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Detection of tick-borne encephalitis virus RNA in patient samples at different stages of infection

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SUMMARY

Objectives: The aim of the study was to evaluate the efficiency of molecular diagnostics of tick-borne encephalitis (TBE) and to correlate viral RNA (vRNA) detection with the clinical and laboratory data.
Methods: Clinical samples from 1125 patients from South Bohemia, Czech Republic, a highly endemic TBE region, were screened for TBE virus (TBEV) RNA by RT-qPCR. Samples included blood, serum, cerebrospinal fluid (CSF), and urine.
Results: TBEV RNA was detected in 14 patients with clinically proven TBE. TBEV RNA was most frequently detected in sera during early infection (11/37 patients tested, 29.7%) but decreased with rising IgG antibody response (3/228, 1.3%). Detection in CSF and urine was infrequent (1/30, 3.3% and 1/52, 1.9%, respectively). Additionally, five patients initially not diagnosed with TBE were retrospectively found to have TBEV RNA in serum, indicating possible underdiagnosis, particularly in mild or atypical presentations. The study also highlighted the diagnostic challenge of an immunocompromised patient whose delayed antibody response hindered timely diagnosis. In such cases, RT-qPCR could significantly shorten the diagnostic timeline.
Conclusions: These findings underscore the value of early RNA detection in improving the diagnosis of TBE and may in the future facilitate the early administration of potential treatment, thereby improving patient outcomes.

Introduction

Tick-borne encephalitis (TBE) is an infection of the central nervous system caused by the tick-borne encephalitis virus (TBEV), genus *Orthoflavivirus*, family *Flaviviridae*.[1] The disease is endemic in temperate climates from western Europe to eastern Asia and is transmitted to humans predominantly by tick bites .[2] More than 10,000 cases are reported worldwide annually. Recently there have been cases of autochthonous transmission in countries previously considered risk-free, such as the UK, the Netherlands, and Belgium. Therefore, this infection is becoming an increasing concern in Europe.[3]

The typical course of the disease is biphasic. The first, initial, phase is characterised by mild, non-specific symptoms (fever, fatigue, headache, joint and muscle pain etc.) and coincides with the spread of the virus from the site of infection via the bloodstream. With the development of specific antibody response, the viral RNA in the blood is usually no longer detectable.[4] After the symptoms of the first phase have disappeared for a few days, the disease progresses to a neurological phase in most patients. The second phase of the infection is characterised by neurological symptoms caused by the entry of the virus into the central nervous system and subsequent replication as well as immune-mediated pathology.[2]

The diagnosis of TBE is typically based on the presence of clinical symptoms of CNS inflammation and laboratory criteria (positive IgM

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and IgG serology, IgM antibodies in the cerebrospinal fluid (CSF), TBEV RNA detection, or virus isolation),[5] nevertheless, these parameters (apart from direct detection of the virus in blood) do not encompass the initial phase of infection, during which the symptoms are rather non-specific and antibodies are not yet present.

The utility of RT-qPCR detection of viral RNA (vRNA) in blood to distinguish the first phase of TBE from other febrile infections with similar symptoms (e.g. anaplasmosis, CMV infection, other arboviral infections), has been suggested several times in the past.[4,6] However, current work on the direct detection and characterisation of TBEV strains in human patients has been limited, with few studies conducted mainly on small patient cohorts and confined to certain geographical areas.[7–9] This report presents the first cohort of patients from the Czech Republic, in whom the virus was detected in various clinical materials (blood, serum, urine, CSF) at different disease stages. The results of molecular detection are correlated with serological and other laboratory data as well as with the clinical picture of the patients. Furthermore, the practical utility of early-phase molecular diagnostics of TBE is demonstrated through several specific clinical cases.

Materials and methods

Patients and samples

The clinical specimens for TBEV RNA detection (whole blood, sera, CSF, and urine) were obtained in two ways: prospectively and retrospectively. Prospective sampling included adult patients examined in the outpatient unit of the Infectious Diseases Department of Hospital Ceske Budejovice, South Bohemian Region, Czech Republic (region highly endemic for TBE) between 2020–2022 who presented with febrile illness during the TBE season. Additional samples were obtained retrospectively from the clinical residuals of all serum samples tested for the presence of specific anti-TBEV antibodies. These tests were performed in the Laboratory of Virology at Hospital Ceske Budejovice during the period 2020–2022 as part of routine clinical diagnostics.

Altogether 1125 patients were sampled: 112 (10.0%) within the prospective and 1013 (90.0%) in the retrospective part of the study, resulting in a total of 1137 serum samples (95 sampled prospectively, 1042 retrospectively), including repeated sampling, 30 samples of full uncoagulated blood, 30 samples of CSF, and 52 samples of urine. Whole blood and urine were only collected from patients who agreed with the additional sampling and their clinical status allowed the sampling. CSF was available only in cases the samples were taken within the routine diagnostic procedure.

