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ORIGINAL RESEARCH

TaqMan qPCR and IgM Detection in Samples of Patients with Tick-Borne Encephalitis Virus Infection in Northeast China

Menghan Yu[1], Xiaoshu Fu[1], Yanli Zhu[2], Miao Li[3], Min Wu[1], Entong Zhou[1], Hanwen Dong[1], Likun Wang[1], Ruoxi Wang[4], Fang Liu[2], Chunlai Jiang[1], Xiangwei Kong[5], Weiheng Su[1,6]

[1]National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun, People's Republic of China; [2]People's Hospital of Jiangyuan District, Baishan, Jilin Province, People's Republic of China; [3]Department of Neurosurgery, China-Japan Union Hospital, Jilin University, Changchun, People's Republic of China; [4]School of Public Health, Capital Medical University, Beijing, People's Republic of China; [5]Changchun University of Science and Technology, Changchun, People's Republic of China; [6]Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education, School of Life Sciences, Jilin University, Changchun, People's Republic of China

Correspondence: Weiheng Su, National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun, People's Republic of China, Email suweiheng@jlu.edu.cn; Xiangwei Kong, Changchun University of Science and Technology, Changchun, People's Republic of China, Email tenbpro@163.com

Purpose: Tick-borne encephalitis virus (TBEV) infections result in severe central nervous system diseases in humans across Asia and Europe. In China, cases of tick-borne encephalitis are primarily caused by the Far East subtype of TBEV, which exhibits a distinct disease course compared to other extensively studied subtypes. However, there is limited knowledge regarding the nucleic acid and serological diagnostic characteristics of patients infected with the TBEV in China, which is the focus of investigation in the present study.

Methods: This study established a TaqMan qPCR approach to detect TBEV RNA in the serum with optimal specificity, sensitivity, and precision. Using TaqMan qPCR and ELISA assay for TBEV IgM detection, serum samples from 63 hospitalized patients bitten by ticks in Northeast China were investigated for diagnostic characteristics.

Results: Twenty-five patients were positive for viral RNA; nineteen patients were positive for IgM, and nine were positive for both viral RNA and IgM. Through comparative analysis, TBEV RNA copies were negatively correlated with the virus incubation period. IgM levels were positively correlated with the clinical symptom scores of patients. The severity of clinical symptoms and the length after the tick bite could be used to predict the IgM occurrence. Furthermore, IgM levels and viral RNA copies were not correlated in double-positive patients.

Conclusion: Both nucleic acid and serological detection methods exhibited distinct windows for detecting TBEV infection, with some overlap, and were associated with specific correlated factors. This study provided novel insights into the diagnosis and course of TBEV-induced tick-borne encephalitis in China.

Keywords: tick-borne encephalitis, TBE, tick-borne encephalitis virus, TBEV, TaqMan qPCR, IgM

Introduction

Tick-borne encephalitis (TBE) is a rapid-onset infectious central nervous system disease caused by the tick-borne encephalitis virus (TBEV), with 10,000–12,000 cases reported annually worldwide.[1] Human infections with TBEV are typically transmitted via tick bites. TBEV is primarily classified into three subtypes: European (TBEV-Eu), Siberian (TBEV-Sib), and Far East (TBEV-FE). TBEV-Eu is primarily found in Europe, whereas TBEV-Sib is prevalent in Russia and extends to Eastern Europe. TBEV-FE is primarily distributed throughout Asia, including China, Japan, and eastern Russia.[2]

Patients infected with different TBEV subtypes exhibit two different disease courses: monophasic and biphasic.[3] Generally, infection with TBEV-Eu and TBEV-Sib leads to a biphasic course, whereas infection with TBEV-FE leads to a monophasic course.[4] In the monophasic course, patients start with an incubation period of 4–28 days (average, 8 days),

