroinvasiveness and small plaque size of clones Ek84dHa-1 and Ek84dHa-2.

Clones Ek84dIr-3 and Ek84dIr-4 both had three amino acid substitutions (Table 2). Two of these (Gly_{28}→Asp, Asp_{67}→Gly) would change the lpc of the E protein but, probably, dramatic changes of the virus properties would not be observed since they were compensating each other.

Thus, persistence of the strain EK-328 in HAE/CTVM8 cells can result in appearance of lpc-mutants in the viral population with decreased neuroinvasiveness for mice.

Clone M93dIr had 2 amino acid substitutions. Ile/Thr_{426}→Thr was a reversion to the EK-328 strain and Lys_{124}→Glu negated additional positive charge of the protein E due to substitution Glu_{122}→Gly. This clone showed mixed but mostly large plaque phenotype and low neuroinvasiveness for laboratory mice. A similar clone, 160/57, was obtained earlier in our laboratory during plaque cloning and one reverse passage of the small plaque from variant M in PEK cells. It had large plaque size (7 mm) and low neuroinvasiveness for laboratory mice (Romanova et al., 2007).

Properties of EK-328 and variant M viral populations after 120 days of persistence in IRE/CTVM19

Interesting changes in the strain EK-328 and variant M plaque phenotype occurred during long-term persistence in the *I. ricinus* cell line (IRE/CTVM19) (Fig. 4). Appearance of bright plaques of different sizes from <1 mm to 3-4 mm was observed for strain EK-328. The proportion of plaques of different sizes varied with time. After 120 d.p.i., bright plaques of 1 mm and 3 mm in the ratio 1:3 were observed (Table 3). Variant M was characterised by changing plaque phenotype from bright <1 mm plaques to pale plaques with blurred edges of 1.5-2 mm and 6 mm (Fig. 4). After 120 d.p.i. in the tick cell line, variant M formed plaques of 6 mm, 3 mm and 1 mm in the ratio 1:1:4 (Table 3).

Variants obtained after the 120-day persistence of strain EK-328 and variant M in the IRE/CTVM19 tick cell line (5000EK and 5000M) showed a change in plaque phenotype in PEK cells in comparison to the parental variants. Variant 5000M retained the low neuroinvasiveness of the parental variant (Table 3). Neuroinvasiveness of the variant 5000EK decreased compared to the parental strain EK-328: the LD_{50} increased from 1.9 to 2.3 $\log_{10}$PFU/ml, but this increase was not statistically significant.

Whole genome nucleotide sequences were obtained for both variants, 5000EK and 5000M. Substitutions are shown in Table 3.

For variant 5000EK, two nucleotide substitutions (A_{1172}→T (protein E); T_{5343}→C (protein NS3)) were discovered. The substitution in the E protein led to the lpc-mutation Asp_{67}→Ala. The T_{5343}→C nucleotide substitution was silent and does not change the NS3 amino acid sequence.

We discovered four nucleotide substitutions in variant 5000M in comparison to parental variant M, one of which was synonymous (T_{7920}→C/T). Variant 5000M retained a mutation in the E protein (Glu_{122}→Gly) and the phenotype of the parental variant, but had a reverse substitution in the E protein (Ile/Thr_{426}→Thr). This virus also had two heterogeneous nucleotides A_{579}→ A/G and C_{6208}→ C/T, which could lead to heterogeneous amino acid positions in the prM (Ile_{37}→ Ile/Met) and NS3 proteins (His_{537}→ His/Tyr).

The Ile/Thr_{426}→Thr substitution in the E protein was a reversion to the parental strain EK-328. Amino acid substitution Ile_{37}→ Ile/Met in the prM protein seems to be unique among TBEV full genome GenBank sequences (183 entries, 28.03.2017). Ile_{37}→ Ile/Met is located in the pr part of the prM protein but not in the cleavage sequence. The His_{537}→ His/Tyr substitution was located in the helicase domain of the NS3 protein. A virus with such a substitution in a similar context was isolated from a human brain in Siberia (accession number AEQ77279, Kulakova et. al., 2012).

During the adaptation of the virus population we observed a situation when we clearly saw phenotypic changes in the population but did not see any changes in consensus sequence with Sanger sequencing. This could happen because the viral population is composed of a vast number of different genotypes that lead to similar phenotypes. It is very important to analyse a viral population as a whole because even small subpopulations can drastically affect population properties (Romanova et al., 2007). We took clones from total populations of 5000EK and 5000M variants for better understanding of the composition of the viral population. All clones were plaque-purified once and then multiplied in the IRE/CTVM19 cell line. We studied plaque phenotype, neuroinvasiveness and nucleotide sequence of the clones.

We selected three clones from the 5000EK variant population. All of these clones had medium plaque size (3-3.5 mm, Table 4). Neuroinvasiveness of 5000EK-2 was similar to the parental population, while 5000EK-1 showed lower neuroinvasiveness for mice than the parental variant (Table 4).

Analysis of the E protein nucleotide sequences of both clones showed that 5000EK-1 with decreased neuroinvasiveness had an additional substitution (His_{157}→Tyr) (Table 4). It is possible that due to this unique substitution its neuroinvasiveness was lower than that of the parental strain.