Concerning the results of the serological testing and the clinical data, the patients were divided into the following groups: 1) patients with confirmed TBE (N=265): 1a) patients sampled repeatedly with documented seroconversion during the duration of the study (N=15); 1b) patients with only IgM positivity and no further sampling, i.e. probable TBE patients in the early phase of the disease (N=22); 1c) patients with both IgM and IgG positivity in the first sample (N=228); 2) patients tested IgM negative/IgG positive i.e. vaccinated or with a probable history of TBE (N=42) 3) patients tested IgM/IgG negative including patients with other aetiology of symptoms (N=818) (Table 1). Data on clinical history, symptoms, and laboratory findings (e.g. C-reactive protein levels, liver function tests, complete blood count) for 19 TBEV RNA-positive patients were retrospectively collected from the hospital information system.

Case definition

TBE cases were identified based on the ECDC case definition[10] implemented by the European Commission,[11] which we expanded to include the presence of typical clinical symptoms of the first phase (summer febrile illness without neurological symptoms). The disease phase upon sampling and severity of the neurological phase were evaluated based on clinical findings: 1) first phase was defined as febrile illness with other accompanying non-specific symptoms (e.g. headache, arthralgia, myalgia, chills, fatigue, diarrhoea) but no neurological impairment, 2) second phase was defined by the presence of neurological symptoms (meningitis,encephalitis, encephalomyelitis) in the presence of CSF inflammation (CSF leukocytes > 5×10^6 cells/l).

Sample processing

RNA was extracted from 200 µl of the samples using MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific) and KingFisher Apex automated isolator (Thermo Fisher Scientific) according to the instructions of the manufacturer. The detection of vRNA in the urine samples was extensively optimised in terms of sample collection and storage: use of urine conditioning buffer (UCB; Zymo Research), storage conditions (−80 °C with/without UCB, room temperature with/without UCB); sample concentration and RNA extraction. The optimisation steps were tested using negative urine samples spiked with known amounts of TBEV (strain Hypr) in a dilution row ranging from 200 plaque-forming units (PFU)/ml of urine to 0.002 PFU/ml of urine. Based on the results, a combination of the previously described RNA extraction protocol [11] with 10x sample concentration proved to gain the highest sensitivity. Briefly, the urine samples (total volume 30–50 ml) were 10x concentrated using 30 kDa Amicon Ultra Centrifugal Filters (Millipore). RNA was isolated from 140 µl of the concentrated sample using QIAamp Viral RNA Kit (Qiagen) with modifications according to Gorchakov et al.[12]

Serological antibody analysis

The presence of specific TBEV antibodies in serum samples was determined in the Laboratory of Virology at Hospital Ceske Budejovice as part of routine diagnostics using Chorus TBEV (IgM/IgG) ELISA Test (DIESSE Diagnostica Senese Società Benefit S.p.A.) following the manufacturer's instructions.

RT-qPCR detection

Using SuperScript III Platinum master mix (Invitrogen), a one-step reverse transcription-quantitative polymerase chain reaction

Table 1
Proportion of patients with detected TBEV RNA according to clinical presentation and the presence of antibodies to TBEV in serum.

	Confirmed or probable[a] TBEV infection			Vaccinated and TBE history	Serologically TBE negative
	Documented seroconversion[b]	IgM+/IgG-	IgM+/IgG+	IgM-/IgG+	IgM-/IgG-
Patients tested	15	22	228	42	818
TBEV RNA positive	10	1	3	1	5
Proportion	66.7%	4.5%	1.3%	2.4%	0.6%

[a] Probable TBEV infection – patients with single serum sample available which was specific anti-TBEV IgM positive and IgG negative and symptoms were consistent with TBE.
[b] Patients with documented seroconversion during the study – i.e. tested TBEV serologically negative in the initial sampling(s), and IgM positive and IgG positive in one or more of the subsequent samples.

(RT-qPCR) was used to detect the virus in 5 µl of the extracted RNA as described by Gäumann et al.[13] A synthetic single-stranded DNA standard was used to create a calibration curve to allow relative quantification of the viral genome copy numbers in the samples.

Virus isolation

Selected RT-qPCR-positive samples were inoculated on a six-well plate with A549 cells (ATCC) grown to 80% confluence in dedicated media (DMEM Low Glucose w/ Stable Glutamine w/ Sodium Pyruvate,10% BOFES (Biosera), 1% L-glutamine (Biosera), 1% antibiotics (penicillin/streptomycin/amphotericin B) (Biosera)). The cells were followed each day for signs of cytopathic effect (CPE) indicating virus replication. Samples were taken after CPE was evident or after 6 days post inoculation and tested for the presence of the vRNA by RT-qPCR, as described above. Viral titres were determined by plaque assay on A549 cells according to previously established protocols.[14]

Sanger sequencing

For samples positive in RT-qPCR, cDNA was generated using High-Capacity RNA-to-cDNA Kit (Thermofisher Scientific) and random octamers. Subsequently, 4 µl of cDNA were used as a template in a conventional PCR reaction using primers 3 F and 3 R [8] and PrimeSTAR Max DNA Polymerase (Takara). PCR products were Sanger sequenced from both ends (Eurofins). The obtained sequences were checked manually, assembled, and included in the phylogenetic analyses.

