

RESEARCH PAPER



Evervac: phase I/II study of immunogenicity and safety of a new adjuvant-free TBE vaccine cultivated in Vero cell culture

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ABSTRACT

Approximately 10,000 cases of tick-borne encephalitis (TBE), a serious disease of the central nervous system caused by tick-borne encephalitis virus (TBEV), are registered worldwide every year. Vaccination against TBE remains the most essential measure of preventing the disease. Unlike available TBE vaccines, a new inactivated lyophilized candidate vaccine Evervac is produced in Vero continuous cell culture and its final formulation does not include aluminum-based adjuvants. To study the safety and immunogenicity of Evervac, healthy adults 18–60 y of age were immunized twice at 30-d intervals. The study was single-blind, randomized, comparative, controlled, and was conducted in TBE-endemic areas. The commercial lyophilized vaccine TBE-Moscow was used as a comparison treatment. The subjects were observed for incidence, severity, and duration of adverse reactions. It was shown that the severity of local and systemic reactions in the Evervac vaccine group was mild to moderate. There were no significant differences in the incidence of adverse reactions between the Evervac and TBE-Moscow vaccine groups. Immunization with Evervac produced a significant increase in geometric mean titer (GMT) of anti-TBEV antibodies in both initially seronegative and seropositive recipients. The seroconversion rate for the initially seronegative recipients was 69% (GMT = 1:214) after the first dose and reached 100% after the second dose. In these parameters, there were no significant differences between the study and control vaccine groups. Thus, the adjuvant-free Vero-based vaccine Evervac was well tolerated, had low reactogenicity, induced a pronounced immune response, and was overall non-inferior to the commercial adjuvanted TBE vaccine used as a control.

ARTICLE HISTORY

Received 12 February 2020
Revised 24 March 2020
Accepted 14 April 2020

KEYWORDS

Inactivated vaccine; tick-borne encephalitis; tick-borne encephalitis vaccine; reactogenicity; immunogenicity; vaccine safety; flavivirus

Introduction

Tick-borne encephalitis (TBE) is a severe infectious disease of the CNS caused by tick-borne encephalitis virus (TBEV), a member of the *Flavivirus* genus of the *Flaviviridae* family. Three main phylogenetically distinct TBEV subtypes are Far Eastern, European, and Siberian. All subtypes of the virus circulate in Russia, although the Siberian subtype is predominant.¹ TBE-endemic area spreads across Eurasia, including several European countries, Russia, China, Mongolia and Japan.² Currently, natural foci of the infection remain highly active and the area of circulation of TBEV is expanding. In the Russian Federation, more than 60 million people live in TBE-endemic regions and 2000–3000 cases of the disease are registered annually.³

Prophylactic vaccination is the most effective form of protection against TBE. Currently on the market, there are six inactivated TBE vaccines based on the European and Far Eastern TBEV strains. According to modern data, most neutralizing epitopes of TBEV surface antigen (protein E) are common between different subtypes, and antibodies induced upon immunization with the available TBE vaccines provide cross-protection against different

strains of all three TBEV subtypes.^{4–6} Most TBE vaccines have an adult formulation and a formulation for children. Pediatric versions of the vaccines contain half the amount of antigen and are administered in half the volume of the adult formulation (0.25 ml instead of 0.5 ml).² TBE vaccines registered in Europe and Russia use primary culture of chicken embryo fibroblasts for virus propagation.⁷ The Chinese vaccine uses a primary culture of hamster kidney cells instead.⁸ All of these vaccines' formulations include aluminum hydroxide gel as adjuvant.

According to the World Health Organization (WHO) recommendations, the use of primary cell cultures in the production of antiviral vaccines should be limited and, if possible, replaced with continuous cell lines (CCLs). CCLs have a number of advantages: they are less likely to be contaminated by infectious agents, more stable, and allow the use of certified standardized cell bank systems in the manufacturing process, which can lead to a better safety profile of the preparations obtained by this technology.^{9,10} Currently, certified CCLs are widely used as substrates in the viral vaccines production.^{11,12} One of the more popular cell substrates are Vero cells, which are used in the production of live and inactivated polio vaccines, inactivated

rabies vaccines, rotavirus vaccines,^{13,14} as well as for a number of vaccine candidates, for example, against influenza and Chikungunya fever.^{15,16} In recent years, Vero cell line has been actively used to produce flavivirus vaccines.^{17,18}

An earlier version of the adjuvanted TBE vaccine using CCL 4647 as a substrate was developed at the Chumakov Federal Scientific Center.^{19,20} Subsequently, Vero cell-derived inactivated whole-virion lyophilized vaccine Evervac based on the TBEV strain Sofjin was developed by this manufacturer.^{21–23} From the available TBE vaccines, it differs in the absence of aluminum hydroxide adjuvant. Preclinical trials of Evervac vaccine have demonstrated its safety and immunogenicity in laboratory animals.

The main objective of this study was to assess the tolerability, safety, reactogenicity, and immunogenicity of Evervac vaccine in healthy volunteers in comparison with lyophilized TBE-Moscow vaccine based on the same TBEV strain Sofjin, used as a control.²⁴

Materials and methods

Study design

The randomized, controlled, comparative phase I/II study was conducted in volunteers aged 18–60 according to the Clinical Trial Protocol No. KЭ-BAK-I/II-002/15 v.1.0 of 05.16.2016.²⁵ This study was conducted in accordance with the Declaration of Helsinki, ICH GCP, and Russian regulations. The study protocol, an informed consent form, and other documents requiring preliminary consideration were approved by the Ethics Committee under the Ministry of Health of the Russian Federation. The study was conducted in TBE-endemic regions of Siberia in two clinical centers: Perm State Medical University named after Academician E.A. Wagner (Perm) and the Healthcare Unit No. 163 of FMBA of Russia (Novosibirsk). In total, 100 healthy recipients 18–60 y of age from Perm and Novosibirsk regions participated in the trial. Evervac vaccine was manufactured by the Chumakov Federal Scientific Center, which was the sponsor of the clinical trial.