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followed by occurrence of symptoms, such as high fever, headache, general discomfort, muscle pain, and joint pain, etc., and even neurological symptoms like neck stiffness, meningitis, and paralysis in severe cases. This symptomatic phase may last for 5–7 days (up to 12–14 days).[5] The biphasic course of TBE also starts with an incubation period of 2–28 days, followed by a prodromal phase of viremia (first phase, 2–10 days) with flu-like symptoms.[4] After an asymptomatic interval period that lasts for 2–7 days, patients experience the neurological phase (second phase, a few days to two month), in which approximately 20–30% of TBEV-infected patients experience recurrent fever and neurological symptoms, including nystagmus, consciousness confusion, and central nerve paralysis.[6–8] Neurological complications during the neurological phase can have long-term effects on the body and, in some cases, can result in permanent neurological sequelae, with a mortality rate as high as 40%.[9]

In China, the majority of cases are concentrated in the northeast regions, particularly in mountainous areas, such as Jilin, Inner Mongolia, and Heilongjiang provinces. All reported cases in China are caused by TBEV-FE infection.[5] In China, Asia and Russia, TBEVs are predominately transmitted by *Ixodes persulcatus*, in contrast to transmission by *Ixodes ricinus* in Europe.[10] Currently, the diagnosis of patients infected with TBEV in China mainly follows the National Occupational Tick Borne Encephalitis Diagnosis Criteria.[5] This involves considering whether there is a history of tick bites, sudden fever, typical clinical manifestations of acute central nervous system injury, positive specific serological tests, and referring to the results of onsite TBE epidemiological investigations for comprehensive analysis. Other diseases must be ruled out before a diagnosis can be made.

Immunoglobulin M (IgM) is the first antibody to appear in response to the initial antigen exposure. A positive IgM test result is an indicator of acute or recent infection. In general, IgM is more specific than IgG in terms of cross-reactivity with other flaviviruses.[11] Steininger conducted both IgM and IgG tests for TBEV in patients from a highly endemic area in southern Germany. IgM ELISA tests showed high clinical sensitivity and specificity, whereas TBEV IgG was detected in only 55% cases by analyzing paired serum and cerebrospinal fluid samples.[12] In addition, researchers have reported that induced IgM can persist in the serum for several months in individuals who have recently been vaccinated against TBEV, making serological testing less comprehensive.[13] However, owing to the late occurrence of antibodies, which are generally detectable only after the onset of neurological symptoms, there is a risk of misdiagnosis in patients in the early stages of the disease, potentially delaying optimal treatment.[14]

Although antibodies may not appear early in the disease course, the presence of the virus in serum samples can be detected. Some European countries have reported the use of RT-qPCR to detect the viral RNA load of TBEV in patient sera. Saksida determined the levels of TBEV RNA in serum samples obtained from 80 patients during the first phase of TBE in Slovenia and analyzed the association between phylogenetic clades and viral RNA load or disease severity.[15] Schwaiger also tested positive for TBEV RNA in the cerebrospinal fluid of patients with TBEV.[16] However, there are currently limited reports of patients infected with TBEV in China using RT-qPCR.

In this study, TaqMan qPCR and serological IgM detection were used to analyze serum samples from patients bitten by ticks around the Changbai Mountains in Jilin Province, China. Correlation and logistic regression analyses were performed for serum viral load, antibody levels, symptoms, and duration, laying the foundation for diagnosing whether patients bitten by ticks were infected with TBEV.

Materials and Methods

Viruses and Clinical Samples

The inactivated TBEV-FE Senzhang strain (GenBank accession no. JQ650523) was preserved in the laboratory. Inactivated Zika virus and inactivated dengue virus were generously provided by Dr. Qiming Liang from Shanghai Jiao Tong University, China. Fresh clinical serum samples from patients bitten by ticks (range, 1–30 d from bite) were obtained from the Department of Neurology at the People's Hospital of Jiangyuan District, Baishan, Jilin province, China in 2023. This study was conducted under Biosafety Level 3 conditions according to China directive for management of human pathogenic microorganisms.

Criteria to Determine Clinical Symptom Score

A scoring system for quantifying the severity of illness in patients with TBE was used, predominantly based on the acute disease severity assessment questionnaire described previously.[17] Two modifications, especially in this study, were presence of skin redness or swelling or lymphadenopathy, which was given 0.5 point, and the presence of feeble or muscle soreness, which was given 1 point.