Next generation sequencing

Using Primal Scheme online primer design tool,[15] a set of 27 primer pairs (Suppl. Table 1), each amplifying an approximate 500 bp segment of the TBEV genome with approximately 100 bp overlap between amplicons was designed based on an alignment of all available full-genome sequences of European subtype TBEV strains. Due to the variable length of TBEV 3′UTR sequences, the previously validated primer pair TBE_LJ_37 [9] was included to expand the range of our coverage in this region. Equimolar amounts of each primer were mixed in two primer pools for multiplex PCR of non-overlapping amplicons (final concentration 0.015 µM per primer). Reverse transcription, PCR amplification and library preparation were conducted according to manufacturer's protocol using the NEBNext ARTIC SARS-CoV-2 Companion Kit (Oxford Nanopore Technologies) substituting the provided SARS-CoV-2 primer sets for our TBEV primers. MinION flow-cell and library preparation followed Oxford Nanopore Protocol SQK-LSK109. Sequencing was run until each barcoded sample reached a minimum of 10,000 pass reads. Subsequent analysis was conducted within the Linux environment using Ubuntu version 22.04.3 LTS. Pass read POD5 files were demultiplexed and basecalled using the Dorado super-high-accuracy algorithm v.3.6 and quality checked using samtools and FastQC. The ARTIC pipeline was used to trim and polish sequences using the Medaka algorithm and to assemble reads using Minimap2 against the primal-scheme alignment consensus.

Obtained sequences were deposited in the GenBank database under the following accession numbers; partial E protein sequences: PQ356372-PQ356377, complete coding nucleotide sequences (CDS): PQ356378-PQ356382 (Suppl. Table 2).

Phylogenetic analyses

Partial nucleotide sequences coding the TBEV E protein as well as the complete CDS were analysed. The sequences were reviewed, assembled, aligned, trimmed, and analysed using Geneious Prime software (v. 2024.0.5.). Datasets were built using the obtained sequences of sufficient length and quality and additional sequences from the GenBank database. The most suitable nucleotide substitution model was selected using IQ TRee 2[16] based on the Bayesian information criterion. Phylogenetic analyses were performed using the maximum likelihood algorithm (PhyML v.3.3.20180621).[17]

Statistical analyses

Statistical analyses were performed using GraphPad Prism 10.2.3. Fisher's exact test was used for the comparison of frequencies, linear regression, and Spearman ranking test for testing relationships between continuous variables. Differences with p-values < 0.05 were considered statistically significant.

Ethics

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Local Hospital Ethical Committee of Hospital Ceske Budejovice (Reference Number 102/20). Patients whose samples were obtained in the study on the early detection of the TBE agreed with their participation and signed an informed consent form. The Ethical Committee waived the need for written informed consent for the patients from whom residuals of routinely collected serum specimens were used.

Results

TBEV RNA detection – patient comparison

Out of 265 confirmed TBE cases, TBEV RNA was detected in 14 patients in different clinical materials (Table 1). The highest detection rate was observed in the group of patients initially seronegative, who later seroconverted (66.7%; 10 positive of 15 tested). In general, the samples taken at disease onset (typically IgM negative or borderline, IgG negative) were positive for RNA in sera/blood, subsequently RNA diminished from the bloodstream when the IgG titres increased. Correspondingly, the RNA detection rate in the group of IgM/IgG-positive patients was comparatively low (1.3%; 3/228) (Table 1). All the patients in the group with a reported history of vaccination had high IgG titres (average of 132 AU/ml; range 29.5 to > 200 AU/ml), and none were positive for TBEV RNA.

Based on the negative correlation between antibody titre and vRNA detection efficiency, we added a patient group 3), consisting of retrospectively analysed serum samples from patients who tested negative for IgM and IgG antibodies and had not undergone repeated testing. This group included a large portion of patients with diseases of other aetiology causing symptoms similar to the initial phase of TBE. TBEV RNA was detected in 5 out of 818 tested (0.6%) patients from this group, indicating acute TBEV infection which was not later tested/confirmed serologically (Table 1).

TBEV RNA detection – sample comparison

In the prospective part of the study, serum, whole blood, CSF, and the urine samples were collected from a subset of patients. Only one patient tested positive in whole blood resulting in almost identical Ct values in RT-qPCR for serum and whole blood samples (37.3 and 36.6 respectively) (Suppl. Table 2). A single sample of CSF was positive for TBEV RNA in an IgM/IgG positive patient who was already RNA negative in sera sampled in parallel (Table 2).

The methodology for urine testing was extensively optimised using TBEV spiked previously negative urine samples. The final method combining sample concentration with modified RNA extraction according to Gorchakov et al.[12] resulted in the detection limit of 20 PFU/ml of the primary urine sample (before concentration). The urine conditioning buffer had no positive effect on the

Table 2
Numbers of patient samples positive / tested (%) for TBEV RNA in the study according to the sample type and results of serological testing.