The study was blind to the extent that participants, clinicians evaluating adverse effects, and laboratory analysts were unaware of which vaccine was administered to volunteers. In the first part of this study, the tolerability and safety of Evervac were evaluated in 10 healthy volunteers, while another 10 participants were immunized with the control TBE-Moscow vaccine, manufactured by the Chumakov Federal Scientific Center. Once the safety of the first dose of the vaccines has been confirmed by the sponsor and a local safety committee, the second part of the study was started, in which 80 more volunteers were enrolled. Participants from the initial safety assessment groups received second doses of the study and control vaccines 30 d after the first dose and were included in the final analysis.

Subjects

Healthy men and women between 18 and 60 y of age, who successfully underwent physical and laboratory examination, without registered prior TBEV infections and not vaccinated against TBE within the last 3 y were eligible for inclusion. Exclusion criteria were clinically significant deviations in

blood and/or urine laboratory tests; previous vaccination against other flavivirus infections; serious adverse events associated with previous vaccination; history of chronic infectious or autoimmune diseases; recent use of immunosuppressants or immunomodulators; vaccination with any live vaccine within 4 weeks or with an inactivated vaccine within 2 weeks before the study; acute infections within the last 4 weeks; medical history of substance abuse; pregnancy or breastfeeding. Women of childbearing potential had to have negative pregnancy test and be abstinent or use adequate contraception with an efficiency of more than 90%.

Randomization was performed using the sealed envelope system. After assigning a randomization number to participants, an opaque envelope with a participant number was given to a healthcare worker who administered the vaccine. Only this healthcare provider could open the envelope in the absence of other members of the research team. Inside the envelope was a tear-off sticker with the randomization number of the participant and the name of the corresponding vaccine. The participants did not know which vaccine they were receiving. After the vaccination, a separate clinician observed the participant for possible adverse events. The analyst who tested the sera was provided only with the code number of the participant and thus was also blinded. The distribution of participants into groups was carried out using a random number generator (Microsoft Office Excel, the RAND function, followed by sorting in ascending order.

Vaccines

The study vaccine Evervac (EV) was produced from the TBEV strain Sofjin; the continuous cell line Vero was used as a substrate for virus reproduction. Master cell bank of Vero cells was prepared using the passage 139 from the ampoule No. 0519 of the VeroWHO reference cell bank obtained from the WHO. Currently, this cell bank is also used for the production of BiVak polio vaccine licensed in the Russian Federation.²⁶ To inactivate the virus, 0.02% formaldehyde was added to the virus-containing cell culture fluid. The inactivated culture fluid was clarified, concentrated by ultrafiltration, and purified by gel chromatography. The finished form of the vaccine is a lyophilized preparation containing $0.75 \pm 0.15 \mu\text{g}$ of inactivated TBEV antigen, 250 μg of human albumin, 37.5 mg of sucrose and 5 mg of gelatose. Water for injection is used to dissolve the vaccine before use.

As a comparison treatment, we used cultural purified inactivated lyophilized vaccine against TBE (TBE-Moscow vaccine (MV)) manufactured by the Chumakov Federal Scientific Center. This vaccine is based on a primary culture of chicken embryo cells as a substrate for TBEV reproduction. The lyophilized vaccine is supplied with an ampoule of aluminum hydroxide gel ($0.8 \pm 0.2 \text{ mg/ml}$) as a solvent and an adjuvant.

Both vaccines contain the specific antigen of the TBEV strain Sofjin (Genbank KC806252).²⁴ The antigen titer in the vaccines is not less than 1:128 by enzyme-linked immunosorbent assay (ELISA) when using the “VectoTBE-antigen” kit (Vector-Best, Novosibirsk, Russia). EV and MV vaccines do not differ significantly in the quantity of the TBEV antigen or

excipients per dose, except for adjuvant, which is absent in EV vaccine. For both vaccines, the protein E content is standardized at the level of $0.75 \pm 0.15 \mu\text{g}$ per 0.5 ml.

Both groups of recipients eventually received two doses of corresponding vaccines with an interval of 30 d. Vaccines were administered intramuscularly into the deltoid muscle immediately after dissolution in 0.5 ml of the corresponding solvent (water for injection for EV and aluminum hydroxide gel for MV).

Safety and reactogenicity

Assessment of the tolerability, reactogenicity and safety of the vaccines was carried out based on the registration of adverse events (AE) (i.e., local and systemic reactions), assessment of neurological status, thermometry, biochemical and clinical blood tests, general urine analysis, total blood IgE levels, and registration of the incidence of serious adverse events (SAE). Any adverse and unexpected sign, including a deviation from the norm of laboratory indicators, a symptom or a disease occurring after vaccination and observed within the duration of the trial, was considered an AE.

Subjects were under clinical observation for at least 30 min after vaccination and recorded any reactions or AEs within the duration of the trial in self-observation diaries. At the preliminary safety assessment stage, first cohort of subjects was monitored for 24 h after vaccination in the hospital and then daily outpatient monitoring was carried out for 6 d. At the second stage of the study, the severity of the observed local and systemic reactions was evaluated directly after vaccination, for the next 6 d – on an outpatient basis, and then according to the self-observation diaries. Monitored local reactions were itching, burning, soreness, redness, swelling, hyperemia, infiltration or pain at the injection site, and systemic were fever, irritability, fatigue, eye pain, arthralgia, myalgia, paresthesia, lymphadenopathy, headache, dizziness, nausea, vomiting, abdominal pain and diarrhea. Assessment of the severity of AEs was scored on a 4-point scale in accordance with the standards required by the Russian regulations:

Grade 0 – no reaction: local reactions – no symptoms, systemic reactions – no symptoms, temperature – up to 37.0°C ;

Grade 1 – weak reaction: local reactions – hyperemia up to 50 mm in diameter or infiltrate up to 25 mm, systemic reactions – mild symptoms, temperature – from 37.1°C to 37.5°C ;

Grade 2 – medium reaction: local reactions – hyperemia of more than 50 mm or an infiltrate of 26–50 mm, systemic reactions – symptoms that significantly impair normal daily activity, temperature – from 37.6°C to 38.5°C ;

Grade 3 – strong reaction: local reactions – infiltrate more than 50 mm, systemic reactions – symptoms that impede normal daily activity, temperature – more than 38.6°C ;

To assess the allergenic properties of the vaccines, total serum IgE titers of the participants were determined using total-IFA-BEST IgE ELISA kit (Vector-Best, Novosibirsk, Russia). The sensitivity of the kit is 2.5 IU/ml, the measurement range is 0–800 IU/ml as stated by the manufacturer.