Variant 5000M demonstrated plaque size heterogeneity (Table 3). We cloned one of the large plaques (5000M-1) and studied it in more detail (Table 5).

5000M-1 formed 6 mm plaques in PEK cells and showed high neuroinvasiveness for mice. The LD_{50} of the M variant was over 3 log_{10}PFU/ml, while for 5000M-1 it was 1.5 log_{10}PFU/ml, similar to that of the strain EK-328. We determined the full genome sequence of the 5000M-1 clone to understand the reason for these phenotypic changes (Table 5). 5000M-1 had 14 nucleotide reversions to the EK-328 strain (original isolate from the pool of *I. persulcatus* ticks) from variant M, and two unique nucleotide substitutions at positions 3024 and 9306. Variant 5000M had six additional nucleotide substitutions from variant M, which were not shared by

5000M-1. Biological properties such as plaque size and neuroinvasiveness of 5000M-1 were also closer to strain EK-328 than to its parental variant 5000M (no data shown). We consider that such a large number of nucleotide reversions were unlikely to have occurred under our experimental conditions, so this clone could be a direct descendant of the strain EK-328, which was used in experiments with serial passages in *H. marginatum* ticks (Chunikhin and Dzhivanyan, 1979), rather than a descendant of variant 5000M or variant M.

Discussion

In natural foci, long-term persistence of the virus in ticks includes not only the periods of tick activity, but also preservation of the virus during diapause, moulting and oviposition. The biochemical processes that occur during these stages of the tick life cycle may affect the genetic composition of the viral population. In the present study we analysed phenotypic and genetic properties of the Siberian TBEV subtype population during long-term persistent infection of usual and unusual vector ticks and of cell lines derived from unusual and non-vector tick species.

TBEV infection of ticks

According to our data, TBEV of Siberian subtype successfully replicated and persisted both in the main virus vectors (*I. ricinus* and *I. persulcatus*) and in *D. reticulatus* ticks and the highest virus titres were observed in *D. reticulatus* ticks. We can assume that level of virus reproduction and duration of virus persistence in the tick are important, but not decisive, factors that determine the vector competence of ticks.

We are aware that in our experiments we infected ticks by the unnatural percoxal route that allowed us to inject the virus directly into the tick haemocoel, bypassing the intestinal barrier. However, in our opinion, this circumstance could not greatly affect the resulting picture of the long-term persistence of TBEV in the tick, as previous studies have demonstrated *Dermacentor* ticks can acquire and transmit TBEV during experimental feeding on mammalian hosts (Alekseev and Chunikhin, 1992; Belova et al., 2013b).

In our experiments we used size of plaques in PEK cells as an indicator of changes in the composition of the viral population during persistent infection. Apparently, all changes may not be noticeable when assessing only the plaque size. We are also aware that lpc-mutations are not the only reason for a small-plaque phenotype in PEK cells and vice versa. However, plaque size is an easy-to-observe indicator, and appearance of small plaques is a marker of the presence of lpc-mutants in a viral population.

Previously it has been shown that adaptation of TBEV to *H. marginatum* ticks leads to selection of a variant with unchanged high neurovirulence, but decreased neuroinvasiveness, for mice (Romanova et al., 2007). Therefore, in the present work only neuroinvasiveness for mice by i/p inoculation was used to describe and compare virulence features of the original strain and obtained virus variants. Neuroinvasiveness of the original strain EK-328 adapted to mice CNS was rather high (0.9 $\log_{10}$PFU, Table 1). One passage of this strain in PEK cells decreased the virus neuroinvasiveness (1.9 $\log_{10}$PFU, Table 1). This phenomenon was shown in several experiments (Shevtsova et al., 2017). Strain EK-328 after one passage in PEK cells was used for persistent infection of ticks. However, all virus variants obtained after persistence in ticks showed neuroinvasiveness similar to those of the original strain EK-328 adapted to the CNS of mice (from 0.3 to 1.2 $\log_{10}$PFU, Table 1).

It has been shown that serial passage of TBEV in ticks leads to selection of variants with reduced plaque size in PEK cells, reduced neuroinvasiveness and characteristic changes in properties of the virion due to nucleotide substitutions in the E protein (Labuda et al., 1994; Romanova et al., 2007). In our experiments, only plaque phenotype changes were observed during the adaptation of strain EK-328 to persistent infection of different tick species, and only in some cases. We did not observe reduced neuroinvasiveness for mice of the variants with heterogeneous plaques; this could have been due to a low proportion of the mutants being present in the viral population. We also did not detect any changes in nucleotide sequence of the genome fragment encoding E protein of the variants adapted to persistent infection of

D. reticulatus ticks. The Sanger method of sequencing allows detection of nucleotide mutants in a virus population when present at a rate of 20-25% or more (Solmone et al., 2009). This methodological restriction could explain the fact that we did not find substitutions in the E protein sequences of the analysed variants. The mutants may have been present in the total population at a rate too low for detection and/or the nucleotide substitutions may have been localised in various positions that did not affect the consensus sequence (Domingo et al., 2012).