	IgM-/IgG-	IgM+/IgG-	IgM+/IgG+	IgM-/IgG+	Vaccinated IgM-/IgG+	Total
Sera	12/819 (1.5%)	5/30 (16.7%)	4/228 (1.8%)	1/25 (4.0%)	0/35 (0.0%)	**22/1137 (1.9%)**
Blood	0/22 (0.0%)	1/2 (50%)	0/3 (0.0%)	0/3 (0.0%)	0/0 (0.0%)	**1/30 (3.3%)**
CSF	0/4 (0.0%)	0/4 (0.0%)	1/22 (4.5%)	0/0 (0.0%)	0/0 (0.0%)	**1/30 (3.3%)**
Urine	0/21 (0.0%)	1/3 (33.3%)	0/27 (0.0%)	0/1 (0.0%)	0/0 (0.0%)	**1/52 (1.9%)**
Total	**12/866 (1.4%)**	**7/39 (17.9%)**	**5/280 (1.8%)**	**1/29 (3.8%)**	**0/35 (0.0%)**	**25/1249 (2.0%)**

Specific IgM and IgG anti-TBEV antibodies were determined in serum samples.

detection efficiency of TBEV RNA compared to plain urine samples stored at −80 °C immediately after sampling. In the prospectively sampled group, 52 samples of urine were collected from 51 patients and tested in parallel to serum samples. From the total of 4 patients RNA-positive in sera in this group, only a single patient was RNA-positive in the urine sample. No other urine sample was RNA-positive.

The number of TBEV genome copies in the positive samples varied between 6 $\times 10^2$ and 2 $\times 10^6$ copies/ml of sera/blood. The positive CSF sample reached 5 $\times 10^4$ viral genome copies/ml and the urine sample 45 genome copies/ml of the original sample. Apart from the reduced probability of vRNA detection in blood after TBEV-specific IgG seroconversion (IgM positive IgG negative: 5/30, 16.7%; IgM and IgG positive: 4/228, 1.8%; Fisher's exact test; $p < 0.01$), we also observed a statistically significant negative correlation between the IgM or IgG titre and TBEV genome copy number (Spearman ranking test; r=0.41 and 0.42 respectively; $p < 0.05$). From two of the serum samples with high TBEV RNA concentrations (TbN146, Ct = 27; 69CB2020/S1 Ct = 31) we succeeded in isolation of the virus in an A548 cell culture. The replication of the virus in several subsequent passages was confirmed by RT-qPCR and plaque assay. The strains are deposited under the codes BCCO50_0519 and BCCO50_0520 in the Collection of Arboviruses in the Biology Centre Collections of Organisms.

Patient clinical data

Detailed clinical data for 19 out of the 20 TBEV RNA-positive patients were available in the hospital information system (Supp. Table 3). Those patients were in different stages of the disease, with fourteen (73.7%) in the first (initial) phase, and five (26.3%) with developed neurological symptoms. Overall, patients in the cohort were generally younger and healthy individuals (median age 41 years (IQR 33–62), 47.4% reported no chronic diseases, Charlson Comorbidity Index (CCI) of 0–2 in 63.2% of patients). None of the patients reported previous TBE vaccination. History of a tick bite was reported in 78.9%. The median incubation period from the tick bite to the onset of symptoms was 7 days (IQR 1.8–12.3) and the median duration of symptoms prior to testing was 4.5 days (IQR 3.0–6.3).

The presenting symptoms of the initial phase are summarised in Table 3 and the laboratory findings in Table 4. Notably, a significant proportion of patients (4, 21.1%) presenting with symptoms associated with TBE were prescribed antibiotics. In some cases, patients presented with atypical symptoms including chills (5, 26.3%) or respiratory symptoms such as sore throat or cough (4, 21.1%).

A significant proportion of patients (8, 42.1%) were hospitalised for the early phase symptoms, with a median hospital stay duration of 3 days (range 1–7 days). Additionally, fifteen patients (78.9%) developed the neurological phase, with ten cases of meningitis (66.7%), four cases of meningoencephalitis (26.7%) and one case of severe encephalomyelitis with bulbar syndrome (6.7%). Four patients either did not progress to the second phase (2, 10.5%) or had missing data regarding further development (2, 10.5%). There were no recorded deaths in the cohort. There were no statistically significant correlations found among the laboratory and clinical data and Ct/viral loads, except for the correlation of antibody titres and TBEV genome copy numbers described above.

Table 3
Demographic and clinical data of the 20 patients positive for TBEV RNA in one or several samples.

Demographic Data	Number of patients / Median[a]	% of total / IQR[b]
Male (n=20)	9	45.0
Female (n=20)	11	55.0
Tick Bite History (n=19)	15	78.9
Median Age (years) (n=20)	41[a]	33–62[b]
Median Incubation Period (days) (n=19)	7[a]	1.8–12.3[b]
First Phase Hospitalisation (n=19)	8	42.1
Progression to Second Phase (n=19)	15	78.9
Meningitis (n=15)	10	66.7
Encephalitis (n=15)	4	26.7
Encephalomyelitis (n=15)	1	6.7
Abortive (n=19)	2	10.5
Not Known (n=19)	2	10.5
First Phase Presenting Symptoms (n=19)	Number of patients	% of total
Fever	16	84.2
Headache	12	63.2
Malaise, Fatigue	10	52.6
Gastroenteritis Symptoms	10	52.6
Diarrhoea	8	42.1
Arthralgia/Myalgia	7	36.8
Chills	5	26.3
Respiratory Symptoms (Sore throat/Cough)	4	21.1

Laboratory parameters available for fourteen patients in the early phase of the infection are presented in Table 4.
[a] Median.
[b] IQR = interquartile range.

