



Functional characterization and immunogenicity of a novel vaccine candidate against tick-borne encephalitis virus based on *Leishmania*-derived virus-like particles

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ABSTRACT

Tick-borne encephalitis virus (TBEV) is a major cause of neurological infections in many regions of central, eastern and northern Europe and northern Asia. In approximately 15% of cases, TBEV infections lead to the development of severe encephalitis or meningitis. The main route of TBEV transmission is tick bites; however, ingestion of dairy products from infected animals (goats, cattle and sheep) is also a frequent cause of the disease. Therefore, vaccination of livestock in virus endemic regions could also contribute to the decrease in TBEV infection among humans. Although few vaccines against TBEV based on inactivated viruses are available for humans, due to high costs, vaccination is not mandatory in most of the affected countries. Moreover, there is still no vaccine for veterinary use. Here, we present a characterization and immunogenicity study of a new potential TBEV vaccine based on virus-like particles (VLPs) produced in *Leishmania tarentolae* cells. VLPs, which mimic native viral particles but do not contain genetic material, show good immunogenic potential. For the first time, we showed that the protozoan *L. tarentolae* expression system can be successfully used for the production of TBEV virus-like particles with highly efficient production. We confirmed that TBEV recombinant structural proteins (prM/M and E) from VLPs are highly recognized by neutralizing antibodies in *in vitro* analyses. Therefore, VLPs in combination with AddaVax adjuvant were used in immunization studies in a mouse model. VLPs proved to be highly immunogenic and induced the production of high levels of neutralizing antibodies. In a challenge experiment, immunization with VLPs provided full protection from lethal TBE in mice. Thus, we suggest that *Leishmania*-derived VLPs may be a good candidate for a safe alternative human vaccine with high efficiency of production. Moreover, this potential vaccine candidate may constitute a low-cost candidate for veterinary use.

1. Introduction

Infectious diseases remain the leading cause of morbidity and mortality in humans and animals worldwide. Respiratory viral infections and arboviral infections represent the major categories of emerging viral infections globally. Flaviviruses are vector-borne positive sense RNA viruses that can emerge unexpectedly in human populations and cause

serious diseases that are medically important. Tick-borne encephalitis virus (TBEV), an important representative of this group, can cause a disorder of the central nervous system that may lead to serious medical complications, including meningitis and meningoencephalitis (Dumpis et al., 1999). The main route of TBEV transmission is tick bites; however, other routes, such as the consumption of unpasteurized milk and milk products from infected animals such as goats, cows and sheep, remain

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important (Růžek et al., 2010).

The geographical range of TBEV, in the past confined to East Asia and Eastern Europe, is quickly increasing; at present, it is detected in almost all of Europe (Yoshii, 2019; Mansbridge et al., 2022). Recently, TBEV has also been reported in North Africa (Khamassi Khbou et al., 2020; Fares et al., 2021). The incidence of TBE has increased over 400% during the past 20 years in Europe, which makes tick-borne encephalitis (TBE) the second most serious disease transmitted by ticks (Donoso Mantke et al., 2011). According to World Health Organization data, 10,000–12,000 tick-borne encephalitis cases are reported each year (World Health Organization, 2017).

TBEV is a small enveloped, single-stranded RNA virus with a positive-polarity RNA genome of approximately 11 kb (Růžek et al., 2019). The viral RNA contains a single open reading frame (ORF), which is translated to a large polyprotein cleaved co- and post-translationally by cellular and viral proteases to yield three structural (E, C and M) and seven nonstructural proteins involved in the replication cycle of the virus within a cell (Barrows et al., 2018). Two viral proteins (glycoprotein E and the small membrane protein M) play a major role in viral entry into target cells. Envelope E glycoprotein, as the most exposed structural element of virions, participates in the assembly of infectious particles and plays a role in viral entry, since it allows interactions with specific cell surface receptors and induces fusion between the viral envelope and the host cell membrane. It is composed of three structural domains and a transmembrane domain that is required for anchoring the protein in a lipid membrane. Domain I contains an *N*-glycosylation site and fusion-loop peptide is located in the domain II (Lattová et al., 2020). Domain I and II together are responsible for E protein dimerization. Immunoglobulin-like domain III is the most likely candidate for interactions with cellular receptors. It has also been shown that during infection, most neutralizing antibodies are directed against domain III of glycoprotein E (Zhang et al., 2017). The prM/M glycoprotein is a small membrane protein that is cleaved to the pr peptide and M protein present in mature virions during maturation of viral particles. One *N*-glyco-site is present in the pr fragment. The exact role of the prM protein in flaviviruses has not been fully determined, but it is believed to be a chaperone-like protein assisting in proper folding of E glycoprotein. This protein is also required for pH-dependent rearrangements during virion maturation and protection from premature fusion with cellular membranes (Roby et al., 2015).

Despite numerous strategies of research, there is currently no licensed therapeutic agent available for the treatment of TBEV infections. Patients diagnosed with TBE infection are usually treated to alleviate the symptoms. As there are no treatment procedures available, it is important to search for innovative prevention methods and potential therapies. Vaccination is the most effective means of disease prevention. Five vaccines against TBE based on inactivated virus are currently on the market; in the EU, two vaccines are marketed: FSME-Immun® by Pfizer and German Encepur® by Novartis. Both vaccines are based on formaldehyde-inactivated European subtype whole virus particles. Although vaccines are safe and highly effective, some drawbacks exist, and the vaccination schedule requires three doses to stimulate the development of a protective antibody response. Additionally, booster vaccinations are required every 3–5 years to maintain protective immunity, especially in the elderly population; vaccine failures even after a complete series of vaccine doses have been reported. Moreover, the production of inactivated vaccines carries the inherent risk of utilizing large quantities of potentially highly pathogenic viruses (Lehrer and Holbrook, 2011). Due to the high costs and required multiple doses, vaccination coverage in humans remains low in several endemic countries.

Currently produced human TBEV vaccines are not approved for veterinary use, and production cost limits their potential use for immunization of animals. A candidate vaccine for veterinary use has been developed, but it is also based on inactivated TBEV and has not yet been approved for clinical use (Salát et al., 2018). Given all the drawbacks of

existing vaccines, there is an urgent need for the improvement of existing TBEV vaccines and the introduction of new cheap vaccines that would be widely available for humans and could also be used for vaccination of animals to prevent the routes of transmission and reduce the number of reservoirs of virus in the environment. The intensive efforts of many laboratories mainly concentrate on recombinant vaccines such as DNA vaccines or virus-like particles (VLPs) (Růžek et al., 2019; Barrett et al., 2003).