Blood sampling

Blood for clinical and biochemical blood tests, as well as for IgE assay, was drawn before vaccination (d 0), 2 and 30 d after the first dose; and 2 and 28 d after the second dose. Blood samples for immunogenicity assessment were taken from on d 0, 30 d after the first dose and 28 d after the second dose of the vaccine.

Immunogenicity

Vaccine immunogenicity was assessed by the development of antibodies to TBEV (anti-TBEV IgG) using ELISA “VectoTBE-IgG” kit (Vector-Best, Novosibirsk, Russia) according to the manufacturer’s instructions. It has been previously shown that titers of anti-TBEV IgG induced by several licensed TBE vaccines and determined by this test system correlate well with the levels of neutralizing antibodies to TBEV.²⁷ Validation of this test system was carried out by the manufacturer in accordance with the requirements of Russian regulatory agencies and GMP guidelines. Antiviral immune response was evaluated using the following indicators: titer of anti-TBEV IgG, geometric mean titer of virus-specific antibodies (GMT), seroconversion factor (fold increase in GMT compared to the initial level), and seroprotection rate (calculated as the percentage of recipients with antibody titers reaching $\geq 1:100$ after immunization).

Statistical analysis

EV and MV vaccines are identical in the vaccine TBEV strain and do not differ significantly in the content of the viral antigen (protein E) and excipients per dose. An assumption was made that anti-TBEV antibody titers induced by both vaccines would be distributed normally. Thus, anti-TBEV antibody titer values in both groups were postulated to represent a sample from the same population (null hypothesis). The sample size for this study was calculated based on standard deviations from the average antibody titers obtained in a clinical trial of TBE vaccines produced from the Far Eastern TBEV subtype, where VectoTBE-IgG test system was used to analyze the sera.²⁸ The average value and standard deviation of antibody titers for EV vaccine were expected to deviate from the assumed values by no more than 30%. The calculation was carried out assuming a significance level of 0.05 and a power of 80%. The calculation showed that the minimum sample size for detecting differences in immunogenicity of the vaccines was 44 people in each group. Given the possibility of participants withdrawing from the study, the group size was increased to 50 subjects. The main laboratory parameters obtained during the study were processed according to the rules of descriptive statistics. The values are presented as the mean or medians and standard deviations, depending on the nature of distribution of the parameters. Immunogenicity analysis was performed for all participants receiving two doses of the vaccines and following other study procedures. Geometric mean titers of anti-TBEV-antibodies (GMT) and 95% confidence intervals (CIs) were calculated for the immunogenicity analysis. The homogeneity of the groups by the characteristic of sex was analyzed using the Fisher exact test

(FET), while the two-tailed Student test was used for age, weight and height differences, and immunogenicity analysis. The incidence of AEs or SAEs and other reactions was compared using FET. Comparison of the severity of AEs (mild, moderate, severe) between groups was carried out using the non-parametric Mann–Whitney test. Statistical processing of the results was carried out by generally accepted methods of variation statistics using the Microcal Origin 8.0. software. *P*-values less than 0.05 were considered significant.

Results

Demographic and anthropometric data

A total of 100 participants (53 women and 47 men) aged 18–59 y (mean age 27.9 ± 9.8 y) were enrolled in this study. All subjects were Caucasian. The participants were randomized into two groups of 50 and vaccinated with either EV or MV vaccines. Both groups were homogeneous regarding sex, age, weight, and height. The main demographic and anthropometric characteristics of the study participants are presented in Table 1.

Tolerance, reactogenicity and safety of Evervac and Moscow-TBE vaccines

At the initial evaluation of the tolerability and safety of EV and MV vaccines in 20 participants (10 in each group), local reactions were recorded in one recipient of EV vaccine and in one recipient of MV vaccine. These reactions were observed on the d 1 after vaccination and manifested as pain at the injection site during palpation. Systemic reactions were recorded in three subjects in each of the groups on d 1–3 after vaccination and manifested as an increase in body temperature, weakness, headache, and mild myalgia. The reactions were predominantly mild, lasted 1–3 d, and did not require any medical intervention. Only one subject was prescribed 500 mg paracetamol to reduce the fever. There were no SAEs or any post-vaccination complications. Incidence and nature of local and systemic reactions did not differ in both comparison groups. After the evaluation of the results of this preliminary stage, the second part of the trial was started. Since both the participants at the preliminary and following stages were treated identically, hereafter, the data is presented for combined groups ($N = 50$ in each group).

In total, over the entire period of the study, AEs were registered in 10 participants in the EV vaccine group and in

Table 1. Demographic and anthropometric characteristics of participants (full analysis).

Parameter	Statistic	Evervac (EV)	TBE-Moscow (MV)
Number of subjects	<i>N</i> total = 100	50	50
Sex	Female/Male	30/20	23/27
Age, y	Mean \pm SD	27.1 ± 9.2	28.8 ± 10.5
	Interval	18–50	19–59
Height (cm)	Mean \pm SD	170.0 ± 10.0	172.3 ± 9.0
	Interval	154 – 196	152 – 190
Weight (kg)	Mean \pm SD	67.3 ± 11.7	69.0 ± 12.9
	Interval	48–96	45–97

SD, standard deviation.

12 participants in the MV vaccine group. These AEs were considered to be related to vaccination (Table 2).

During the study, the only local reaction reported was pain at the injection site upon palpation. In total, it was recorded in seven subjects (7%, 3 female and 4 male, aged 21–50). These reactions were observed only after the first vaccination on d 1–2, were mild, and resolved within 1–3 d. EV and MV vaccines did not differ in the rate of local reactions (FET).