Sequencing and phylogenetic analyses

Of the total of 20 RNA positive samples, amplification and Sanger sequencing of the viral E protein coding fragment was possible in 8 samples (Ct values ranging 27–35; 10^6-10^3 genome copies/ml). From 6 samples we obtained TBEV E protein-coding sequences of suitable length and quality for phylogenetic analyses (Fig. 1).

Most of the patient sequences (in red) were placed into two clusters (Fig. 1). One comprising sequences of TBEV strains isolated from ticks sampled in 2000 in locality Borovany in the South Bohemian region (blue background), the second one formed by sequences of strains isolated from ticks and rodents at another South Bohemian locality Kaplice-Zdar (green background) almost 40 years ago (the sequence obtained from patient 69CB2020 is 100% identical to published sequences of TBEV strains 235, T133, 76; JF501410, JF501412, EF113081). The sequence from patient TbN146 falls into a different part of the same cluster with a sequence from Germany, while sequence TbN79 is placed close to these clusters. The sequence obtained from patient Vr981 seems to be genetically distant from all others, forming a separate cluster with sequences from the western part of Germany and Switzerland. Interestingly, the patient did not report any history of travelling.

Additionally, four complete CDS (positions 130–10,929 in the TBEV reference genome: strain Neudoerfl, NC_001672.1) were obtained by Nanopore sequencing directly from the serum samples. A

Table 4
General laboratory findings in the first phase of TBE.

Laboratory parameter (n=14)	Median	IQR[a]	Number of patients	% of total
Leukocytes (cells x 10^9/l)	3.50	2.98–4.20	-	-
Leukopenia (< 4.0 ×10^9/l)	-	-	**10**	71.4
Leukocytosis (> 10.0 ×10^9/l)	-	-	0	0.0
Neutrophils (cells x 10^9/l)	2.05	1.60–2.68	-	-
Neutropenia (< 2.0 ×10^9/l)	-	-	**7**	50.0
Neutrophilia (> 7.0 ×10^9/l)	-	-	0	0.0
Lymphocytes (cells x 10^9/l)	0.90	0.80–1.20	-	-
Lymphopenia (< 0.8 ×10^9/l)	-	-	**2**	14.3
Lymphocytosis (> 4.0 ×10^9/l)	-	-	0	0.0
Thrombocytes (cells x 10^9/l)	152.5	128.8–166.0	-	-
Thrombocytopenia (< 150 ×10^9/l)	-	-	**6**	42.9
Thrombocytosis (> 450 ×10^9/l)	-	-	0	0.0
CRP[b] (mg/l)	2.45	0.00–7.33	-	-
> 5 mg/l	-	-	**5**	35.7
> 40 mg/l	-	-	1	7.1
AST[c] (µkat/l)	0.68	0.41–0.90	-	-
> 0.72 µkat/l	-	-	**7**	50.0
ALT[d] (µkat/l)	0.58	0.37–0.71	-	-
> 0.78 µkat/l	-	-	**3**	21.4
GGT[e] (µkat/l)	0.40	0.17–0.74	-	-
> 0.68 µkat/l	-	-	**4**	28.6
LFT[f] elevated	-	-	**8**	57.1

[a] IQR = interquartile range.
[b] CRP = C-reactive protein.
[c] ALT = alanine transaminase.
[d] AST = aspartate aminotransferase.
[e] GGT = gamma-glutamyl transferase.
[f] LFT = liver function test (AST, ALT, GGT).

phylogenetic tree was built based on these sequences and additional 42 sequences from the GenBank database. The analyses confirmed that the sequences from patients belong to two closely related clusters, while Vr981 is genetically distant, clustering with sequences acquired from a sheep in Germany, human patients in Russia (Abssetarov strain), and ticks from Hungary and Finland (Kumlinge strain) (Fig. 2).

Discussion

The primary objective of this study was to determine the proportion of patients with detectable TBEV RNA in different clinical materials at various stages of the disease. This report presents the findings of the first cohort of patients in the Czech Republic in whom the TBEV RNA was detected. The cohort comprised samples from patients examined at the Hospital Ceske Budejovice situated in South Bohemia, a region with a high incidence of TBE.[18,19]

It has been reported that the detection rate in initial blood samples (serum, whole blood or plasma) is high (up to 100%) in the initial phase of the disease. However, the detectability of TBEV RNA is known to decrease during the second phase due to antibody response.[4] The cohort of patients in our study included just a minor proportion of patients in the early phase of the disease (absence of IgM response) resulting in a lower detection rate of viral RNA in blood. Nevertheless, we detected viral RNA also in a substantial part of the patients with specific IgM or, in a few cases, even IgG antibody titres in sera. To the best of our knowledge, there are only four reported cases of TBEV RNA in blood despite both IgM and IgG antibody response.[4,7,20,21] As there is some degree of cross-reactivity among antibodies against different flaviviruses (anti-TBEV especially with antibodies against Louping ill virus, but also, to a lesser degree, against West Nile, Dengue and Usutu virus) and multiple flaviviruses co-circulate in many parts of Europe, serological tests may have inconclusive results.[22–25] The RT-qPCR-based approach used in our study is highly specific for TBEV RNA.[13]