Virus-like particles based on recombinant proteins structurally very similar to the natural virions may provide alternative, specific antigens used for vaccination purposes. Biological carriers in the form of virus-like particles are an innovative approach to the construction of vaccines due to morphological, biophysical and antigenic properties almost identical to those of natural virions as well as the lack of genetic material (Lua et al., 2014). VLPs are spontaneously produced during flavivirus infection or may be produced in various expression systems as an alternative to authentic antigens, eliminating biosafety problems (Russell et al., 1980). As there is no need to work with the virus, VLPs are also much safer to produce than inactivated vaccines. Some VLP-based vaccines against hepatitis B virus and human papilloma virus have been approved by the FDA for use in humans (Lua et al., 2014; Fuenmayor et al., 2017). VLPs based on the prM and E proteins of TBEV are immunogenic and can potentially be used as vaccine antigens (Heinz et al., 1995).

The main eukaryotic platforms for the production of recombinant proteins are mammalian, insect and yeast expression systems. Here, we propose a new TBE vaccine candidate based on virus-like particles produced in unconventional *Leishmania tarentolae* expression system. This system has been previously successfully used for the production of different proteins, especially those that require post-translational modifications, such as glycosylation (Aparecida et al., 2019). For the first time, we showed that an *L. tarentolae* expression system can be successfully used to produce TBEV VLPs with high production efficiency. The system leads to the production of recombinant TBEV VLPs with mammalian-type *N*-glycosylation patterns.

The vaccine was tested in mice, and we demonstrated its safety and effectivity. The produced VLPs elicited good titers of neutralizing antibodies, making them good candidates for a safe alternative human vaccine with low cost and high efficiency of production. Moreover, this potential vaccine candidate may represent a low-cost candidate for veterinary use to protect susceptible animals from symptomatic TBE or to vaccinate small ruminants to prevent milk-borne TBEV infections in humans.

2. Materials and methods

2.1. Plasmids

The construction of the genes used for the production of recombinant proteins is summarized in Fig. 1. Sequences of TBEV structural prM and E proteins (Neudoerfl strain) were separated by the sequence of P2A self-cleavage peptide to provide efficient separation of prM and E proteins. Additionally, a linker of 3 amino acids was added following the P2A sequence to reduce spherical hindrance in the structure of proteins. The construct was obtained by gene synthesis using *L. tarentolae*-adapted codons (GeneArt Thermo Fisher Scientific). Synthesized genes were ligated into SalI and NotI restriction sites in the pLEXY_I-blecherry3 vector (Jena Bioscience).

For the production of antigens used for assessing antibody titers in postimmunization sera in HEK293T cells, plasmids coding for full-length prM-E proteins and E protein without a transmembrane domain were used. The prM-E construct was used to obtain mammalian-derived TBEV VLPs, as these proteins are expressed together in mammalian cells from such particles.



Fig. 1. Schematic illustration of the amino acid sequence of the prMP2AE construct used for the construction of the *L. tarentolae* stable cell line. ssL –

signal sequence LMSPA1 phosphatase from *L. mexicana*, region 1–23 in the amino acid sequence of this protein, (GenBank accession number: CAA87090.1); prM – pre-membrane protein of TBEV, region 114–281 in the amino acid sequence of Neudoerfl strain polyprotein (GenBank accession number: AAA86870.1); GSG – linker sequence; P2A – self-cleavage peptide from swine *Teschovirus-1*, region 979–997 in the amino acid sequence of polyprotein of this virus (GenBank accession number: NP_653143.1); ss – signal sequence for E protein of TBEV from transmembrane domains of the prM protein, region 212–281 in the amino acid sequence of the Neudoerfl strain polyprotein; E – envelope protein of TBEV, region 282–776 in the amino acid sequence of the Neudoerfl strain polyprotein.

2.2. *L. tarentolae* cultivation and protein expression

Recombinant prMP2AE proteins were expressed using *L. tarentolae* cells in the inducible expression system LEXSY according to the guidelines of the manufacturer (Jena Bioscience). Briefly, a plasmid was introduced into cells by electroporation to obtain a stable cell line. Transfected cells were subjected to polyclonal selection by bleomycin (100 µg/mL). Recombinant cell lines were cultured in selective medium with hemin at 26 °C under aerated conditions and protected from light. For recombinant protein expression, cells were induced by adding tetracycline (15 µg/mL) and grown in agitated culture for 72 h.

2.3. SDS-PAGE and western blotting

Analysis of protein expression and purification was conducted using SDS-PAGE. Samples were run in reducing or nonreducing conditions on 10–20% gradient Tris-glycine gels in Tris-glycine SDS running buffer. After electrophoresis, the gel was used for either Coomassie staining or western blotting. Coomassie staining was performed using Imperial™ Protein Stain (Thermo Fisher Scientific). For western blotting, the proteins were transferred onto PVDF membranes using wet overnight transfer in buffer containing 25 mM Tris-Base and 150 mM glycine. After the membrane was blocked with 5% nonfat milk in TBS-T (TBS buffer with 0.1% Tween-20 (v/v)), proteins were detected with a specific monoclonal anti-Flavivirus group antigen antibody (4G2) (Absolute Antibody) (1:2000 dilution), monoclonal anti-TBEV E protein antibody 19/786 kindly provided by Professor Matthias Niedrig (1:1000 dilution) or in-house produced polyclonal rabbit serum anti-prM protein (1:1000 dilution) followed by anti-mouse or anti-rabbit peroxidase HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) (diluted 1:3000). Blots were developed using a Super Signal™ West Pico Plus Substrate system (Thermo Fisher Scientific) using the Chemidoc system Alliance™ Q9-Series (UVITEC).

2.4. Ultracentrifugation in sucrose density gradient

The medium from induced cells was collected and ultracentrifuged through a 20% (w/w) sucrose cushion in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.4) for 3 h at 130,000×g. The supernatant was removed, and the pellet was resuspended overnight in PBS with protease inhibitors. Subsequently, a sample was treated with or without 1% Triton X-100 on ice for 1 h, overlaid on a 20–60% (w/w) sucrose gradient in TNE buffer and ultracentrifuged for 16 h at 135,000×g. A total of 7 fractions were collected and analyzed by western blotting and Coomassie staining as described above.