In infected ticks, different organs and tissues may have some impact on the viral population depending on the location of virus replication. Further experiments to study TBEV persistent infection in detail were performed in the more homogeneous system of tick cell lines.

TBEV infection of tick cell lines

We showed that the kinetics of replication of Siberian TBEV subtype, isolated from *I. persulcatus*, were similar in cells from vector (*I. ricinus*) and non-vector (*H. anatolicum*) ticks. Similar results were obtained previously when titres of TBEV of European and Siberian subtype at 5 and 6 d.p.i. were comparable in ticks of five species of the genera *Ixodes* and *Dermacentor* (Belova et al., 2014). However, it has been shown that, after 10 d.p.i., TBEV (strain Neudörfl, European subtype) titres in tick cell lines derived from the main virus vector *I. ricinus* were 100-1000 times higher than virus titres in tick cell lines derived from non-vector ticks *I. scapularis, R.* (*B.*) *microplus, H. anatolicum, R. appendiculatus,* and *O. moubata* (Ruzek et al., 2008b). It is safe to assume that different TBEV subtypes or different virus strains regardless of subtype can vary in their ability to replicate in cells of unusual tick species.

During this study, we obtained a number of clones from viral populations adapted to tick cell lines (see Table 2 and Table 4), and the most common feature amongst them (7 out of 10 clones) was appearance of lpc-mutations. Only clone Ek84dIr-1 had a substitution that decreased lpc of the E protein (Lys_{296}→Met). Clones Ek84dIr-3, Ek84dIr-4 and M93dIr had sets of two charge-compensating mutations. Similar clones were obtained earlier during cloning and passage of tick-adapted variant M in PEK cells (Romanova et al., 2007).

Previously, it has been reported that lpc-mutants can appear during TBEV adaptation to ticks (Labuda et al., 1994; Romanova et al., 2007; Khasnatinov et al., 2009) and, moreover, at least some of the mutants have had an advantage for replication in ticks (Khasnatinov et al. 2009). In our experiment, two different processes determined properties and genotype of the clones: selective pressure during persistent infection of tick cell lines and the cloning process itself, which was basically virus multiplication in PEK cells at extremely low MOI (see Materials and Methods). This process can lead to rapid changes in the viral population (Romanova et al., 2007). In our work, not only the majority of clones but also all the sequenced adapted variants had lpc-mutations. Thus lpc-mutations can be a result of selective pressure on the viral population in tick cell lines. Virus variants with additional substitutions (compensating and/or other substitutions), found in this study, could be really present in the viral population, and/or they could be fixed in the viral population during cloning.

In our experiments we also found four clones with E protein Tre_{115}→Ala substitution (Table 2) in viral populations adapted to IRE/CTVM19 and HAE/CTVM8. It was proposed that this substitution could be adaptive in *I. ricinus* ticks and could distinguish viruses adapted to *I. ricinus* from the ones adapted to *I. persulcatus* (Khasnatinov et. al., 2009). Our data provide additional evidence that this substitution benefits virus in *I. ricinus* cells. We also found clones with this substitution in the HAE/CTVM8 cell line derived from *H. anatolicum.*

It is important to note that the E protein Asp_{67}→Ala mutation (discovered in the 5000EK consensus sequence as well as in 5000EK-1, 5000EK-2, 5000EK-3, Ek84dIr-3, Ek84dIr-4 clones) is similar to the one found in viruses isolated from ticks (Khasnatinov et. al., 2009). Theses authors showed that the replacement of Asp_{67}→Gly increases virion affinity to the cell glycosaminoglycans, decreases virus neuroinvasiveness for mice and gives the virus a replication advantage in ticks. It can be assumed that the Asp_{67}→Ala substitution may also provide such benefits in tick cells *in vitro* and can be responsible for reduced plaque size and decreased neuroinvasiveness of the variant 5000EK.

Clone 5000M-1 of variant M adapted to the IRE/CTVM19 cell line had genotype (Table 5) and phenotype almost identical to strain EK-328, parental to variant M. Previously we showed that 17 passages in *H. marginatum* ticks was not enough to completely remove parental strain EK-328 from the viral population, despite the fact that it was less adapted to replication in *H. marginatum* ticks, and it could be found in the variant M population after 5 passages in mouse CNS (Romanova et al., 2007). In the present work we have shown that an additional passage in PEK cells and 120 days of persistence in the IRE/CTVM19 cell line also did not lead to elimination of the parental strain. Moreover, under suitable conditions it could become a major part of the viral population. Therefore, virus variants with properties vastly different from the majority can persist within the viral population for a very long time.

We found some interesting mutations during our examination of the consensus sequence of 5000M variants adapted to the IRE/CTVM19 cell line. Firstly, position 426 in the E protein changed from Ile/Thr heterogeneity (with Ile in majority) to Thr. Generally, TBEV strains of European genotype, with *I. ricinus* as a main vector, have Ala in this position, while strains of Siberian and Far-Eastern genotype with *I. persulcatus* as a main vector have Thr. Previously, during adaptation of strain EK-328 to *H. marginatum* ticks (i.e. variant M), Ile appeared in this position. In our study, during persistence of the variant M in the *I. ricinus* cell line, Thr that is characteristic of the parental Siberian genotype, but not of the European one, appeared in the 426 position. This fact does not contradict an assumption of Ala preference in *I. ricinus* ticks, as Ile to Ala mutation requires 2 nucleotide substitutions which may take a longer adaptation time.