Furthermore, efforts have been made to detect TBEV RNA in non-invasively obtained samples like urine, as this approach was successfully applied to mosquito-borne flaviviruses.[12,26,27] TBEV RNA has been previously documented in urine, but in only six isolated cases.[7,20,28,29] In these six cases, the patients were in the developed neurological phase, and three of them were immunosuppressed. The virus was present in urine, despite antibodies in the sera, except in one patient who had undergone B-lymphocyte depletion therapy, resulting in an impaired antibody response. In addition, a recent study that attempted to investigate the benefit of PCR detection from saliva and urine, with no positive results, suggests that these materials have limited applicability for TBEV direct detection.[30] Nevertheless, this work has focused solely on the neurological phase and has analysed a small number of samples (2 samples of concentrated saliva, 4 samples of concentrated urine). Our study presents an additional case of a patient with TBEV RNA positivity in urine during the early neurological phase of the disease (IgM positive, IgG negative), fourteen days after the first symptoms onset. This was the only positive urine sample out of 52 tested, despite substantial efforts to make the detection method as sensitive as possible. Therefore, we suggest that TBEV RNA is rarely present in urine, or in quantities below the detection limit.

To date, several clinical trials conducted in different European countries have shown that CSF is not a suitable material for routine PCR diagnosis of TBE. Out of more than five hundred CSF samples tested in studies published to date, the presence of TBEV RNA in CSF was detected in only five cases. These were patients in the early stage of the neurological phase of the disease. In three cases, the patients were seronegative,[4,31] and in two cases IgM antibodies were present.[32,33] In all other studies with large numbers of CSF samples examined, the results were negative.[7,21,34] Only one patient tested positive for TBEV RNA in CSF in our cohort. The most probable explanation for the inability to achieve higher detection rates from the CSF is that the virus is associated with the brain parenchyma and rarely enters the CSF. This positive patient was a 65-year-old immunocompetent male with meningoencephalitic form of TBE and no major comorbidities. The CSF was taken on day 21 of symptom duration, at which time the patient was already seropositive in both IgM and IgG antibodies.

The direct molecular genetic detection of TBEV has a big diagnostic potential in patients with impaired humoral immunity, who tend to develop a delayed antibody response. In these patients, RT-qPCR leads to earlier and more precise diagnosis, thus saving the healthcare costs associated with differential diagnosis and leading to better patient management. To date, four cases of severe tick-borne encephalitis have been reported in patients undergoing rituximab (anti-CD20 antibody) therapy, in which a markedly delayed antibody response (up to three months following disease onset) has been documented.[31,35] Our patient cohort included a 40-year-old individual with primary progressive multiple sclerosis undergoing biologic therapy with ocrelizumab (another anti-CD20 B-cells depleting antibody). The patient did not seroconvert until day 26 of symptom duration, at which point tetraplegia with bulbar syndrome had developed, and the patient stayed in the resuscitation unit. In this particular case, PCR diagnostics could have shortened the diagnostic period by three weeks, thus preventing unnecessary transfers, empiric antibiotic treatment and repeated patient investigation.

It was somewhat unexpected that there were five patients in whom the diagnosis of TBE was not established by routine clinical and serological testing, and TBEV RNA was only detected