Systemic reactions in total were recorded in 15 subjects (15%, 9 female and 6 male, aged 19–46) only after the first vaccination, were mostly mild and manifested in the form of fever, headache, weakness, fatigue, myalgia and chills (Table 3). All reactions were observed 1–3 d after vaccination, did not disturb the daily activities of participants, and mainly resolved within 1–3 d. Only two subjects from the EV vaccine group were prescribed a single dose of paracetamol (500 mg at night). There was no difference in the frequency and severity of systemic reactions between the studied vaccines (FET).

No severe AEs were observed during the study and there were no differences in the frequency and nature of local and systemic reactions recorded in EV and MV groups (FET). The physiological parameters (body temperature, blood pressure, heart rate, respiratory rate) recorded before vaccination and within 7 d of observation after each dose of the vaccines remained within the normal range; there were no deviations in the neurological status of the vaccine recipients. No clinically significant changes in hematology and blood and urine biochemistry were observed during the course of the study, there were no significant differences between the study and control groups (Student *t*-test).

The studied vaccines did not demonstrate any allergenic properties – serum IgE levels of subjects from both vaccine groups did not undergo significant changes during the course of the study. Total serum IgE titers of participants at the time points indicated in the ‘Materials and Methods’ section ranged from 0 to 149.1 IU/ml. The average titers of total serum IgE did not differ significantly between EV and MV vaccine groups and were in the range of 29–44 IU/ml (data not shown).

Table 2. Local and systemic reactions after vaccination with Evervac and TBE-Moscow vaccines.

Adverse events	Severity	Evervac (<i>N</i> = 50)	TBE-Moscow (<i>N</i> = 50)
		no. (%)	no. (%)
Local reactions	Mild	2 (4)	5 (10)
Systemic reactions	Mild	6 (12)	6 (12)
	Moderate	2 (4)	1 (2)
Total		10 (20)	12 (24)

N, number of subjects in each group.

Table 3. Types of systemic reactions after vaccination with Evervac and TBE-Moscow vaccines.

Systemic reaction	Evervac (<i>N</i> = 50)	TBE-Moscow (<i>N</i> = 50)
	no. (%)	no. (%)
Fever	6 (12)	5 (10)
Headache	2 (4)	1 (2)
Weakness	4 (8)	1 (2)
Myalgia	3 (6)	1 (2)
Chills	1 (2)	-

N, number of subjects in each group.

The obtained data indicate a good tolerance, low reactivity, and favorable safety profile of Evervac vaccine in subjects aged from 18 to 60 y.

Immunogenicity of Evervac and TBE-Moscow vaccines

The titer of specific antibodies to TBEV (anti-TBEV IgG) was determined in the sera of subjects who were immunized twice and completed the study. While 50 subjects in both groups received the first dose of the vaccines, one subject was excluded from further participation due to the refusal of the second dose. Another subject was disqualified due to the development of an upper respiratory tract infection, which was considered not to be related to the vaccination. Hence, only 49 subjects in both EV and MV groups were included in the final immunogenicity analysis.

Baseline anti-TBEV IgG determination revealed that a significant proportion of participants (51% of total, 20 and 30 in the EV and MV groups, respectively) were initially TBEV-seropositive. Therefore, we analyzed the immunogenicity of the vaccines separately for the initially seronegative and seropositive participants.

For the initially seronegative subjects, GMTs were 2.59 log (1:389) after the first dose and 3.16 log (1:1445) after the second dose of EV vaccine, while for MV vaccine after the first and second vaccination GMTs were 2.69 log (1:490) and 2.95 log (1:891), respectively (Table 4). Between the first and the second vaccination in the EV-vaccinated subjects GMT increased 3.7-fold, while in the subjects vaccinated with MV the increase was 1.8-fold. The level of seroprotection was 69% after the first and 100% after the second immunization with EV, and 36.8% and 94.7%, respectively, in those vaccinated with MV. Thus, for the initially seronegative subjects, on d 30 after the first injection, there were statistically significant differences in the seroprotection rates (FET, $p = .0394$) between the groups. After the second vaccination (d 58), these differences were not present.

There were 20 initially seropositive individuals in the EV group and 30 subjects in the initially seropositive MV group; their GMTs of anti-TBEV IgG before vaccination did not differ significantly (3.03 for EV and 2.85 for MV, respectively,

see Table 4). For these seropositive vaccine recipients, the seroconversion level was the percentage of recipients whose titers increased more than 4 times compared to the baseline level (d 0).

After the first dose of either vaccine, anti-TBEV antibody titers in seropositive subjects increased, however, to a lesser extent than for the initially seronegative participants, and remained almost unchanged after the second vaccination. GMTs of EV-vaccinated subjects were 1:7690 after the first and 1:7770 after the second vaccination, and 1:3850 and 1:5130, respectively, in those vaccinated with MV. The mean of fold increase comparing to the baseline (seroconversion factor) was 7.2 after the first and 7.3 after the second dose of EV vaccine, and 5.4 and 7.2, accordingly in the group vaccinated with MV vaccine. The seroconversion rates reached 75% and 85% after the first and second immunization with EV and remained unchanged at the 70% level after the second vaccination in MV-vaccinated subjects. There were no differences in any of the abovementioned parameters between EV and MV-vaccinated subjects at all time stages.

Discussion

All currently used commercial vaccines against TBE are manufactured using primary cell cultures as substrates for TBEV reproduction.^{7,8} However, the use of continuous cell lines (CCLs) for the virus reproduction has several advantages, including better standardization, higher stability and safety, and a number of technological advantages, such as the possibility of using microcarrier cell culture bioreactors.¹² In addition, using CCLs provides a solution to the ethical problem associated with sacrificing large numbers of animals to obtain primary cell cultures. These considerations motivated researchers to pursue the development of a new generation of classic inactivated flavivirus vaccines using Vero cell line as a substrate.^{29,30}

The new inactivated vaccine Evervac based on the strain Sofjin of the Far Eastern TBEV subtype was developed at the Chumakov Federal Scientific Center. Vero cell line obtained from the WHO reference cell bank was used as a substrate for the virus reproduction. The vaccine is lyophilized to ensure

Table 4. Immunogenicity of Evervac and TBE-Moscow vaccines for initially seronegative and initially seropositive subjects.