Secondly, in the variant 5000M we found a nonsynonymous mutation ($His_{537} \rightarrow$His/Tyr) in the NS3 protein of the viral replicative complex. As Variant M has several mutations in replicative complex proteins and in the 5`-NTR, which is important for flavivirus replication initiation (Filomatori et al., 2006), we can speculate that the observed mutation in the NS3 protein is a further adjustment of the virus replication machinery rather than a random mutation or a result of genetic drift.

It is important to note that variant 5000M accumulated four nucleotide substitutions (three nonsynonymous) and variant 5000EK only two nucleotide substitutions during 120 days of persistent infection in the IRE/CTVM19 cell line. Thus in our experiments the ability of the variant M population to evolve rapidly was higher than that of the EK-328 strain. The fact that three of the nucleotide substitutions in M variant were nonsynonymous could be a sign of strong selective pressure on the viral population in IRE/CTVM19 cells.

Every single virus variant or clone that we obtained during experiments in tick cell lines had at least one substitution in the E protein (Fig. 5). Moreover, the majority of these viruses were lpc-mutants. Thus, we can say that the E protein is extremely important in adaptation to tick cell lines. We can also assume that tick cell lines favour selection of viral variants with low neuroinvasiveness. We cannot say if the same selection takes place in intact ticks as in tick cell lines. In infected ticks, TBEV can be found in almost all organs and tissues (Stefutkina, 1989), which may exert additional selective effects on the viral population. As a result, the accumulation of lpc-mutants in ticks may occur more slowly than in tick cell lines, and/or during virus persistence in ticks the set of lpc-mutants may be more diverse and not identifiable by analysis of the consensus sequence.

Conclusions

TBEV strain EK-328 of the Siberian subtype effectively replicated and formed persistent infection in ticks and tick cell lines of vector and non-vector species. Thus, the level of virus reproduction and the duration of virus persistence in ticks are important, but not decisive, factors that determine vector competence.

The rate and features of viral population changes are different during persistent infection of ticks and tick cell lines. During persistence of TBEV in *Ixodes* and *Dermacentor* ticks, properties of the viral population remained virtually unchanged. Persistent TBEV infection of the tick cell lines favoured selection of viral variants with low neuroinvasiveness for laboratory mice and substitutions in the E protein that increased its lpc (lpc-mutants).

The original strain adapted to CNS of mice was not eliminated from the viral population during long-term persistence of TBEV in ticks and tick cell lines.

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Figure captions

Fig. 1. The kinetics of TBEV strain EK-328 replication during persistent infection of *Ixodes ricinus, I. persulcatus* and *Dermacentor reticulatus* ticks. *I. ricinus* at 0-2 d.p.i. and *I. persulcatus* at 0 d.p.i. were not tested.