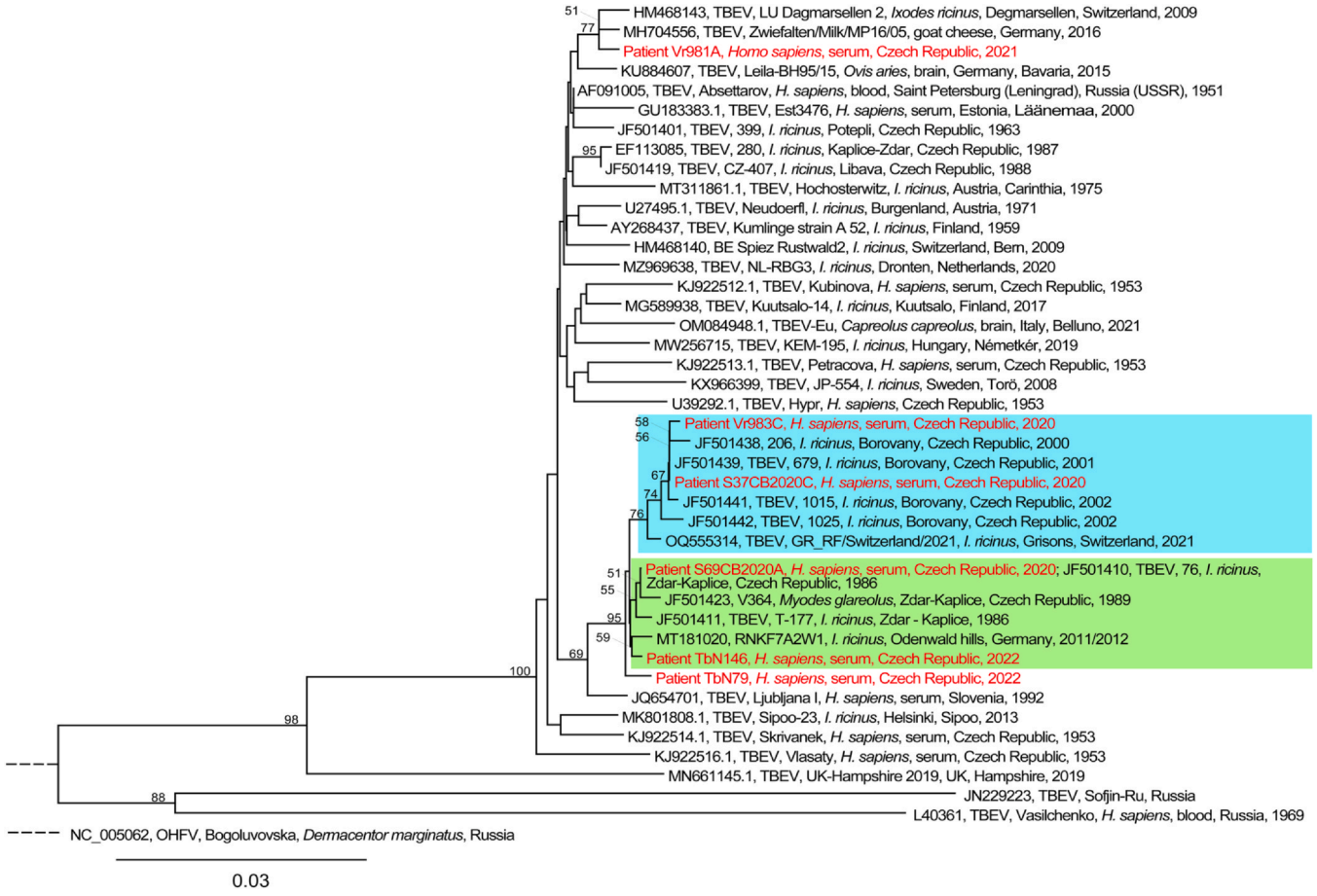

Fig. 1. Maximum-likelihood phylogenetic tree based on an alignment of flavivirus partial (848 bp) envelope protein coding sequence. Six nucleotide sequences (in red) were obtained directly from the patient serum samples in this study and an additional 52 sequences from the GenBank database. Omsk haemorrhagic fever virus sequence (NC_005062) was used as an outgroup. The code indicates GenBank accession number, virus, strain, biological source, place and country of origin, and year of sampling. The tree was generated using the TIM2e+G4 substitution model and 1000 replicates bootstrap analysis. Numbers next to nodes indicate bootstrap support, only values < 50% are shown. The lengths of tree branches correspond to the number of substitutions per site. The scale bar indicates evolutionary distance.

retrospectively as part of this study. These were patients in an initial phase of the infection, two of whom did not attend any follow-up and therefore the subsequent course is unknown (it may be assumed that the infection was mild). In two other cases, the course was biphasic, but antibody testing was not repeated during the neurological phase. The last patient from this group was diagnosed with febrile gastroenteritis. The laboratory findings in this patient were consistent with the initial phase of TBE, including elevated liver tests, low CRP, and bicytopenia in the blood count. However, due to the presence of IgG antibodies only, TBEs was not considered a probable diagnosis. In all these patients, TBE has not been reported to the national surveillance system, prompting the question of whether this infection may be an underdiagnosed disease, particularly in cases where the course is mild or abortive.[36]

The clinical manifestation and laboratory characteristics of the peripheral phase of TBE in our patient cohort were consistent with findings previously described by Bogovič et al.[37] Laboratory findings frequently revealed changes in the blood count, including leukopenia with neutropenia and thrombocytopenia. The minimum values of neutrophil and thrombocytes were 0.8×10^9/l and 91×10^9/l, respectively. Similar values might occasionally mislead clinicians to an inappropriate diagnosis of hemato-oncological disease.

A total of 22.7% of patients presenting with peripheral phase symptoms were prescribed antibiotics. The capacity to identify the virus at this stage would not only enhance the diagnostics, but it would also prevent the prescription of a redundant and potentially harmful empirical antibiotic treatment. This phase of infection with detectable viremia would also be an optimal time for targeted antiviral treatment. To date, no specific antiviral treatment has been approved for use in patients in the EU. The use of specific immunoglobulins remains a current practice in Russia and Kazakhstan, where it seems to be an effective strategy for disease prevention and reduction of disease severity.[38] In Europe, the use of specific immunoglobulin (FSME-Bulin, Encegam) has been discontinued due to concerns that it may result in an antibody-enhanced disease course.[39–41] However, screening and characterisation of monoclonal antibodies from TBEV-infected individuals has recently identified a number of candidates with potential for development as high-efficacy therapeutics.[42] Nevertheless, for the success of any treatment, precise diagnostics in the early (viremic) phase of infection appears to be crucial.

E protein and complete CDS obtained directly from the patient samples formed two clusters with published TBEV sequences from ticks and rodents from two historically described TBEV foci in the same geographical area.[43–45] As shown previously there is no apparent host or geographical signal in the phylogeny of European subtype TBEV strains.[45,46] Interestingly, all the patient samples are distant from the historical patient strains isolated during the 1953 epidemics [47] in Central Bohemia, a neighbouring region in the Czech Republic.