Blood sampling day	Parameter	Evervac (EV)	TBE-Moscow (MV)
Initially seronegative subjects			
		N = 29	N = 19
30 d after the 1st vaccination (d 30)	GMT [95% CI]	2.59 [2.32;2.86]	2.69 [2.06;3.32]
	Seroprotection rate [95% CI]	69.0% [52.2%;85.8%]	36.8% [15.1%;51.9%]
28 d after the 2nd vaccination (d 58)	GMT [95% CI]	3.16 [3.02;3.30]	2.95 [2.74;3.16]
	Seroprotection rate [95% CI]	100% [96.7%;100%]	94.7% [84.6%;100%]
Initially seropositive subjects			
		N = 20	N = 30
Baseline (d 0)	GMT [95% CI]	3.03 [2.75;3.31]	2.85 [2.64;3.06]
30 d after the 1st vaccination (d 30)	GMT [95% CI]	3.89 [3.73;4.05]	3.59 [3.4;3.78]
	Seroconversion rate [95% CI]	75% [52.8%;89.2%]	70% [52%;83.5%]
28 d after the 2nd vaccination (d 58)	GMT [95% CI]	3.89 [3.75;4.03]	3.71 [3.57;3.85]
	Seroconversion rate [95% CI]	85% [63.1%;95.6%]	70% [52%;83.5%]

N, number of subjects in each group; GMT, geometric mean titer of anti-TBEV IgG (expressed as log₁₀) measured by ELISA; 95% CI, confidence interval.

Seroprotection rate – percentage of subjects with an anti-TBEV IgG titer of $\geq 1:100$.

Seroconversion rate – percentage of subjects with a ≥ 4 -fold anti-TBEV IgG titer increase comparing to the previous level.

the high stability of the viral antigen throughout the shelf life and should be dissolved in water for injection before use. The formulation does not contain aluminum-based adjuvants, antibiotics and preservatives. Evervac is intended for immunization of persons 3 y and older, and the primary course of immunization consists of two intramuscular injections with an interval of 1 to 7 months. The revaccination should be carried out 1 y after the first vaccination and continued with 3-y intervals afterward.

The aim of this clinical study was to assess the tolerability, safety, reactogenicity and immunogenicity of Evervac vaccine (EV) during primary vaccination of adults. The commercial TBE-Moscow vaccine (MV), which is also based on the Sofjin TBEV strain, was used as a control. Unlike EV, it contains an adjuvant – aluminum hydroxide gel. MV is the most widely used TBE vaccine in the Russian Federation, and it provides excellent protection against a wide range of different strains of all main TBEV subtypes.^{24,31}

The study did not reveal any differences in the incidence and severity of local and systemic reactions between EV and MV vaccines. Local and systemic reactions for both vaccines were observed only after the first dose, were transient and predominantly mild in nature, and did not cause any subjects to withdraw from the study. There were no statistically significant sex differences in response to vaccination. No adverse reactions were reported after the second dose of either vaccine. The clinical examination parameters and/or laboratory blood and urine test results were not different between the study groups and did not undergo any changes from the corresponding background levels during the trial. The studied vaccines did not reveal any allergenic properties.

Due to both ethical and practical considerations, an inherent limitation of all TBE vaccine studies is the measurement of secondary endpoints, i.e. the antiviral antibody response, and not clinical endpoints (such as protection against the morbidity and mortality). Fortunately, the measurement of antibody response to TBE vaccines has been shown to be an adequate and sufficient alternative. A number of methods can be used to assess the immunogenicity of TBE vaccines during clinical trials; however, determination of virus-specific antibody titers by ELISA or the assessment of neutralizing antibody titers by plaque reduction test is generally employed.^{4,32} In our study, we assessed the anti-TBEV antibody titers of subjects by “VectoTBE IgG” ELISA kit. The antibody titers obtained while using ELISA correlate well with the titers of neutralizing antibodies in vaccinated people,²⁷ and this method is generally accepted in the Russian Federation when conducting clinical studies of TBE vaccines.^{33,34} A titer value of 1:100 in the ELISA is considered as the minimum protective titer of anti-TBEV antibodies, and, accordingly, as the seropositivity threshold.^{35,36}

In accordance with the national ethical standards, clinical trials of TBE vaccines in the Russian Federation are usually carried out in TBE-endemic areas; this study was conducted in Perm and Novosibirsk regions of Siberia with well-documented circulation of TBEV. Populations living in such endemic territories are expected to have a significant percentage of seropositive individuals, both because of previous contacts with TBEV in nature and/or as a result of previous

vaccinations.^{28,37,38} In our study, more than 50% of the participants were found to be initially seropositive to TBEV, and, accordingly, the immunogenicity analysis was carried out separately for initially seronegative and initially seropositive subjects. Statistical analysis demonstrated the comparability of the EV and MV vaccine study groups in relation to the initial titers of anti-TBEV antibodies.

In the group of initially seronegative participants vaccinated with EV vaccine, GMTs exceeded the minimum protective titer more than threefold after the first dose of the vaccine and more than 14-fold after the second dose (1:1445). The seroprotection levels after the first and second vaccination were 69% and 100%, respectively. Comparable data were obtained for the individuals immunized with MV vaccine.

As expected, in the initially seropositive groups after the first injection of either vaccine, antibody titers increased (to a lesser extent than in the initially seronegative groups), and remained almost unchanged after the second vaccination. GMTs of initially seropositive individuals were higher in comparison with the initially seronegative participants at all stages of the study, especially after the first dose of any of the vaccines. These results correspond well to our previous data for Tick-E-Vac and Encevir vaccines, when we observed the differences in antibody titers between initially seronegative and seropositive individuals only after the first immunization against TBE, but not after the second.³⁸

Aluminum hydroxide is one of the most common vaccine adjuvants and it is included into the formulations of all commercial TBE vaccines as well.² In addition to the immunostimulatory effect, aluminum hydroxide provides stabilization of TBEV antigen in adsorbed TBE vaccines. For inactivated TBEV (strain Neudörfl) it was demonstrated that aluminum hydroxide could enhance the antibody response and modulate the spectrum of induced virus-specific and virus-neutralizing antibodies.³⁹ It has been suggested that the particle size of the adjuvant with the adsorbed viral antigen may affect the immunogenic properties of commercial TBE vaccines.⁵ At the same time, there are reports indicating a possible increase in the frequency of local reactions and longer pain at the injection site when using vaccines containing aluminum hydroxide gel.⁴⁰ The possibility of connection between the aluminum hydroxide exposure and the development of certain neurodegenerative disorders has been reported, while several studies concluding that aluminum-based adjuvants are completely safe were criticized for critical weaknesses.^{41,42} This information together with growing public concerns about the safety of vaccines warrants further extensive studies on the safety of aluminum compounds as adjuvants and provides incentives for the development of effective, yet less reactogenic adjuvant-free vaccines, when it is possible.