2.5. Analysis of N-glycosylation

N-glycosylation was analyzed with PNGase F (Thermo Fisher Scientific). The sample of purified VLPs was divided into two equal portions. Samples were incubated in denaturing conditions, and one sample was treated with PNGase F for 16 h at 37 °C, while a second one was an undigested control also incubated for 16 h at 37 °C. After digestion, the samples were analyzed by mobility shift assays with western blotting as described above.

2.6. ELISAs for VLP characterization

ELISA plates were coated overnight at 4 °C with purified VLPs at 5 µg/ml in PBS buffer at pH 7.4. Then, the plate was blocked with 3% BSA (w/v) in PBS-T (PBS buffer with 0.05% Tween-20 (v/v)) for 2 h at RT. Three different primary antibodies were used: mouse monoclonal anti-Flavivirus group antigen antibody (4G2) (Absolute Antibody), mouse monoclonal neutralizing antibody 19/1786 and polyclonal rabbit serum anti-prM protein in dilutions from 1:100 to 1:500,000. The antibodies were diluted in 0.3% BSA w PBS-T, and the plate was incubated for 1 h at RT. Primary antibodies were detected with anti-mouse or anti-rabbit peroxidase HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted 1:1500 in 0.3% BSA in PBS-T. The reaction was visualized with TMB Substrate Solution (Thermo Fisher Scientific). After the reaction was stopped with 0.5 M H₂SO₄, the signal intensity was measured at 450 nm with a plate reader (Tecan).

2.7. Electron microscopy and immunogold labeling

For visualization of particles, fractions from density gradient ultracentrifugation were diluted 1:10 in PBS and deposited on carbon-coated 200 mesh nickel grids. Negative staining was performed with 2% uranyl acetate. For immunogold labeling, grid-deposited particles were blocked with Blocking Solution for Goat Gold Conjugates (Aurion). Grids were washed three times with incubation buffer (PBS buffer with 0.1% BSA-c (Aurion)) and incubated with primary 4G2 or 19/1786 antibodies diluted 1:40 in incubation buffer for 1 h at RT. Following six washes with incubation buffer, labeling was performed with goat anti-mouse IgG conjugated with 6 nm gold particles (Aurion) diluted 1:40 in incubation buffer for 1 h at RT, washed again and fixed with 4% paraformaldehyde. After washing, the grids were stained with 2% uranyl acetate. Samples were analyzed using a transmission electron microscope Tecnai G2 Spirit BioTWIN (FEI) (Faculty of Biology, University of Gdansk, Gdansk, Poland).

2.8. Nanoparticle tracking analysis

Size distribution and concentration analyses were carried out using an NS300 NanoSight NTA (Malvern Panalytical). Samples were prepared by dilution with sterile PBS buffer to reach a concentration of 0.1 mg/ml and were measured with five 60 s tracking repetitions. Data were analyzed using NTA 3.4 Software (Malvern Panalytical).

2.9. Immunization protocol

Groups of 6 female BALB/c mice, 6–8 weeks of age, were immunized subcutaneously with a mixture of antigen and adjuvant. Mice were immunized with 10 µg of antigen in sterile PBS buffer on Days 0, 14 and 28. The total protein content in the VLP antigen for immunization was quantified using a Quick Start™ Bradford Protein Assay (Bio-Rad). AddaVax (InvivoGen) was used as an adjuvant. Antigen was mixed with AddaVax in a 1:1 (v/v) ratio directly before the injection. For the first dose, animals were immunized with 200 µl of antigen-adjuvant mixture administered in two places of injection, 100 µl for each place of injection (10 µg of protein in 100 µl of PBS + 100 µl of AddaVax divided into two portions of 100 µl). For the second and third doses, the volume of antigen-adjuvant mixture was 100 µl administered in one place of

injection (10 µg of protein in 50 µl of PBS + 50 µl of AddaVax). The mice used as a negative control were immunized with adjuvant and PBS buffer only. On Day 42, the mice were sacrificed, and the sera were collected for immunological response analysis. All experiments on animals were conducted by an accredited company (Tri-City Academic Laboratory Animal Centre, Medical University of Gdansk, Gdansk, Poland) in accordance with the current guidelines for animal experimentation. The protocols were approved by the Local Committee on the Ethics of Animal Experiments of the University of Science and Technology in Bydgoszcz (Permit Number: 17/2020). All surgeries were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

2.10. Preparation of antigens for mouse sera titration

HEK293T cells were transfected with plasmids coding for full-length prM-E proteins or E protein and cultivated for 72 h. Next, the cells and medium were collected for analysis and protein or VLP purification. E protein was purified from the cell lysate on Ni-NTA resin. Cells were lysed in buffer containing 300 mM NaCl, 0.5% Triton X-100, 5% glycerol, and 10 mM imidazole, pH 8, and sonicated. The lysate was purified on HisPur Ni-NTA Spin Columns (Thermo Fisher Scientific) according to the manufacturer's instructions. Culturing medium from cells transfected with prM-E protein was used for VLP purification. They were purified by ultracentrifugation as described above.

2.11. Analysis of mouse serum antibody titers by ELISAs

Collected mouse sera were divided into two groups. The antibody response against TBEV was measured using 10 µg/ml mammalian cell-derived TBEV VLPs and 15 µg/ml mammalian cell-derived TBEV E protein. The antibody response for both antigens was measured with ELISAs. After overnight coating, the plates were blocked for 2 h with 3% BSA (w/v) in PBS-T. Serially diluted mouse sera were added to the plate and incubated for 2 h. The binding of antibodies from sera to recombinant proteins was detected with secondary goat anti-mouse HRP-conjugated antibodies (Santa Cruz Biotechnology) (dilution 1:1500) and TMB Substrate Solution (Thermo Fisher Scientific). After the reaction was stopped with 0.5 M H₂SO₄, the signal intensity was measured at 450 nm with a plate reader (Tecan).

The titers of anti-TBEV antibodies were also analyzed by a commercial IMMUNOZYM FSME IgG All-Species kit (Progen GmbH). In this test, IgG antibodies in the sera of immunized mice were quantified according to the manufacturer's guidelines. This test allowed the determination of specific IgG antibodies against TBEV in Vienna Units (VIEU/ml) based on a standard curve and reaction with inactivated virus.