Primers, Probe and Standard Plasmid

Conserved TaqMan qPCR primer pairs (Forward 5′- CGTACAGGACCTGGCAGTA −3′, Reverse 5′- GAGCC TTGGTGTCAACCTT −3′) and probe (5′- FAM- TTTGAACACTCGCTGCTGTCCAAAG -BHQ1 −3′) were designed based on non-structural protein 5 (NS5) gene of more than 85% TBEV strains from GenBank, and were synthesized by the COME Biotech Company (Changchun, China). Especially, the TaqMan qPCR primer pairs and probe sequences correspond to 92% of TBEV-FE strains, which are reported as the exclusive subtype circulating in China [5]. A relatively conserved 323 bp TBEV NS5 gene fragment containing the TaqMan qPCR primer pairs and probe sequences was synthesized and inserted into the PUC57 vector by Sangon Biotech Co. Ltd. (Shanghai, China).

Virus Nucleic Acid Extraction and Reverse Transcription

Virus nucleic acid was extracted from the clinical serum samples and inactivated viruses using the EasyPure® Viral DNA/RNA Kit (TransGen, Beijing, China). The extracted TBEV RNA was then reverse transcribed to cDNA using the TransScript®II One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China), following the manufacturer's instructions.

TaqMan qPCR

The TaqMan qPCR reaction system was prepared using Premix Ex Taq (Takara, Beijing, China). qPCR was performed using the Bio-Rad CFX96 qPCR instrument with the following steps: pre-denaturation at 95 °C for 5 min, followed by amplification for 45 cycles at 95 °C for 5 sec and 60 °C for 30 sec according to the manufacturer's instructions.

For sensitivity analysis, the standard plasmid was 10-fold diluted (10^7–10^1 copies/μL) as DNA templates for TaqMan qPCR amplification. A standard curve was generated using the copy number as the abscissa and the Ct value as the ordinate to obtain the regression equation.

For specificity analysis, the cDNAs of inactivated TBEV, Zika virus, and dengue virus obtained through viral RNA extraction and reverse transcription were used as templates for TaqMan qPCR amplification.

For precision analysis, 10^5 copies/μL and 10^4 copies/μL standard plasmids were used as templates for TaqMan qPCR amplification, with n=6. The amplification reaction was repeated thrice to assess the precision within and between batches.

Detection of TBEV IgM Using Enzyme-Linked Immunosorbent Assay (ELISA)

A TBEV IgM detection ELISA kit (Changchun Institute of Biological Products Co., Ltd., China) was used to test all patient serum samples according to the manufacturer's instructions. Absorbance was measured at 450 nm using a Bio-Rad iMarkTM Microplate Reader (Hercules, CA, USA). The cut-off value was determined by negative control OD value plus 0.1, according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using SPSS statistics v27 (IBM, Armonk, NY, USA) with a two-tailed Student *t* test. Data are presented as the mean ± SD. Linear regression and Pearson's correlation analyses were performed using GraphPad Prism v7 (GraphPad, San Diego, CA, USA). Binary logistic regression analysis was performed using SPSS v27 (IBM, Armonk, NY, USA).

Results

Establishment of TaqMan qPCR Assay for TBEV Detection

A probe and a pair of primers based on the conserved NS5 gene sequences of TBEV were designed. This study utilized TaqMan qPCR to detect the sensitivity of the standard TBEV NS5 plasmid, exhibiting a strong linear relationship across a range of 10^7–10^1 copies per reaction with a minimum detection limit of 10 copies of TBEV (Figure 1A).

To assess the specificity of the TBEV primers used in TaqMan qPCR, cDNAs of TBEV and two other flaviviruses, Zika virus and dengue virus, were compared for amplification. The results revealed that only reactions with TBEV cDNA produced a positive signal, whereas the others did not exhibit amplification (Figure 1B), indicating high specificity.

To validate the precision of the TaqMan qPCR method, standard TBEV plasmids with 10^5 and 10^4 copies were amplified, and statistical analyses of the intra-assay (six biological repeats) and inter-assay (three independent experiments) data were conducted. The intra-assay CVs for the Ct values were 0.51–1.64%, whereas the inter-assay CVs were 1.74% and 1.47%, respectively (Figure 1C), indicating the high precision of this assay.