Previously, we have shown that the immunization of mice with lyophilized TBE-Moscow vaccine without aluminum hydroxide gel does not affect the vaccine's protective properties, induced neutralizing antibody titers and protective immunity spectrum toward different TBEV strains when comparing with the adjuvanted TBE vaccines.^{6,22,31} This fact, possibly, could be attributed to the features of the Sofjin vaccine strain, such as the high protective activity of the viral antigen obtained on its basis.²⁰ This study demonstrated no measurable differences in the intensity of the humoral immune response induced by the TBE vaccine

without adjuvant in comparison with the aluminum-adjuvanted formulation, indicating the possibility of a successful development and implementation of adjuvant-free TBE vaccines.

Since this article describes a pilot phase I/II study, its sample size was enough to detect the differences between the two vaccine groups and between primary seronegative and seropositive individuals, whereas a bigger sample size is required to confirm the results and to obtain additional information. Because the time span of the study was limited to 60 d, a separate prolonged study is necessary to assess the longevity of the immune response to Evervac. As we mentioned previously, in this work only ELISA was used to assess serum anti-TBEV antibody levels, although neutralization test could provide additional information about the antibody spectrum. Lyophilization of a viral antigen ensures its stability over a long period of time,²² while the aluminum hydroxide gel should not be frozen and must be included into the vaccine package in a separate ampoule. The exclusion of aluminum hydroxide from the final formulation of the lyophilized TBE vaccine would ensure a higher degree of standardization, simplify the manufacturing and quality control procedures, and facilitate the storage and transportation of the vaccine to remote regions.

The results of this clinical study of the new adjuvant-free TBE vaccine Evervac demonstrate its safety, good tolerance, low reactogenicity, and high immunogenicity in individuals aged 18 to 60 y. In regards to the studied parameters of the vaccine efficacy, Evervac is equivalent to the commercial TBE-Moscow vaccine.

Conclusion

Healthy adults aged 18 to 60 were vaccinated twice with the new candidate adjuvant-free TBEV vaccine Evervac. The data on the frequency, severity, and duration of local and systemic reactions indicate low reactogenicity and a good safety profile of the vaccine. It was shown that Evervac vaccine induces a pronounced humoral immune response, providing protective titers of anti-TBEV antibodies in 100% of vaccinated subjects.

The candidate vaccine Evervac did not differ from the well-established commercial vaccine TBE-Moscow, used as the control, in any of the studied immunogenicity parameters.

Disclosure of potential conflicts of interest

All co-authors of this work are employees at the Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products of Russian Academy of Sciences, a company that developed and manufactured both the candidate TBE vaccine Evervac and TBE-Moscow vaccine, and was the sponsor of this study.

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References

- Demina TV, Dzhioev YP, Verkhozina MM, Kozlova IV, Tkachev SE, Plyusnin A, Doroshchenko EK, Lisak OV, Zlobin VI. Genotyping and characterization of the geographical distribution of tick-borne encephalitis virus variants with a set of molecular probes. *J Med Virol.* 2010;82(6):965–76. doi:10.1002/jmv.21765. PMID: 20419810.
- Ruzek D, Avšič Županc T, Borde J, Chrdle A, Eyer L, Karganova G, Kholodilov I, Knap N, Kozlovskaya L, Matveev A, et al. Tick-borne encephalitis in Europe and Russia: review of pathogenesis, clinical features, therapy, and vaccines. *Antiviral Res.* 2019;164:23–51. doi:10.1016/j.antiviral.2019.01.014. PMID: 30710567.
- Vorovitch MF, Maikova GB, Chernokhaeva LL, Romanenko VV, Karganova GG, Ishmukhametov AA. Comparison of the immunogenicity and safety of two pediatric TBE vaccines based on the Far Eastern and European virus subtypes. *Adv Virol.* 2019;24:5323428. doi:10.1155/2019/5323428. PMID: 31933642.
- Domnich A, Panatto D, Arbuzova EK, Signori A, Avio U, Gasparini R, Amicizia D. Immunogenicity against Far Eastern and Siberian subtypes of tick-borne encephalitis (TBE) virus elicited by the currently available vaccines based on European subtype: systematic review and meta-analysis. *Hum Vaccine Immunoter.* 2014;10(10):2818–33. doi:10.4161/hv.29984. PMID: 25483679.
- Morozova OV, Bakhvalova VN, Potapova OF, Grishechkin AE, Isaeva EI, Aldarov KV, Klinov DV, Vorovich MF. Evaluation of immune response and protective effect of four vaccines against the tick-borne encephalitis virus. *Vaccine.* 2014;32(25):3101–06. doi:10.1016/j.vaccine.2014.02.046.
- Chernokhaeva LL, Rogova YV, Kozlovskaya LI, Romanova LI, Osolodkin DI, Vorovitch MF, Karganova GG. Experimental evaluation of the protective efficacy of tick-borne encephalitis (TBE) vaccines based on European and Far-Eastern TBEV strains in mice and in vitro. *Front Microbiol.* 2018 16;9:1487. doi:10.3389/fmicb.2018.01487. PMID: 30061869.
- Kollaritsch H, Paulke-Korinek M, Holzmann H, Hombach J, Bjorvatn B, Barrett A. Vaccines and vaccination against tick-borne encephalitis. *Expert Rev Vaccines.* 2012;11(9):1103–1119. doi:10.1586/erv.12.86. PMID: 23151167.
- Xing Y, Schmitt H-J, Arguedas A, Yang J. Tick-borne encephalitis in China: a review of epidemiology and vaccines. *Vaccine.* 2017;35(9):1227–37. doi:10.1016/j.vaccine.2017.01.015. PMID: 28153343.
- WHO Expert Committee on biological standardization. Requirements for the use of animal cells as in vitro substrates for the production of biologicals. Geneva; 1998. WHO Technical Report No 878, annex 1. PMID: 9731464.
- WHO Expert Committee on biological standardization. Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. Geneva; 2010. WHO Technical Report Series 978, Annex 3.
- Genzel Y, Reichl U. Continuous cell lines as a production system for influenza vaccines. *Expert Rev Vaccines.* 2009;8(12):1681–92. doi:10.1586/erv.09.128. PMID: 19943763.
- Tapia F, Vázquez-Ramírez D, Genzel Y, Reichl U. Bioreactors for high cell density and continuous multi-stage cultivations: options for process intensification in cell culture-based viral vaccine production. *Appl Microbiol Biotechnol.* 2016;100(5):2121–32. doi:10.1007/s00253-015-7267-9. PMID: 26758296.
- Barrett P, Mundt W, Kistner O, Howard M. Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines. *Expert Rev Vaccines.* 2009;8(5):607–18. doi:10.1586/erv.09.19. PMID: 19397417.
- Toovey S. Preventing rabies with the Verorab[®] vaccine: 1985–2005 twenty years of clinical experience. *Travel Med Infect Dis.* 2007;5(6):327–48. doi:10.1016/j.tmaid.2007.07.004. PMID: 17983973.
- Ehrllich HJ, Müller M, Oh HML, Tambyah PA, Joukhadar C, Montomoli E, Fisher D, Berezuk G, Fritsch S, Löw-Baselli A, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *N Engl J Med.* 2008;358:2573–84. doi:10.1056/NEJMoa073121.