2.12. Viruses

For the virus neutralization assay and challenge experiment, we used the TBEV strain Hypr (Czech prototype strain originally isolated in Czechoslovakia in 1953 from the blood of a 10-year-old child infected with TBEV) passaged five times in the brains of suckling mice and once in porcine stable kidney (PS) cells before its use in the present study. The virus was provided by the Collection of Arboviruses, Biology Centre of the Czech Academy of Sciences (<https://arboviruscollection.bcco.cz>).

2.13. Virus neutralization assay

Sera were inactivated by heat (56 °C for 30 min) and diluted 1:4 in Leibowitz L-15 medium (Sigma-Aldrich) with 3% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% glutamine (Sigma-Aldrich). Subsequently, 2-fold serial dilutions of the samples in L-15 medium (50 µL/well) were incubated with 10³ PFU/mL of TBEV strain Hypr (50 µL/well) in 96-well plates for 90 min at 37 °C. The virus dose was adjusted to produce a near confluent cytopathic effect with

90–95% cytolysis. Porcine kidney stable cells (PS) were then added (3 × 10⁴ cells in 100 µL per well). After 5 days of incubation, the cytopathic effect was examined using an inverted microscope (Olympus). The highest serum dilution that inhibited the cytopathic effect of the virus was considered the endpoint titer. Samples with a titer of 1:20 and higher were considered positive for the presence of anti-TBEV neutralization antibodies. The data represent the mean values from two independent experiments performed in duplicate.

2.14. Challenge experiment

Ten female BALB/c mice, 6 weeks of age (Envigo), were immunized according to the immunization protocol described above with a mixture of antigen and adjuvant. The other ten mice injected with adjuvant only served as a control group. For evaluation of the protective effect of vaccination, all immunized and control mice were infected intraperitoneally with TBEV (10³ PFU per mouse, strain Hypr) 18 days after the third dose injection. The morbidity and survival of the infected mice were evaluated daily during a four-week experimental period. Mice were euthanized when severe signs of TBE neuroinfection occurred. The challenge experiment was performed in accordance with Czech law and guidelines for the use of laboratory animals. The protocol was approved by the Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Ministry of Agriculture of the Czech Republic and the Committee on the Ethics of Animal Experimentation at the Veterinary Research Institute (Approval No. 26674/2020-MZE-18134).

2.15. Statistical analysis and graphic design

Statistical analyses were performed using the GraphPad Prism 9.3.1 software.

The graphic design was performed with BioRender.

3. Results

3.1. Expression and characterization of *Leishmania*-derived TBEV VLPs

TBEV prM and E proteins were previously shown to form virus-like particles when expressed together in eukaryotic cells (Allison et al., 1995; Schlich et al., 1996). In the present study, the prMP2AE construct (Fig. 1) based on both proteins was used to produce TBEV VLPs in the *L. tarentolae* expression system. Sequences of the prM and E genes were cloned into the pLEXSY_I-blecherry3 vector. The original signal sequence for the prM protein was replaced with the one from the pLEXSY_I-blecherry3 vector—a signal peptide for LMSAP1 phosphatase from *L. mexicana*, which is naturally secreted from cells. This substitution was made for higher production of protein secreted into the culture medium and to provide proper post-translational processing (Wiese et al., 1995). The sequences of the prM and E protein genes were separated by P2A peptide sequence. The P2A peptide from porcine teschovirus-1 was added to facilitate the separation of proteins and further VLP formation (Fig. S1). The genetic sequence of the P2A peptide was introduced after the prM protein gene, followed by the signal sequence (ss) (second transmembrane domain of prM protein) and the E protein gene. The P2A sequence is preceded by a short, 3 amino acid linker to avoid spherical hindrance (Kim et al., 2011). The genetic sequence of the whole prMP2AE construct was codon-optimized for the *L. tarentolae* expression system.

The expression of recombinant proteins was carried out in cell cultures of recombinant protozoa using an inducible stable cell line of *L. tarentolae* (Kushnir et al., 2005). The production was performed for 72 h after tetracycline induction. Protein expression in the cell extract and culture medium was confirmed by immunoblotting with specific antibodies (Fig. 2). Both prM/M and E proteins were detected in cell extracts at high levels. These proteins were also secreted in substantial amounts

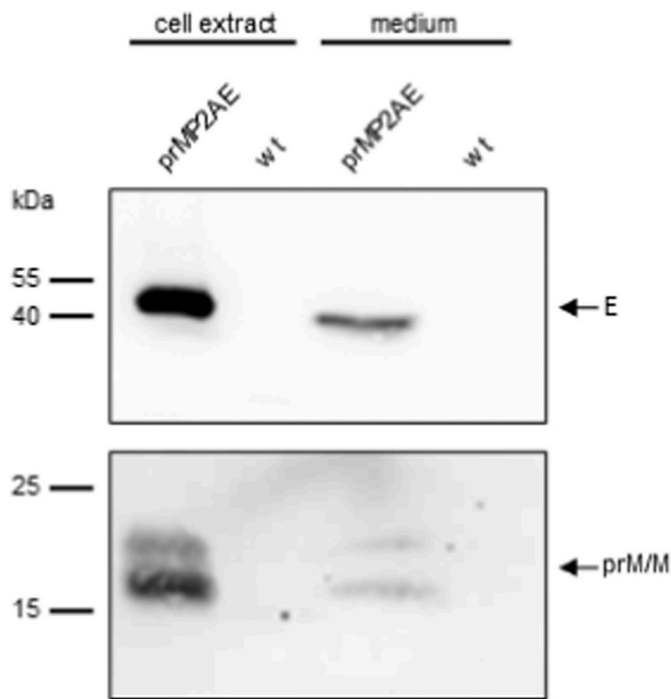


Fig. 2. Analysis of the production of prM and E proteins by *L. tarentolae*. Western blot analysis of prM and E protein production in cell extract and medium with 4G2 anti-E mAbs (top) and L24 anti-prM serum (bottom) under nonreducing conditions. Cell extract and medium from wild-type *L. tarentolae* (wt) were used as a negative control.

into the culture medium, which was chosen for further analyses. The molecular mass of the E protein was determined to be approximately 50 kDa. Two forms of prM/M were detected at approximately 17–20 kDa. The uncleaved prM protein has a theoretical mass of ~26 kDa, a pr fragment of ~17 kDa and a mature M protein of approximately 10 kDa. At least one of the detected forms may correspond to the pr fragment or uncleaved prM protein; in particular, we did not observe any band that could correspond to the M protein. This finding may indicate that the pr fragment is still not fully cleaved from the M protein and that the pr fragment as well as the intact prM protein may be present. As according to prediction using NetPhos-3.1 software (DTU Health Tech) prM protein has 11 sites of high phosphorylation potential, the presence of two bands may also be attributed to phosphorylated and unphosphorylated forms of this protein.