TBEV RNA Analysis in Serum Samples from Hospitalized Patients

Sixty-three patients residing in the Changbai Mountain area of northeast China, who exhibited symptoms following tick bites, were hospitalized in the Department of Neurology at the People's Hospital of Jiangyuan District (Baishan, Jilin province, China) in 2023 and were enrolled in this study. The basic demographic data, clinical characteristics, and laboratory findings of the 63 patients were shown in Table S1.

To detect potential viral RNA copies, all 63 serum samples were analyzed using the established TaqMan qPCR assay. Of the 63 patients, 25 tested positive, with an average TBEV RNA level of $10^{3.36}$ copies/mL. There were 2.5 folds more

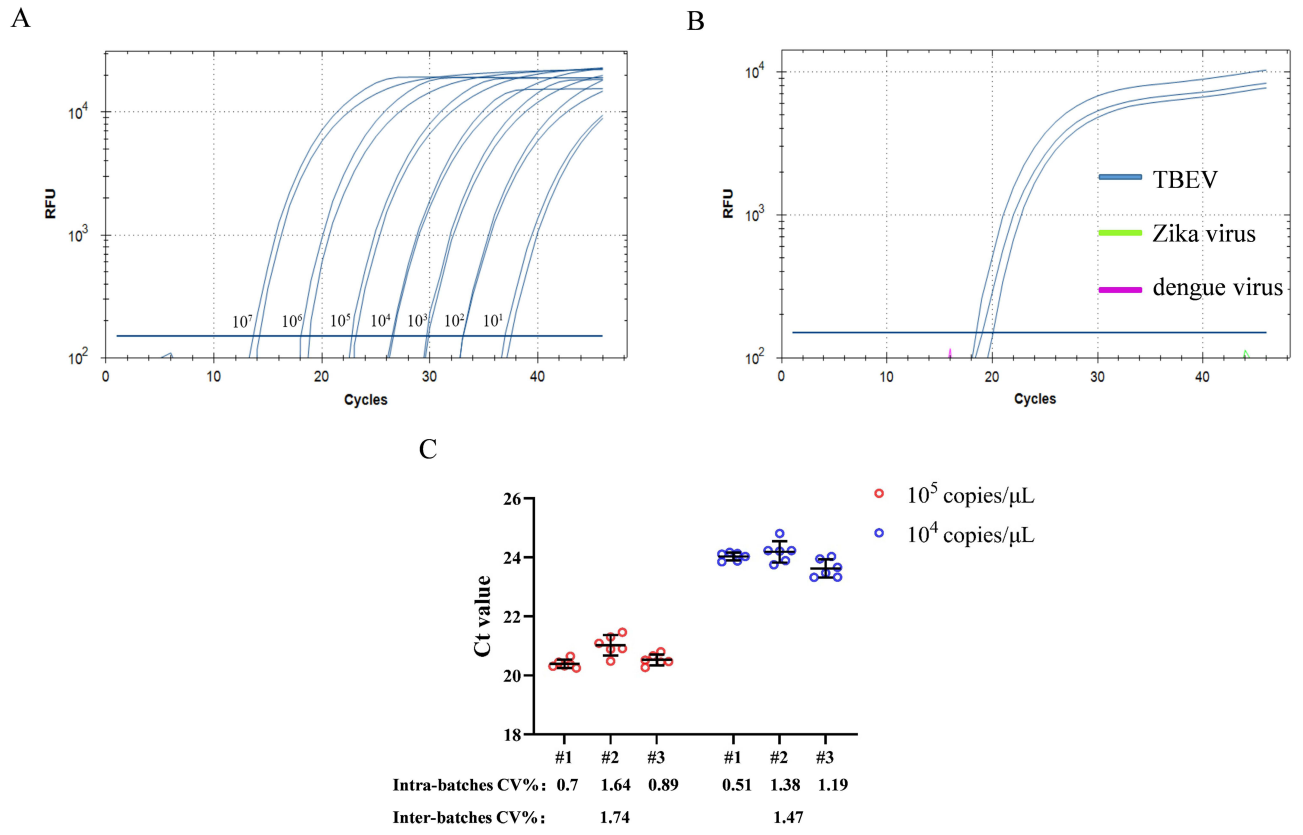

Figure 1 Establishment of TaqMan qPCR for TBEV detection. (**A**) Sensitivity detection of different dilutions of TBEV plasmid. 10^7–10^1 copies of plasmids were marked beside amplification trajectories (n = 2). (**B**) TaqMan qPCR amplification curves of cDNA from TBEV, Zika virus, and dengue virus (n = 3). There was not any amplification for cDNA template