16. Kumar M, Sudeep AB, Arankalle VA. Evaluation of recombinant E2 protein-based and whole-virus inactivated candidate vaccines against chikungunya virus. *Vaccine*. 2012;30(43):6142–49. doi:10.1016/j.vaccine.2012.07.072. PMID: 22884660.
17. Monath TP, Liu J, Kanasa-thasan N, Myers GA, Nichols R, Deary A, McCarthy K, Johnson C, Ermak T, Shin S, et al. A live, attenuated recombinant West Nile virus vaccine. *Proc Natl Acad Sci USA*. 2006;103:6694–99. doi:10.1073/pnas.0601932103. PMID: 16617103.
18. Tauber E, Kollaritsch H, von Sonnenburg F, Lademann M, Jilma B, Firbas C, Jelinek T, Beckett C, Knobloch J, McBride W, et al. Randomized, double-blind, placebo-controlled Phase 3 trial of the safety and tolerability of IC51, an inactivated Japanese encephalitis vaccine. *J Infect Dis*. 2008;198:493–99. doi:10.1086/590116. PMID: 185888481.
19. Elbert BL, Terletskaia EN, Timofeev AV, Mironova LL, Khapchaev U, Amosenko FA, Svitkin YV, Alpatova GA, Krutyanskaya GL. Inactivated vaccine against tick-borne encephalitis (TBE) derived from heteroploid continuous monkey cell line. *Vaccine*. 1989;7(5):477. doi:10.1016/0264-410x(89)90183-7. PMID: 2815981.
20. Elbert BL, Vorovich MF, Terletskaia EN, Lisitsina EA, Atanadze SN, Sidorovich IG, Khozinskii VV, Tugizov S, Kushch AA, Khapchaev I, et al. Study of protective activity of preparations of TBE virus grown in different cell cultures. *Zh Mikrobiol Epidemiol Immunobiol*. 1992;1:25–28. PMID: 1414102. (in Russian).
21. Khapchaev Y, Khoretonenko MV, Timofeev AV, Grachev VP. Reproduction of TBE virus Sofjin strain in Vero cells cultured on microcarriers under conditions of pseudo-suspension. *Biotechnology*. 2003;5:32–39. (in Russian).
22. Vorovich MF, Khapchaev Y, Prilukova NS, Nagireva LI, Grachev VP. Russian inactivated liophilised TBE vaccine. *Biopreparations Prev Diagnosis Treat*. 2004;2:17–20. (in Russian).
23. Vorovich MF, Kiktenko AV, Khapchaev Y, Grachev VP. The new inactivated vaccines against TBE virus. *Zhurnal Infektsionnoy Patologii*. 2012;19(3):13. (in Russian).
24. Vorovitch MF, Kozlovskaya LI, Romanova L, Chernokhaeva LL, Ishmukhametov AA, Karganova GG. Genetic description of a tick-borne encephalitis virus strain Sofjin with the longest history as a vaccine strain. *Springerplus*. 2015;4:761. doi:10.1186/s40064-015-1561-y. PMID: 26682114.
25. State register medicines. accessed 2020 Jan 28. https://grls.rosminzdrav.ru/CiPermissionReg.aspx?PermYear=0&DateInc=&NumInc=&DateBeg=&DateEnd=&Protocol=%d0%9ad0%ad-%d0%92%d0%90d0%9a-I%2fII-002%2f15+&RegNm=&Statement=&ProtoNum=&idCISstatementCh=&Qualifier=&CiPhase=&RangeOfApp=&Torg=&LFDos=&Producer=&Researcher=&sponsorCountry=&MedBaseCount=&CiType=&PatientCount=&OrgDocOut=2&Status=&NotInReg=0&All=0&PageSize=8&order=date_perm&orderType=desc&pagenum=1.
26. Khapchaev YK, Ivanova OE, Vorovitch MF, Romanenko VV, Makarov AE, Drozdov SG, Ishmukhametov AA. Comparative randomized study of tolerability, reactogenicity, immunogenicity and safety of the vaccine “BiVac Polio” (Oral Poliovirus vaccine type 1, 3). *Epidemiol Vaccinal Prev*. 2017;16(3):58–64. doi:10.31631/2073-3046-2017-16-3-58-64. (in Russian).
27. Chernokhaeva LL, Maikova GB, Rogova Y, Romanenko VV, Ankudinova AV, Kilyachina AS, Vorovich MF, Karganova GG. Comparison of results obtained in ELISA and neutralization test in assessment of the protection of population from tick-borne encephalitis. *Vopr Virusol*. 2018;63(1):36–40. doi:10.18821/0507-4088-2018-63-1-36-40. (in Russian).
28. Vorovitch MF, Maikova GB, Chernokhaeva LL, Romanenko VV, Ankudinova AV, Khapchaev Y, Karganova GG, Ishmukhametov AA, Drozdov SG. Immunogenicity and safety of the adult TBE vaccine “Tick-E-Vac”. *Vopr Virusol*. 2017;62(2):73–80. doi:10.18821/0507-4088-2017-62-2-73-80.
29. Pereira RC, Silva AN, Souza MC, Silva MV, Neves PP, Silva AA, Matos DD, Herrera MA, Yamamura AM, Freire MS, et al. An inactivated yellow fever 17DD vaccine cultivated in Vero cell cultures. *Vaccine*. 2015;33(35):4261–68. doi:10.1016/j.vaccine.2015.03.077. PMID: 25862300.