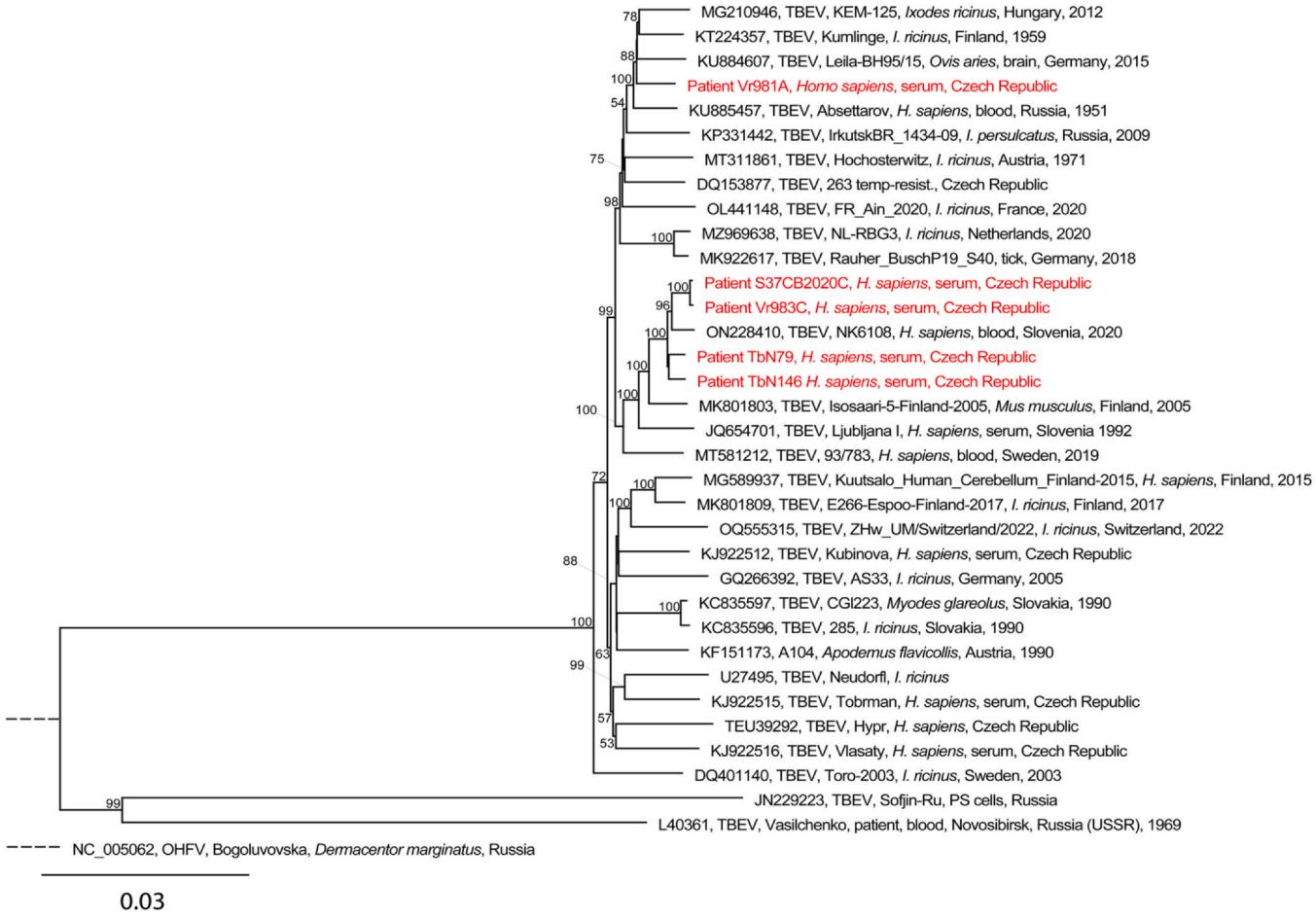

Fig. 2. Maximum-likelihood phylogenetic tree based on an alignment of flavivirus complete coding sequences (10,245 bp). Five nucleotide sequences (in red) were obtained directly from the patient serum samples in this study and additional 30 sequences from the GenBank database. Omsk haemorrhagic fever virus sequence (NC_005062) was used as an outgroup. The code indicates GenBank accession number, virus, strain, biological source, place and country of origin, and year of sampling. The tree was generated using the TIM2e+G4 substitution model and 1000 replicates bootstrap analysis. Numbers next to nodes indicate bootstrap support, only values < 50% are shown. The lengths of tree branches correspond to the number of substitutions per site. The scale bar indicates evolutionary distance.

Conclusions

In summary, direct molecular detection of TBEV RNA allows diagnosis of the initial, viremic, phase of TBEV infection, which is especially valuable in patients with delayed antibody response. This approach not only improves diagnostic accuracy but also has the potential to significantly improve patient outcomes through timely and targeted therapeutic intervention, as there are several promising targeted anti-TBEV approaches in development.[42,48] In addition, the detection of vRNA provides a tool to monitor the epidemiologic situation regarding the spread of TBEV strains that differ in virulence and could influence the course of the disease.

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Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2025.106481.

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