To confirm that recombinant proteins form VLPs in the culture medium, we conducted further analyses. The formation of higher density structures was first confirmed by ultracentrifugation of VLPs from the culture medium in a sucrose density gradient. Seven fractions were harvested and analyzed by immunoblotting. Both proteins were detected in fractions with approximately 36–44% sucrose concentration (Fig. 3a). According to Schalich et al. (1996), the buoyant density of TBEV VLPs is approximately 1.14 g/cm³, which is in agreement with our results, as a sucrose density of 36% (w/w) corresponds to approximately 1.15 g/cm³. Furthermore, we analyzed the detergent sensitivity of the obtained VLPs. VLPs were treated with the strong nonionic detergent Triton X-100 and again ultracentrifuged in a sucrose density gradient (Fig. 3b). After treatment, the majority of both proteins were detected in fractions with a lower sucrose density and/or the proteins did not efficiently enter the gradient. As Triton breaks down higher protein and membrane structures, the results may indicate that some complex, enveloped particles are being formed. Coomassie staining of the collected fractions showed that ultracentrifugation allowed VLP purification (Fig. 3c). The fractions with the highest concentrations of VLPs were combined, and the protein concentration was determined by the

Bradford method. The efficiency of VLP production was approximately 7–10 mg per 1 L of *Leishmania* culture.

To finally confirm VLP formation, we performed transmission electron microscopy analysis. The analyzed samples contained spherical particles with a diameter of approximately 50–60 nm (Fig. 3d). Additionally, the quality of the obtained particles was verified with immunogold labeling. Two specific monoclonal antibodies against the E protein, a 4G2 antibody against the fusion loop epitope and a 19/1786 neutralizing antibody that binds to the conformational epitope between the DI-DIII domains of the E protein, were used (Füzik et al., 2018). Both antibodies reacted with VLPs, suggesting that these epitopes are properly exposed on the surface of the produced particles.

Moreover, nanoparticle tracking analysis (NTA) was performed to assess the size distribution and concentration of purified VLPs. The analysis showed that the population of particles was homogenous in size (Fig. 3e). The mean hydrodynamic diameter was calculated to be 159.5 ± 2.0 nm. Since the same analysis performed on purified medium from wild-type *L. tarentolae* showed only the presence of much smaller particles (Fig. S2), this suggests that analyzed particles are proper VLPs particles.

The estimated number of VLPs purified from 1 L of culture was calculated to be 9.1×10^{10} . NTA also allowed the stability assessment of VLPs. Analysis was carried out on two samples, one freshly purified and one stored at 4 °C for 18 months after purification. There was only a slight change in particle distribution between the samples, which may suggest that the VLPs can be successfully stored for long periods of time. This was also confirmed by ELISA, western blotting and Coomassie staining, which did not show differences between the freshly purified sample and the one stored at 4 °C for 18 months after purification (Fig. S3).

Purified VLPs were subjected to further functional analyses. The antigenic properties of VLPs were assessed by ELISAs using the same antibodies as in immunogold labeling: the specific monoclonal antibodies 4G2 and 19/1786 as well as anti-prM polyclonal serum (Fig. 4a). ELISAs clearly indicated that the produced VLPs are specifically and strongly recognized by the 19/1786 antibody. As the 19/1786 antibody is a neutralizing antibody, the strong binding with VLPs may suggest the proper conformation of the E glycoprotein. The detection with anti-prM serum was not as efficient in the ELISA test. Strong recognition by this serum in a previous Western blot assay may suggest that the produced and purified VLPs may be a combination of mature or only partially mature particles. The produced VLPs were also weakly recognized by the 4G2 antibody; thus, we believe that the fusion loop is covered by the prM protein or hidden in the produced VLPs.

Furthermore, N-glycosylation of prM/M and E proteins present on VLPs was analyzed by treatment with endoglycosidase PNGase F (Fig. 4b). Both the E and prM (pr fragment) proteins possess one N-glycosylation site. In both cases, a shift in molecular mass was observed by western blots after enzyme treatment, which proved that both proteins are glycosylated. Moreover, both forms of the prM/M protein were affected by PNGase F treatment, which confirmed that the detected prM/M proteins were in the form of pr fragments or uncleaved prM proteins. However, taken together, the efficient expression and the data from functional analysis suggest that VLPs may have high potential as good immunogens. Therefore, VLPs purified from the cell culture medium were used for immunization studies in an animal model.

3.2. Immunogenicity of *Leishmania*-derived TBEV VLPs

For determination of the immunogenicity of TBEV VLPs, a group of BALB/c mice were immunized subcutaneously with 3 doses of 10 µg of VLPs in combination with an adjuvant on Days 0, 14 and 28. AddaVax, a squalene-based oil-in-water nanoemulsion, was used as the adjuvant to improve the immunogenic response. AddaVax is an analog of the MF59 adjuvant licensed for human use in Europe. Blood samples were taken before each immunization and 14 days after the last vaccination (on Day