30. Woods CW, Sanchez AM, Swamyc GK, McClain MT, Harrington L, Freeman D, Poore EA, Slifka DK, Poer DD, Amanna IJ, et al. An observer blinded, randomized, placebo-controlled, phase I dose escalation trial to evaluate the safety and immunogenicity of an inactivated West Nile virus Vaccine, HydroVax-001, in healthy adults. *Vaccine*. 2019;37:4222–30. doi:10.1016/j.vaccine.2018.12.026. PMID: 30661836.
31. Chernokhaeva LL, Rogova YV, Vorovitch MF, Romanova L, Kozlovskaya LI, Maikova GB, Kholodilov IS, Karganova GG. Protective immunity spectrum induced by immunization with a vaccine from the TBEV strain Sofjin. *Vaccine*. 2016;34(20):2354–61. doi:10.1016/j.vaccine.2016.03.041.
32. Pollabauer EM, Pavlova BG, Low-Baselli A, Fritsch S, Prymula R, Angermayr R, Draxler W, Firth C, Bosman J, Valenta B, et al. Comparison of immunogenicity and safety between two pediatric TBE vaccines. *Vaccine*. 2010;28:4680–85. doi:10.1016/j.vaccine.2010.04.047. PMID: 20433803.
33. Ankudinova AV, Romanenko VV, Vorovich MF, Kovtun OP, Eshunina MS, Kiktenko AV, Kilyachina AS, Averyanov O. Results of a clinical immunogenicity and safety trial of Tick – E – Vac 0.25 ml vaccine (pediatric dosage). *Vestnik Uralskoi Meditsinskoi Akademicheskoi Nauki*. 2014;5(51):49–55. (in Russian).
34. Feldblum V, Okuneva IA, Danilina TV, Menshikova MG, Pavrov KA, Trofimov DM, Ershov AE. Assessment of reactogenicity, safety and immunogenicity of the domestic vaccine ‘EnceVir’ with the reduced antigenic load in the immunization of children aged 3–17 years accordingly to routine scheduled vaccination (report 2). *Epidemiol Infect Dis*. 2015;20(3):28–32. (in Russian).
35. Paulke-Korinek M, Kundi M, Laaber B, Brodtraeger N, Seidl-Friedrich C, Wiedermann U, Kollaritsch H. Factors associated with seroimmunity against tick borne encephalitis virus 10 years after booster vaccination. *Vaccine*. 2013;31(9):1293–97. doi:10.1016/j.vaccine.2012.12.075. PMID: 23306371.
36. Onishchenko GG, Krivulya SD, Fedorov Y, Pakschina ND, Skudareva ON, Kotova EA, Chernyavskaya OP, Ivanova AA, Arumova EA, Zhukov VI, et al. Sanitary and epidemiological rules SP 3.1.3.2352-08 Tick-borne viral encephalitis prevention. *Bulletin of normative acts of federal executive bodies. Moscow: Rospotrebnadzor*; 2008. p. 36.
37. Shoutova NA, Shkouratova OV, Rouzavina Y, Vlasova NM, Stavitskaya N, Vorobyova MS, Ladyzhenskaya IP, Solyanik RG. Studying immunologic activity and reactogenicity “EnceVir” vaccine immunization using express-scheme. *Sibirskii Meditsinskii Zhurnal*. 2009;24(2–2):30–33. (in Russian).
38. Maikova GB, Chernokhaeva LL, Rogova YV, Kozlovskaya LI, Kholodilov IS, Vorovitch MF, Karganova GG, Romanenko VV, Eshunina MS, Ankudinova AA, et al. Ability of inactivated vaccines based on far-eastern tick-borne encephalitis virus strains to induce humoral immune response in originally seropositive and seronegative recipients. *J Med Virol*. 2019;91(2):190–200. doi:10.1002/jmv.25316. PMID: 30204244.
39. Zlatkovic J, Tsuchnikas G, Jarmer J, Koessl C, Stiasny K, Heinz FX. Aluminum hydroxide influences not only the extent but also the fine specificity and functional activity of antibody responses to tick-borne encephalitis virus in mice. *J Virol*. 2013;87(22):12187–95. doi:10.1128/JVI.01690-13. PMID: 24006434.
40. Haag CK, Dacey E, Hamilton N, White KP. Aluminum granuloma in a child secondary to DTaP-IPV vaccination: A case report. *Pediatr Dermatol*. 2019;36(1):e17–e19. doi:10.1111/pde.13732. Epub 2018 Nov 28. PMID: 30488642.
41. Bergfors E, Trollfors B. Sixty-four children with persistent itching nodules and contact allergy to aluminium after vaccination with aluminium-adsorbed vaccines – prognosis and outcome after booster vaccination. *Eur J Pediatr*. 2013;172(2):171–77. doi:10.1007/s00431-012-1841-2. Epub 2012 Oct 11. PMID: 23052615.
42. Masson JD, Crépeaux G, Authier FJ, Exley C, Gherardi RK. Critical analysis of reference studies on the toxicokinetics of aluminum-based adjuvants. *J Inorg Biochem*. 2018;181:87–95. doi:10.1016/j.jinorgbio.2017.12.015. Epub 2017 Dec 28. Review. PMID: 29307441.