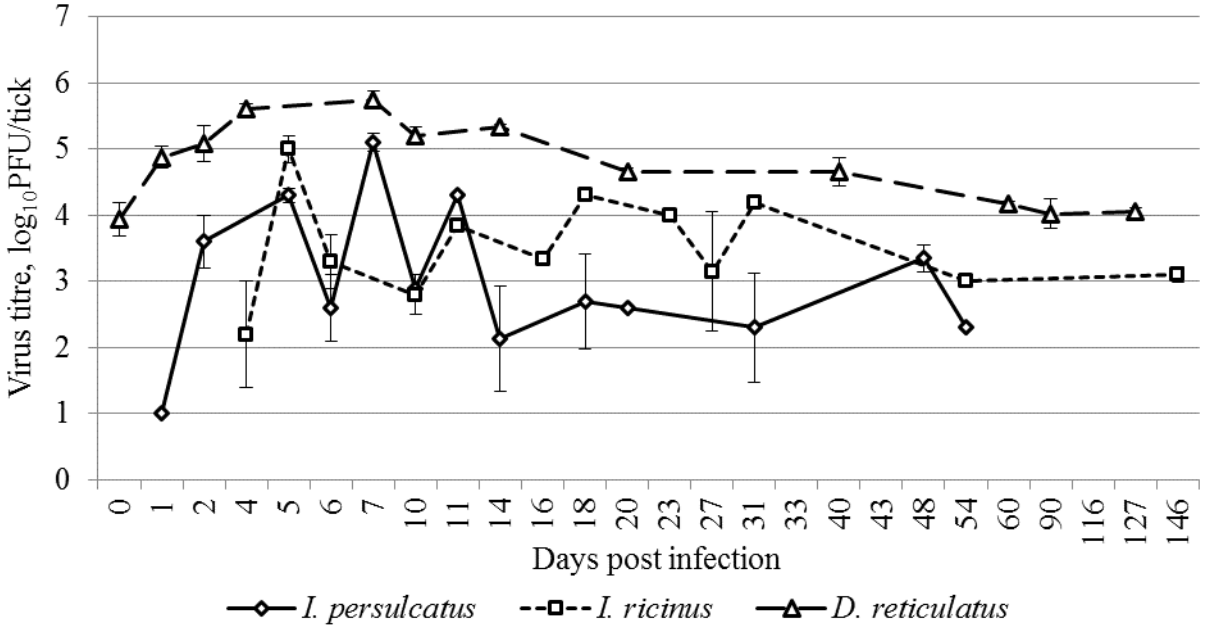
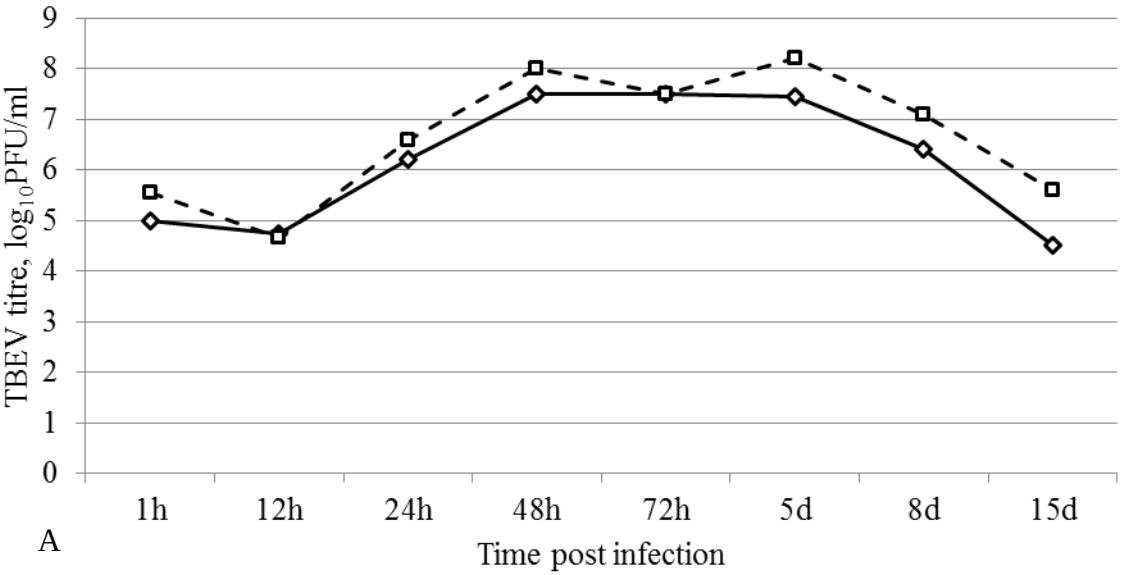
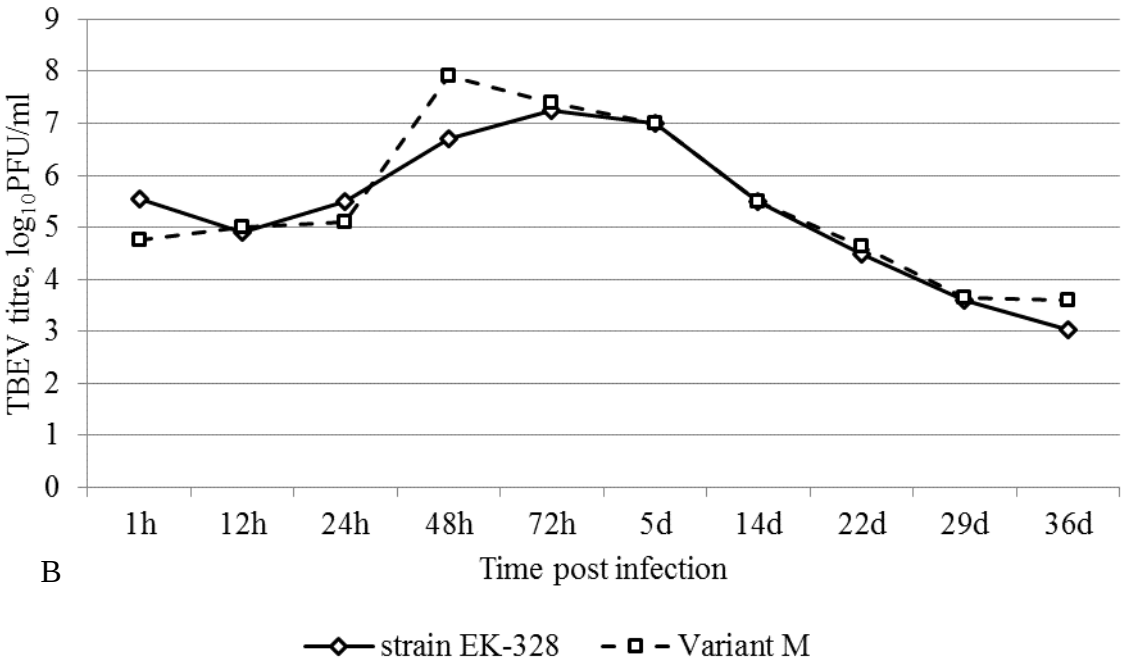


Fig. 2. The kinetics of TBEV strain EK-328 and variant M replication in tick cell lines derived from *Hyalomma anatolicum* (HAE/CTVM8) (A) and *Ixodes ricinus* (IRE/CTVM19) (B).