of Zika virus or dengue virus. (**C**) Variation analysis of TaqMan qPCR assay based on 10^4 and 10^5 copies/μL plasmid templates (n = 6), respectively. The CV% values were indicated.

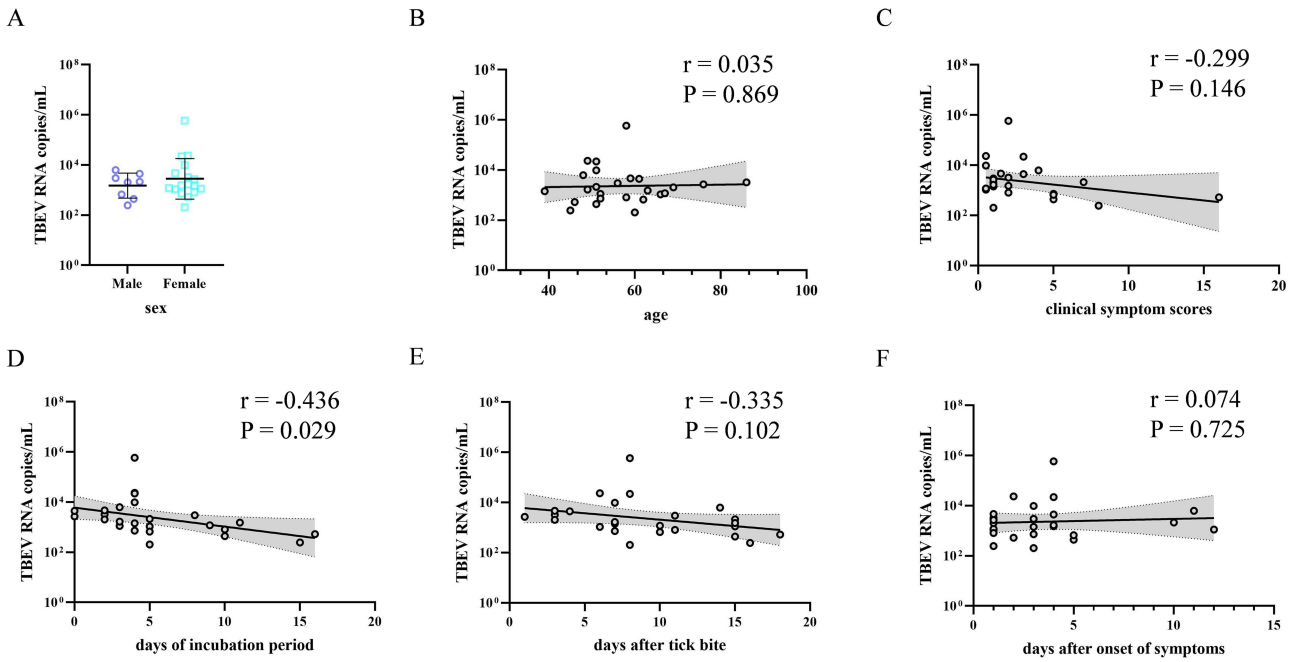

Figure 2 Pearson correlation analysis of TBEV RNA copies to other factors. Pearson correlation and linear fitting of TBEV RNA copies to sex (**A**), age (**B**), clinical symptom scores (**C**), days of incubation period (**D**), days after tick bite (**E**), and days after onset of symptoms (**F**). All samples indicated in this figure were viral RNA positive.

female patients than male patients (18 vs 7) (Figure 2A), but the average serum viral RNA level in female patients and male patients had no significant difference. The majority of positive patients were aged between 40 and 70 years (Figure 2B). Most TBEV RNA-positive patients exhibited relatively mild symptoms (clinical scores less than 5) (Figure 2C). Notably, the patients received symptomatic treatments. The incubation period (latency from tick bite to onset of symptoms) for most patients was within 5 d (Figure 2D).