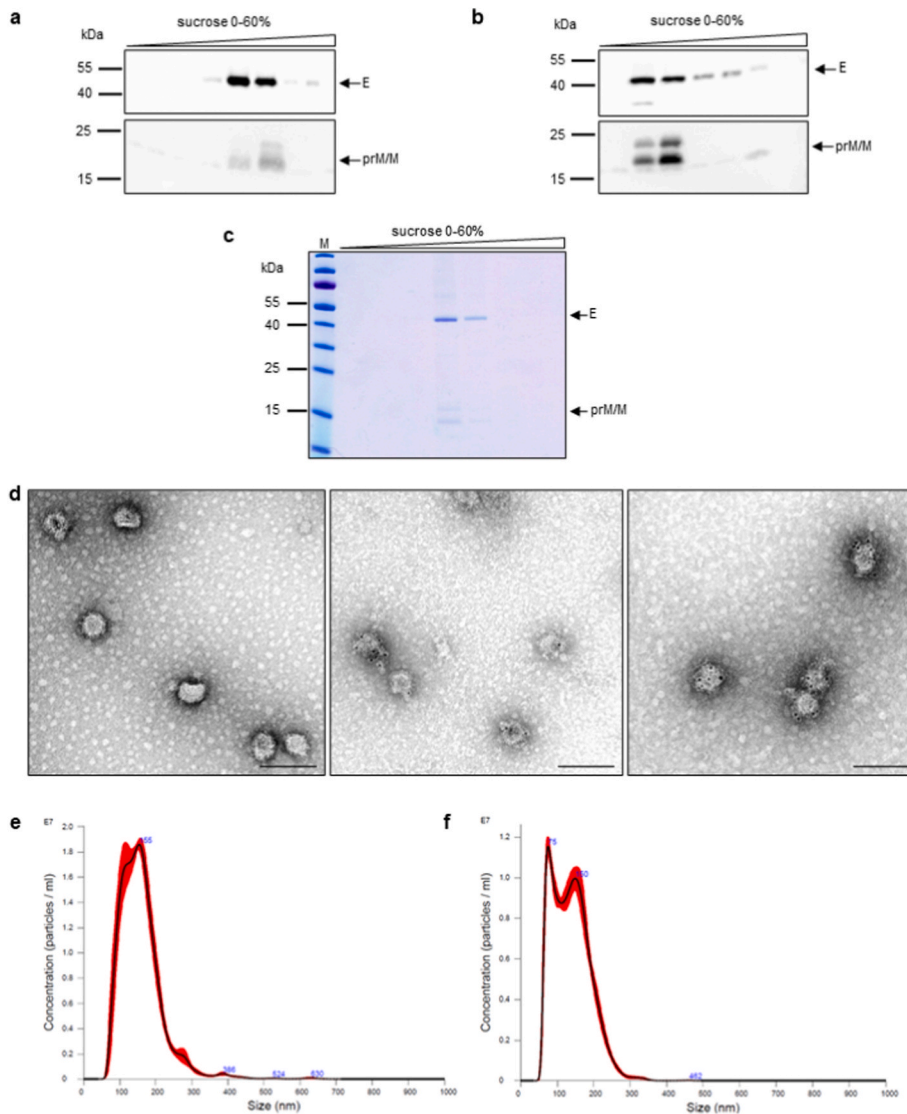


Fig. 3. Analysis of virus-like particle formation. **a** Western blot analysis of fractions collected after ultracentrifugation in a sucrose density gradient (0–60% sucrose/TNE) with 4G2 anti-E Abs and L24 anti-prM serum under nonreducing conditions. **b** Western blot analysis of fractions collected after ultracentrifugation in a sucrose density gradient (0–60% sucrose/TNE) with 4G2 anti-E Abs (*top*) and L24 anti-prM serum (*bottom*) under nonreducing conditions. Prior to ultracentrifugation, the sample was treated with Triton X-100. **c** Coomassie staining of fractions collected after ultracentrifugation in a sucrose density gradient (0–60% sucrose/TNE) of samples not treated with Triton X-100 under reducing conditions. M – molecular marker. **d** Transmission electron micrographs of virus-like particles. After ultracentrifugation in a sucrose density gradient, the particles were negatively contrasted with 2% uranyl acetate and analyzed with a transmission electron microscope (*left*). For confirmation of the specificity of the observed particles, immunogold labeling with 4G2 (*middle*) and 19/1786 (*right*) anti-E mAbs and secondary Abs conjugated with 6 nm gold particles was performed. Scale bar 100 nm **e** Analysis of the size distribution and quantification of freshly purified VLPs (*left*) and VLPs after 18 months of storage at 4 °C. Histograms show the average size distribution of the measured particles. Numbers in blue indicate the size of particles from each peak, and the red surface corresponds to the standard deviation values. Numbers on the y-axis are the results from samples with a concentration of 0.1 mg/ml VLPs.

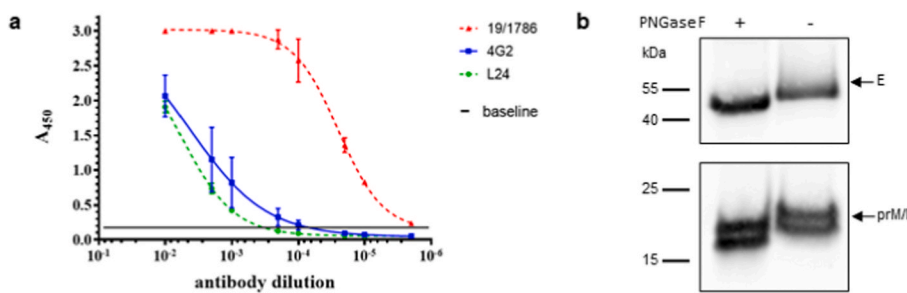


Fig. 4. Functional analysis of purified VLPs. **a** Recognition of particles with specific antibodies. The 4G2 and 19/1786 anti-E mAbs and L24 anti-prM serum were used. The plate was coated with 5 µg/mL VLP protein. For each antibody, the mean from two independent experiments performed in triplicate is shown. Error bars indicate standard deviations. **b** Western blot analysis of prM and E protein mobility shifts after PNGase F treatment under reducing and denaturing conditions. The 19/1786 anti-E mAbs (*top*) and L24 anti-prM serum (*bottom*) were used.

42). Mice in the control group were immunized with the same schedule only with PBS buffer in the presence of AddaVax adjuvant. Animals did not show any side effects during vaccination. Sera were pooled, and the humoral response elicited by immunization was analyzed by determining specific antibody titers using an ELISA test (Fig. 5). VLPs (Fig. 5a) and E protein (Fig. 5b) produced in mammalian cells were used as antigens for titration. The obtained results confirmed that full immunization with the produced VLPs resulted in high antibody titers reaching 1×10^5 . Analyses of antibody levels after each immunization showed that the titer in the experimental group began to grow after the

second immunization, while the level of antibodies in sera from the control group did not show significant changes. Similar levels of antibodies for these two antigens may suggest that most of them are directed against the E protein. The commercially available test based on inactivated virus allowed estimation of antibody levels in the sera of vaccinated mice in Vienna Units (Fig. 5c). In the experimental group, the specific antibody concentration was approximately 55 VIEU/mL, while in the control group, it was equal to approximately 5 VIEU/mL. The level of antibodies after final immunization was significantly higher than in the control animals in all tests. Subsequently, the neutralizing potential