A



B

Fig. 3. Virus titre during long-term persistence of the TBEV strain EK-328 and variant M in an *Ixodes ricinus* tick cell line (IRE/CTVM19).

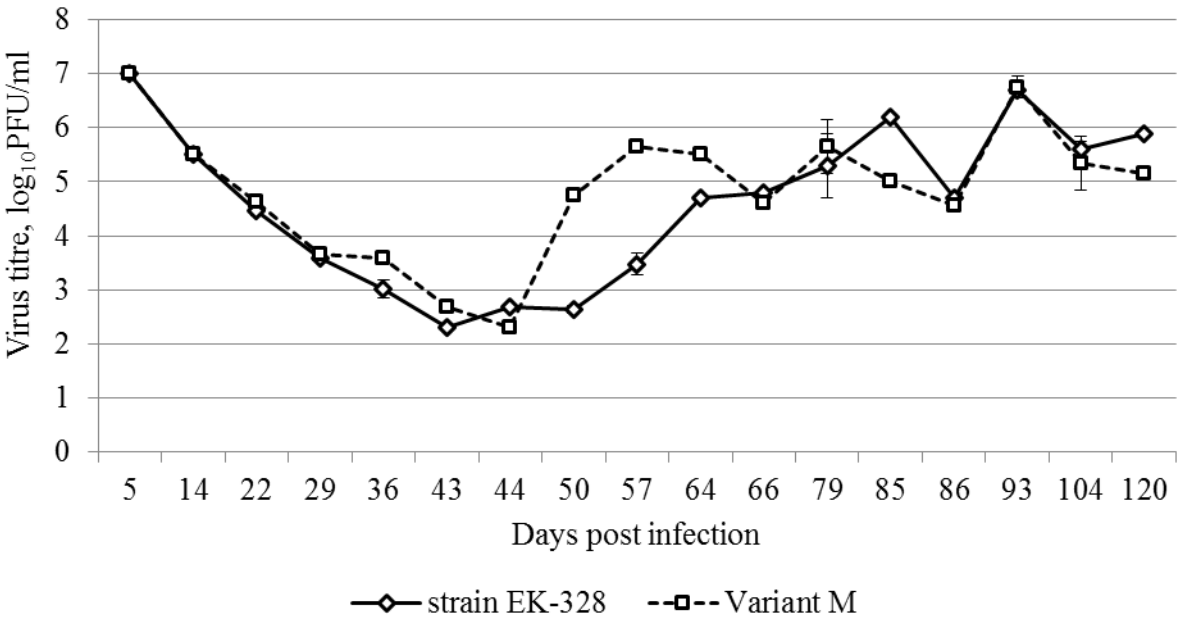


Fig. 4. Changes of the TBEV strain EK-328 and variant M plaque phenotype during long-term persistence in an *Ixodes ricinus* tick cell line (IRE/CTVM19).

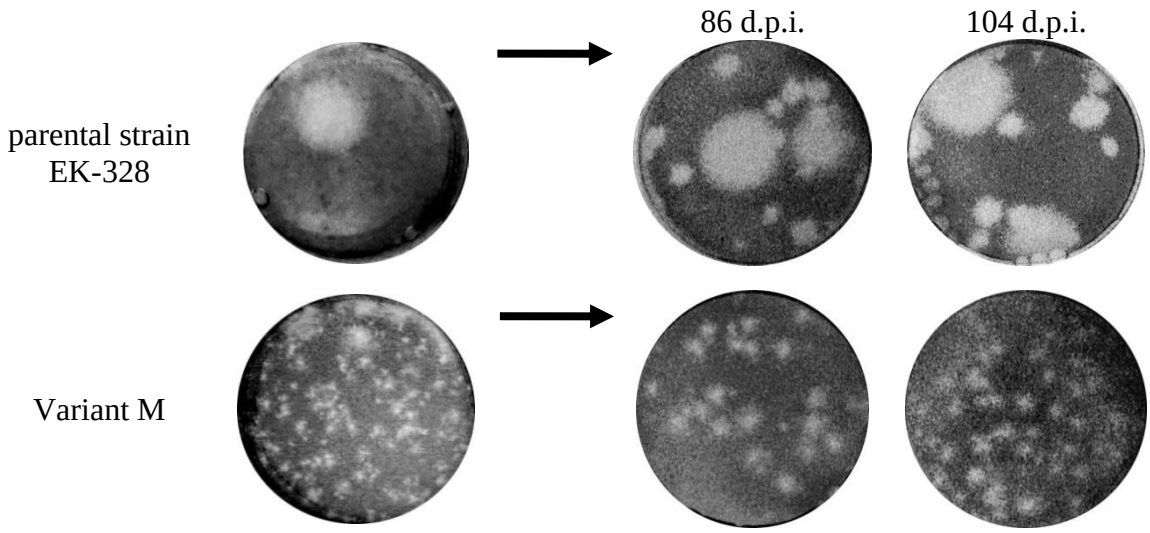
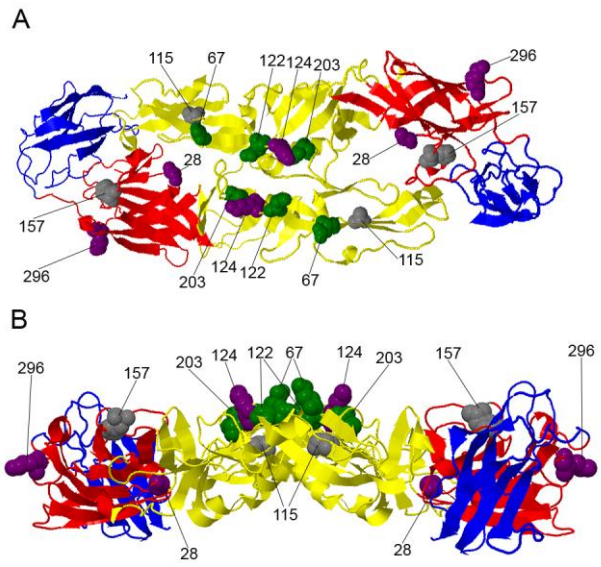