Pearson correlation analysis revealed that the TBEV RNA copies were negatively correlated with the virus incubation period (P = 0.029) (Figure 2D), but showed no significant correlation with other factors (Figure 2B–F). Based on the data analysis results (Table 1), patients (n = 5) with higher levels of viral RNA copies ($10^{3.79}$–$10^{5.77}$) in the serum all had an incubation period within 4 d, and their clinical symptom scores were within 4. Among them, 80% had symptom onset within 4 d, and 80% tested IgM-negative.

To investigate the factors associated with TBEV RNA positivity, binary logistic regression analyses were performed, revealing that none of the factors examined in this study were statistically significant (Figure S1).

TBEV IgM Analysis

IgM is an immunoglobulin elicited early and a biomarker for TBE diagnosis that was assayed in this study using ELISA. Among the 63 patients, 19 tested positive for TBEV-specific IgM. There was no significant difference in the number and OD levels of IgM-positive patients between males and females (Figure 3A). Pearson correlation analysis indicated that the IgM level in patients was positively correlated with the clinical symptom scores (P = 0.032) (Figure 3B), but was unrelated to age (Figure 3C), days after tick bite (Figure 3D), incubation period (Figure 3E), and days after onset of symptoms (Figure 3F). Additionally, the onset of symptoms in patients was slightly delayed (Figure 3F) compared to that in qPCR-positive patients (Figure 2F).

Patients with high IgM levels (OD 1.125–1.642) had clinical symptom scores of at least 5 and low or none virus RNA copies (maximum $10^{3.33}$ copies/mL) (Table 1). Additionally, among patients with high IgM levels, clinical symptom scores ranged from 5 to 14, higher than those of patients with higher virus RNA copies (symptom scores 0.5–4).

Binary logistic regression analyses were conducted to explore associated factors and predict the probability of IgM positivity. The results indicated that clinical symptom scores and days after tick bite significantly influenced IgM

Table 1 Patients with High TBEV RNA Copies or High IgM Levels

	ID	Sex	Age	Days after onset of symptoms	Incubation period	Days after tick bite	Clinical symptom scores	Lg TBEV RNA copies	ELISA OD value
High TBEV RNA copies	28	Female	58	4	4	8	2	5.77	/
	27	Female	49	2	4	6	0.5	4.37	/
	34	Female	51	4	4	8	3	4.34	0.195
	30	Female	51	3	4	7	0.5	3.99	/
	4	Male	48	11	3	14	4	3.79	/
High IgM levels	43	Male	62	5	5	10	5	2.82	1.642
	52	Female	52	3	4	7	11	/	1.561
	57	Male	59	12	0	12	14	/	1.484
	17	Male	51	10	5	15	7	3.33	1.171
	23	Male	51	5	10	15	5	2.64	1.125