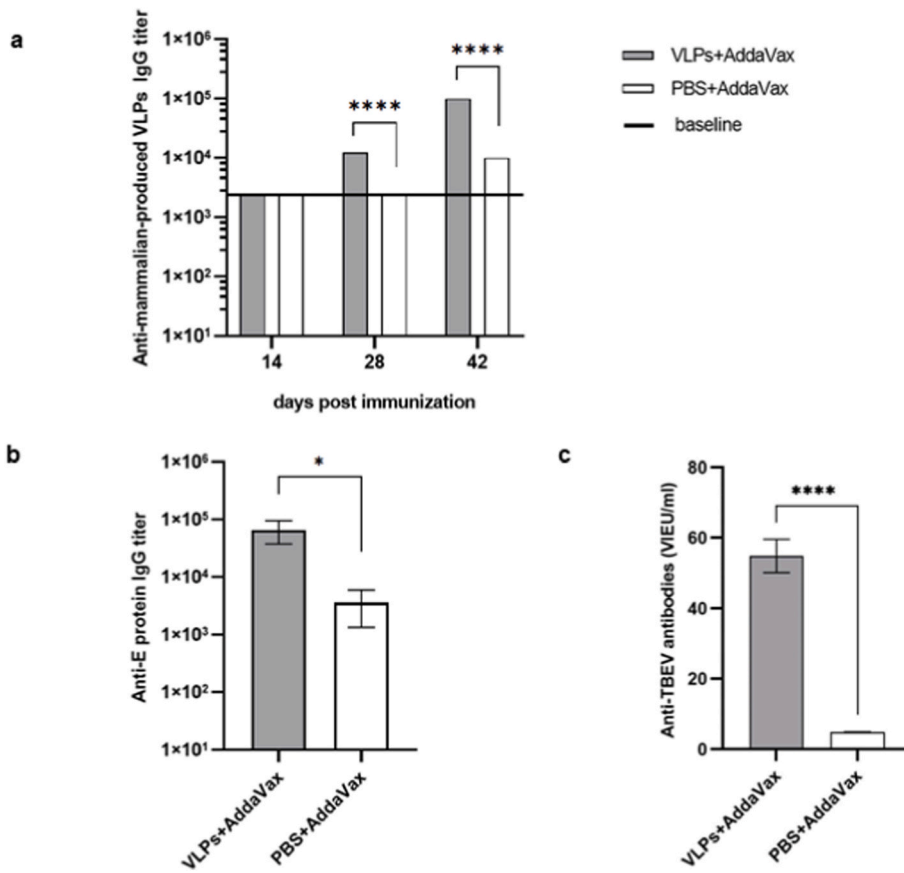


Fig. 5. Analysis of the humoral response after immunization with *Leishmania*-derived VLPs in BALB/c mice. VLPs + AddaVax refers to a group immunized with antigen, and PBS + AddaVax is a control group vaccinated only with adjuvant. **a** The results show antibody titers in mouse sera before every immunization and 14 days after the last immunization. Mammalian-derived VLPs (10 µg/mL) were used as an antigen. Baseline was established as the serum antibody level prior to the vaccination. P values were calculated using the multiple *t*-test (****, $P < 0.05$). **b** Titers of anti-E antibodies in sera of immunized mice 14 days after the last immunization. The plate was coated with 15 µg/mL purified E protein. The P value was calculated using the unpaired *t*-test (*, $P < 0.05$). Antibody titers were calculated as the highest serum dilution for which absorbance value was higher than the mean background value plus two standard deviations (a, b). **c** The concentration of anti-TBEV antibodies in mouse sera 14 days after the last immunization based on the standard curve. The P value was calculated using the unpaired *t*-test (****, $P < 0.0001$). The data represent the values from three independent experiments performed in duplicate, and error bars indicate standard deviations.

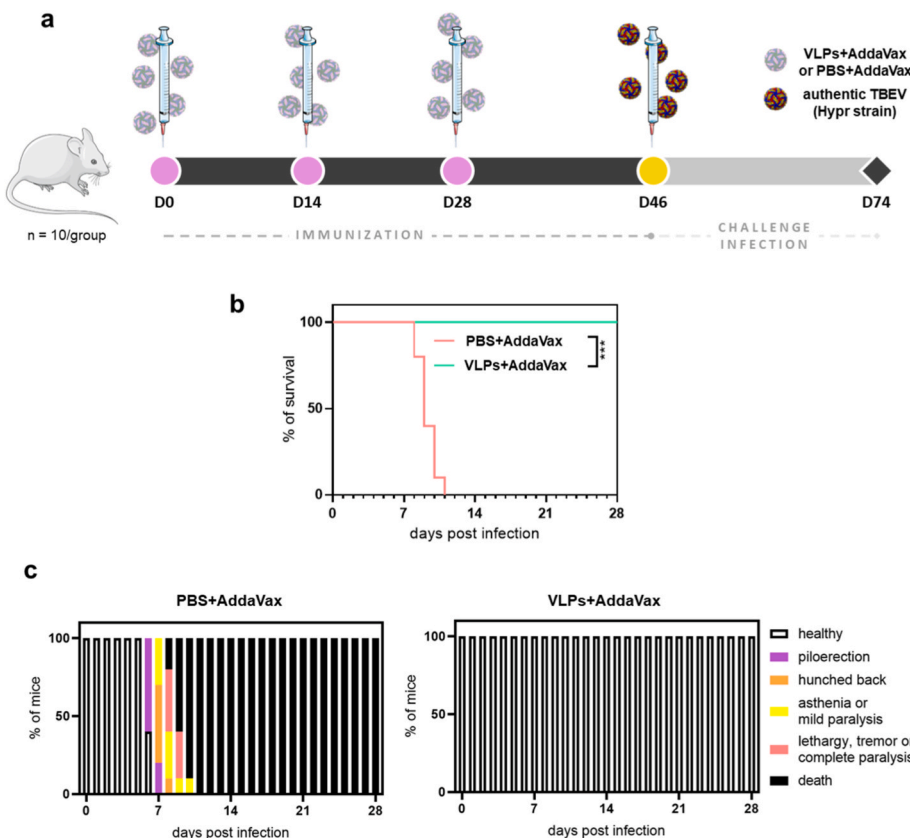


Fig. 6. Efficacy of vaccine candidate in challenge experiments. **a** Experimental protocol. Mice were immunized with three doses of the vaccine candidate (VLPs + AddaVax) two weeks apart. A control group was injected with the adjuvant only (PBS + AddaVax). Eighteen days after injection of the third dose, the mice were challenged with authentic TBEV. Morbidity and survival were assessed during a four-week experimental period. (Figure created with Servier Medical Art, available at www.servier.com). **b** Kaplan-Meier survival curve. The P value was calculated using the Mantel-Cox test (***, $P < 0.001$). **c** Histograms show disease progression in the control group receiving adjuvant alone (left) and in the group immunized with the vaccine candidate (right).