Fig. 5. Substitutions in the E protein of TBEV after adaptation to persistent infection of tick cell lines, showing surface (a) and side (b) views. Subunits coloured as domain I (red), domain II (yellow), and domain III (blue). Highlighted amino acids: green – substitutions increasing local positive charge (lpc-substitutions), purple – substitutions decreasing local positive charge, grey – substitutions that do not change local positive charge of the E protein molecule. Numbering of the substitutions is done according to the TBEV E protein.



Tables

Table 1. Plaque phenotype in PEK cells and neuroinvasiveness for mice (LD_{50}) of the TBEV strain EK-328 variants adapted to persistent infection of different tick species.

Variant number	Passage history	TBEV titre log_{10}PFU/tick	Plaque size, mm[a]	LD_{50}, log_{10}PFU[b]
original strain EK-328	11 passages in mice	-	6-8	0.9
EK-328	11 passages in mice, 1 passage in PEK cells	-	6-8	1.9
Ir15d	EK-328 + 15 days in unfed *I. ricinus*	2.7	8-10	0.3
Ir146d	EK-328 + 146 days in unfed *I. ricinus*	3	8-10	nd
Ip18d	EK-328 + 18 days in unfed *I. persulcatus*	3.2	7-8 (96%), <1 (4%)	0.8
Ip48d	EK-328 + 48 days in unfed *I. persulcatus*	3.2	8-10	nd
Dr14d	EK-328 + 14 days in unfed *D. reticulatus*	4.1	7-8	0.7
Dr40d	EK-328 + 40 days in unfed *D. reticulatus*	5.3	8-10	nd
Dr128d	EK-328 + 128 days in unfed *D. reticulatus*	3.2	8 (50%), 1 (50%)	0.8
Dr128d104hF	EK-328 + 128 days in unfed and 104 hours in feeding *D. reticulatus*	5.4	7 (50%), 2.5 (50%)	1.2
Dr128d7dF	EK-328 + 128 days in unfed and 7 days in feeding *D. reticulatus*	6.3	8-10	nd
Dr135d	EK-328 + 135 days in unfed *D. reticulatus*	2.7	3-4	nd

[a] size of plaques on PEK cell culture was measured at 7 d.p.i. The percentage of each plaque size is shown in brackets.

[b] The error in the experimental method was previously determined as 0.5 log_{10}PFU (Kozlovskaya et al., 2010).

nd – not done

Table 2. Properties of the TBEV EK-328 strain clones after 84-93 days of persistence in tick cell lines (compared with parental viruses).

Clone number	Passage history before clone selection	Plaque size[a], mm	LD_{50}, $log_{10}PFU$[b]	Amino acid substitutions in E protein (from parental strain)
parental strain EK-328	10 passages in mice, 1 passage in PEK cells	6-8	1.9	-
Ek84dIr-1	EK-328 after 84 days persistence in IRE/CTVM19 cells	3	2.3	Tre_{115}→Ala *Lys_{296}→Met* [c]
Ek84dIr-2	EK-328 after 84 days persistence in IRE/CTVM19 cells	1	1.1	Tre_{115}→Ala
Ek84dIr-3	EK-328 after 84 days persistence in IRE/CTVM19 cells	1.5	1.9	*Gly_{28}→Asp* **Asp_{67}→Gly**[d] Ala_{463}→Val
Ek84dIr-4	EK-328 after 84 days persistence in IRE/CTVM19 cells	1	nd	*Gly_{28}→Asp* **Asp_{67}→Gly** Ala_{463}→Val
Ek84dHa-1	EK-328 after 84 days persistence in HAE/CTVM8 cells	1	>3	**Asp_{203}→His** Tre_{115}→Ala
Ek84dHa-2	EK-328 after 84 days persistence in HAE/CTVM8 cells	1	nd	**Asp_{203}→His** Tre_{115}→Ala
parental variant M	17 passages of the strain EK-328 in *H. marginatum*, 5 passages in mice, 1 passage in PEK cells	5-8 (1%), <1 (99%)	>3	-
M93dIr	Variant M after 93 days persistence in IRE/CTVM19 cells	6 (75%), 1 (25%)	>3	*Lys_{124}→Glu* Ile_{426}→Thr

[a] size of plaques on PEK cell culture was measured at 7 d.p.i. The percentage of each plaque size is shown in brackets.