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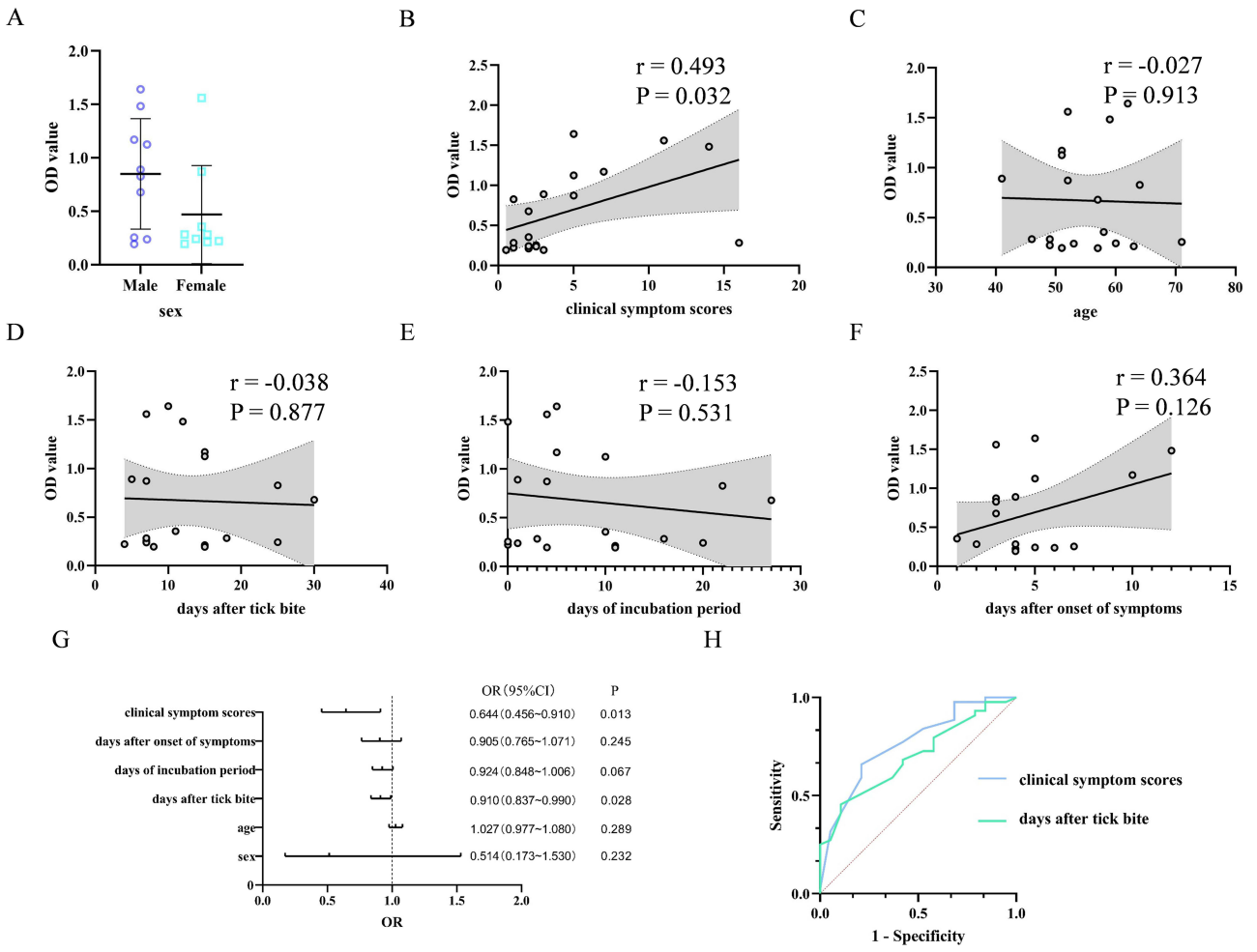

Figure 3 Pearson correlation and binary logistic regression analysis of TBEV IgM level to other factors. Pearson correlation and linear fitting of TBEV IgM level to sex (**A**), clinical symptom scores (**B**), age (**C**), days after tick bite (**D**), days of incubation period (**E**) and days after onset of symptoms (**F**). All samples indicated in this figure were IgM positive. Binary logistic regression analysis (**G**) and ROC curve (**H**) of factors associated with TBEV IgM positivity.

positivity (P = 0.013 and 0.028, respectively), whereas other factors showed no significance (Figure 3G). Furthermore, the receiver operating characteristic (ROC) curve was used to assess the predictive value of the IgM-positive model (Figure 3H). The calculations based on ROC curves revealed that a clinical symptom score exceeding 1.75 (Youden index 0.449) or a tick bite time over 6.5 days (Youden index 0.349) indicated a high risk of testing positive for TBEV IgM (Table S2).