of postimmunization sera was analyzed. The experiment was conducted with the TBEV Hypr strain. The sera from the experimental group were able to neutralize the virus up to a dilution of 1:160. The serum from the control group did not show any neutralizing potential. Finally, a challenge experiment with a lethal dose of TBEV was performed to verify whether immunization with the prepared vaccine would protect animals from the development of TBE (Fig. 6a). All vaccinated and infected mice survived until the end of the experiment at 28 days post-infection (Fig. 6b) and did not show any symptoms of TBE (Fig. 6c). In contrast, mice from the control group started to develop symptoms on the sixth day post-infection and had to be euthanized by the eleventh day after infection. Therefore, we can conclude that VLPs produced in *L. tarentolae* were highly immunogenic, causing effective production of neutralizing antibodies and providing protection against a lethal dose of TBEV, as confirmed in the challenge experiment.

4. Discussion

Despite available vaccines, TBEV is still a major concern in many European and Asian countries (Bogovic, 2015). This report is the first to evaluate the production of TBEV VLPs in the *L. tarentolae* expression system, which provides post-translational processing, including glycosylation processes, very similar to that of mammalian cells. Only a few viral antigens have been previously successfully produced in the *L. tarentolae* system (Breton et al., 2007; Baechlein et al., 2013; Pion et al., 2014; Grzyb et al., 2016; Czarnota et al., 2016; Fischer et al., 2016). *L. tarentolae* cultures can be easily scaled up; therefore, they are good candidates for industrial scale production. The use of this expression system could also lead to significantly lower vaccine production costs than are currently incurred for inactivated vaccines. Production costs in the *L. tarentolae* system are lower than in mammalian cells, due to lower costs of the culture medium as well as lower requirements for cell cultures. The purification procedure can also be carried out more easily and at a lower cost because the culturing medium for *L. tarentolae* has fewer components than the medium for mammalian cells. All these, in turn, could directly translate into greater availability of the TBEV vaccine. Additionally, it may lead to the development of a cheap veterinary vaccine, as immunization of animals can be a way to reduce viral reservoirs and reduce transmission to humans (Salát and Růžek, 2020). In our study, the introduction of additional elements (e.g., the signal sequence from *L. mexicana* and P2A peptide) in addition to the sequences of the TBEV structural proteins made it possible to obtain a very high yield of recombinant particles. Although some studies have shown that the addition of the *L. mexicana* signal peptide can impair the production of some recombinant proteins (Breton et al., 2007; Pion et al., 2014), our results confirmed the data obtained by Wiese et al., 1995) and Grzyb et al., 2016 (Wiese et al., 1995; Grzyb et al., 2016), which indicated that proteins fused with this signal sequence are efficiently produced and successfully secreted into the culture medium. Codons were optimized to obtain the highest possible production efficiency (Breton et al., 2007). Moreover, to our knowledge, the P2A peptide was successfully used for the first time to facilitate the separation of the TBEV prM and E proteins. All these factors allowed not only a high expression efficiency but also a very high secretion of particles into the culture medium, which translates into the ease of their purification using a one-step purification process (Figs. 2 and 3a, c). VLP formation was further confirmed by electron microscopy analysis using immunogold labeling with two different antibodies recognizing the E protein (Fig. 3d). A high level of recognition of particles by neutralizing 19/1786 antibody suggests that the exposition of important epitopes is correct and therefore can induce a strong immunological response (Füzik et al., 2018).

L. tarentolae has been shown to be the first single-cell organism able to produce biantennary *N*-glycans similar to those in higher eukaryotic organisms, lacking only sialylation (Breitling et al., 2002). Both the TBEV prM and E proteins have one *N*-glycosylation site. The role of these

glycans is still not fully understood, but it has been shown that the removal of *N*-glycans from the E protein reduces the infectivity of viral particles (Yoshii et al., 2013). Therefore, it is important that the glycosylation pattern is also maintained in the vaccine candidate antigen so that the immune response induced by the vaccine provides the highest possible level of protection from the native virus. It has also been shown that glycosylation may be important for TBEV VLP secretion (Goto et al., 2005). In this study, the glycosylation profile of proteins composing VLPs was also examined. We confirmed that both the prM and E proteins were fully glycosylated, as shown by PNGase F treatment (Fig. 4b).

The strong immunogenicity potential of *Leishmania*-derived TBEV VLPs was confirmed by immunization of mice. Our study showed that immunization with VLPs results in high antibody titers measured by ELISAs with heterologous-derived antigens from mammalian cells (Fig. 5), and the sera from immunized animals had strong neutralizing properties against the virus. Moreover, immunization with VLPs protected mice from developing any TBE symptoms in experimental infection with a lethal dose of TBEV (Fig. 6). Without a doubt, the high safety profile and strong immunogenic potential of the vaccine antigen characterized in this study call for further investigation of these promising observations.

To our knowledge, this is the first study undertaken to prove that the production of flaviviral VLPs is possible in a system based on the protozoa *L. tarentolae*. We have also shown that the particles produced in this system have strong immunogenic properties and are a good candidate for a cost-efficient and highly effective TBEV vaccine. Further studies, such as the analysis of protection from other virus subtypes and safety studies, need to be conducted to confirm the high potential of this vaccine antigen. However, based on the present study, we believe that the VLP particles described in this report may be good candidates for the production of TBEV vaccines on an industrial scale.

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Author contribution

Conceptualization: M.Z., D.R. and E.K.; Data curation: M.Z., G.B., J. S., P.S. and K.B.; Data analysis and interpretation: M.Z., J.S., P.S., D.R. and E.K.; Funding acquisition: D.R., M.Z. and E.K.; Investigation and assays: M.Z., G.B., J.S., P.S., and K.B.; Methodology: M.Z., P.S. and D.R.; Project administration: E.K.; Supervision: D.R. and E.K.; Visualization: M.Z., J.S. and P.S.; Writing – original draft: M.Z. and E.K.; Writing – review & editing: M.Z., B.S., D.R. and E.K. All authors have read the manuscript drafts and approved the submitted version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2022.105511>.

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