[b] The error in the experimental method was previously determined as 0.5 $log_{10}PFU$ (Kozlovskaya et al., 2010).

[c] substitutions decreasing local positive charge are shown in italics.

[d] substitutions increasing local positive charge (lpc-substitutions) are shown in bold.

nd – not done

Table 3. Characteristics of the TBEV strain EK-328 variants after 120 days of persistent infection in an *Ixodes ricinus* cell line (IRE/CTVM19).

Virus	Passage history	Plaque size, mm[a]	LD_{50}, log_{10}PFU [b]	Amino acid substitutions[d] (compared to the parental strain EK-328)
parental strain EK-328	10 passages in mice, 1 passage in PEK cells	6-8	1.9	-
5000EK	EK328+ 120 days + 1 passage in IRE/CTVM19	3 (25%), 1 (75%)	2.3	(E) **Asp_{67}→Ala** [c]
parental variant M	17 passages of the strain EK-328 in *H. marginatum*, 5 passages in mice, 1 passage in PEK cells	5-8 (1%), <1 (99%)	>3	(prM) Ala→Val (E) **Glu_{122}→Gly** (E) Thr_{426}→Ile/ Thr (NS1) Glu_{280}→Asp (NS2a) Lys_{52}→Asn (NS4a) Ser_{22}→Gly (NS4a) Arg_{41}→Lys
5000M	Variant M + 120 days + 1 passage in IRE/CTVM19	6 (17%), 3 (17%), 1 (66%)	>3	(prM) Ile_{37}→ Ile/Met (E) **Glu_{122}→Gly** (NS1) Glu_{280}→Asp (NS3) His_{537}→ His/Tyr

[a] size of plaques in PEK cells at 7 d.p.i. The percentage of each plaque size is shown in brackets.

[b] data received for variants that persisted in tick cell culture for 120 days without additional passage; the error in the experimental method was previously determined as 0.5 log_{10}PFU (Kozlovskaya et al., 2010).

[c] substitutions increasing local positive charge (lpc-substitutions) are shown in bold.

[d] protein with a mutation is shown in parenthesis.

Table 4. Plaque phenotype and neuroinvasiveness of the TBEV 5000EK clones.

Virus	Plaque size, mm[a]	LD_{50}, $log_{10}PFU$[b]	Amino acid substitutions in E proteinc
5000EK	3 (25%), 1 (75%)	2.3	**Asp_{67}→Ala**[d]
5000EK-1	3	3.9	**Asp_{67}→Ala** His_{157}→Tyr
5000EK-2	3,5	2.5	**Asp_{67}→Ala**
5000EK-3	3	nd	**Asp_{67}→Ala**

[a] size of plaques in PEK cells at 7 d.p.i. The percentage of each plaque size is shown in brackets.

[b] The error in the experimental method was previously determined as 0.5 $log_{10}PFU$ (Kozlovskaya et al., 2010)

[c] in comparison to the strain EK-328

[d] substitutions increasing local positive charge (lpc-substitutions) are shown in bold.

nd – not done

Table 5. Comparison of the nucleotide sequences of TBEV strain EK-328, variant M, variant 5000M, and 5000M-1.

Nucleotide position in viral genome (amino acid position numbered for each protein and mutation[a])	Variant M	Variant 5000M	EK-328	5000M-1
19	G	G	A[c]	A
42	A	A	C	C
470 (Ala_{17}→Val)	T	T	C	C
483	G	G	G/A	G
579 (Ile_{37}→Met)	A	*G/A*[b]	A	A
1337 (Glu_{122}→Gly)	G	G	A	A
2190	T	T	C	C
2249 (Thr_{426}→Ile)	T/C	C	C	C
2362	C	C	T	T
3024	T	T	T	C[d]
3300 (Glu_{280}→Asp)	C	C	G	G
3387	T	T	C	C
3438	A	A	G	G
3672 (Lys_{52}→Asn)	T	T	G	G
4827	A	A	G	G
6208 (His_{537}→Tyr)	C	*C/T*	C	C
6526 (Ser_{22}→Gly)	G	G	A	A
6584 (Arg_{41}→Lys)	A	A	G	G
7437	C	C	G	G
7920	T	*C/T*	T	T
9306	T	T	T	C
10104	T	T	C	C

[a] amino acid substitutions are shown in comparison with EK-328.

[b] nucleotide substitutions unique to 5000M variant are shown in italics.

[c] nucleotides matching between 5000M-1 and EK-328 but not variant M or 5000M are highlighted in grey.

[d] nucleotide substitutions unique to 5000M-1 are underlined.