Further correlation analyses between viral RNA copies and IgM levels were performed in double-positive patients. The results showed that the IgM levels and viral load were not correlated (Figure S2A). Moreover, neither IgM positivity nor RNA positivity affected the detection value of the other (Figure S2B and C). Therefore, these two detection methods can only complement each other in confirming the final infection status of a patient.

Discussion

Most hospitals in China rely mainly on the clinical symptoms presented by patients for TBEV diagnosis. However, because the initial symptoms of patients with TBE are mainly fever and headache, which are similar to the symptoms of influenza and other diseases like Lyme disease, it is difficult to accurately diagnose the patient's infection. Therefore, patient treatment may not be timely or appropriate.[8] Currently, laboratory diagnosis of TBEV primarily involves the detection of specific IgM or IgG antibodies in the serum. However, owing to the late appearance of antibodies, the use of TBEV antibody detection is not widespread. Researchers have used qPCR to test the serum of suspected patients with

TBEV in hospitals, providing assistance in the diagnosis and treatment of patients.[16,18] However, there are limited reports on qPCR detection and analysis of patients infected with TBEV-FE.

In the present study, we established a TaqMan qPCR method for detecting TBEV, with good sensitivity, specificity and precision. The sensitivity of the assay was 10 copies/μL. Subsequently, viral RNA and IgM levels in the serum of 63 suspected TBE patients bitten by ticks in Northeast China in 2023 were tested using both TaqMan qPCR and ELISA.

Statistical analysis revealed 25 qPCR-positive serums. Pearson correlation analysis revealed that the virus RNA copies was not related to patient age or sex, which is consistent with the situation of TBEV-Eu infected patients, according to the findings of Bogovič P.[19] Furthermore, a negative correlation was found between the virus RNA copies and the incubation period (Figure 2E), suggesting that this may be due to the initial stage of infection with a low virus load and weak virulence, resulting in a limited virus replication.

Nineteen patients tested positive for TBEV IgM. None of the patients were vaccinated against TBEV. According to the binary logistic regression analysis, the time of the tick bite and severity of clinical symptoms could, to some extent, predict IgM detection (Figure 3H). When the clinical symptom score was >1.75 or the time of tick bite was >6.5 d, the risk of IgM positivity was high (Table S2). Pearson's correlation analysis showed that IgM antibody levels were only positively correlated with clinical symptom scores (Figure 3C). However, as there is a lack of follow-up investigations for this patient, this inference requires further validation.

Nine patients tested positive for both viral RNA and IgM, demonstrating an overlapping period between the duration of viral RNA and IgM in the serum. However, virus RNA or IgM positivity did not affect the detection of the other one (Figure S2).

In certain studies, it has been reported that patients presenting for medical care during the second phase of TBEV-Eu disease exhibit few TBEV RNA in blood or cerebrospinal fluid samples, but IgM is detectable.[20] This may be because patients infected with TBEV-Eu undergo a biphasic course; the interval of asymptomatic period may result in the clearance of the virus from the serum. In contrast, patients infected with TBEV-FE undergo a monophasic course, and the presence of the virus and antibodies in the serum may occur simultaneously.

Saksida[14] reported that after the appearance of the IgM antibodies, the number of qPCR-positive samples decrease by at least one-third. Bogovič[19] predicted that once IgM is detectable, the virus RNA load would become much lower. However, this was not significantly reflected in the data of this study, possibly because our serum samples were transversely collected and lacked a longitudinal progression assay.

There are still some limitations to this study. The study only tested patients suspected of having a TBE infection who were bitten by ticks in the Changbai Mountains area in 2023. Owing to the relative small number of patients, the analysis still had a certain degree of randomness. Additionally, the analysis only focused on serum samples and lacked follow-up blood investigations as well as other biological samples, such as cerebrospinal fluid, making the impact of the virus on patients during the longitudinal infection process unclear.

Ethical Approval and Informed Consent

The study was conducted according to the principles of the Declaration of Helsinki. Medical Ethics Approval for this study (Approval No. 2023001) was obtained from the Medical Ethics Committee of the People's Hospital of Jiangyuan District, Baishan, Jilin Province, China. Serum samples were collected after obtaining written informed consent from all patients.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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