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DEVELOPMENT OF RECOMBINANT ANTIGEN-BASED ELISA FOR THE DETECTION OF ANTI-TULAREMIA ANTIBODIES IN SWINE AND HUMAN SERA: A PILOT STUDY

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To the present day, a *Francisella* infection is diagnosed by a mandatory combination of two methods: a screening Enzyme-linked Immunosorbent Assay (ELISA) and a confirmatory Western Blot (WB), both based on the use of *Francisella tularensis* subsp. *holarctica* lipopolysaccharides (LPS) as a capture antigen. The purpose of the present work was to obtain and assess three recombinant proteins (FTT1696, FTT0077, FTT0975) as antigens in an indirect ELISA (iELISA) with the final goal to replace the confirmatory WB. Cloning strategy in vector pASG103, expression in *E. coli* and purification of proteins using Strep-system are described in detail in this report. Sera with confirmed antibody titers against *F. tularensis* reacted with all three antigens, which make them suitable for the serological detection of *F. tularensis* in swine and humans.

Key words: ELISA, FTT1696, FTT0077, FTT0975, recombinant proteins, tularemia.

Tularemia is a zoonotic hazardous disease, caused by gram negative, bacterial pathogen *Francisella tularensis,* which shows a facultative intracellular replication [1]. Often, the natural outbreaks of tularemia are connected with dense populations of rodents and lagomorphs. However, the infection has been reported in a wide variety of other mammals, birds, amphibians and arthropods [2, 3]. Hoof animals (including wild boar (*Sus scrofa*) and domestic swine) can also act as asymptomatic carriers of tularemia infection [4], while their role in the epidemiology of the disease is poorly studied. However, some studies from Germany and Czech Republic show the importance of tularemia seroprevalence study in hoof animals, as they do not succumb to the infection and therefore, antibodies can be persist for a longer period of time [4, 5, 6]. Tularemia does not spread from human to human, while the risk of human infection with *F. tularensis* is extremely high after contact with ill animals or investigation of contaminated water, meal, etc., as the infective dose of *F. tularensis* is around 10 bacteria by inhalation [2, 3].

The first official cases of tularemia in Ukraine were reported in the 1940s [8, 9]. These cases were infections in workers in fur and sugar factories. In 1948–1949, deaths to tularemia were reported in all regions of the country. In 1960, the tularemia incidence declined sharply because of the natural reservoires' identification and implementation of special measures by health authorities. Additionally, vaccination of people living in endemic areas was provided and a reduction of root vole populations had been achieved [7, 8]. In the late 1980s and 1990s, new outbreaks were recorded. These outbreaks were accompanied by massive infection of people from the steppe regions of Ukraine —

Odesa and Mykolaiv. Furthermore, a constant activity of tularemia natural foci was registered on Biryuchy Island [9]. According to the official data of the Public Health Center and the Regional Laboratory Centers of the Ministry of Health of Ukraine, 3,086 positive cases of *F. tularensis* were registered from 1941–2008 collected at 1,084 locations from all regions of Ukraine [8]. However, since then, tularemia outbreaks among people usually occurred in the form of several individual cases per year [8]. In March 2019, the most recent case of human tularemia infection in Ukraine was registered in the Okhtyrka region of Sumy oblast [17].

At present, there is a lack of methodologies and commercial tools for tularemia serological surveys in Ukrine. The availability of such test systems is essential for ongoing epidemiological surveillance of natural foci and rapid diagnosis of disease in humans and animals.

The golden standard for the serological studies on the presence of antibodies against *F. tularensis* in blood serum is the usage of lipopolysaccharides of the pathogen as antigen [2, 11]. To obtain such an antigen, it is necessary to maintain a live *F. tularensis* strain in the laboratory, which significantly increases safety procedures in the laboratory. Both aspects could be overcome by the development of a diagnostic method based on recombinant antigens. Recombinant technologies allow to achieve big amounts of target proteins fast and in a safe way. Another important aspect that has to be considered is that *F. tularensis* belongs to a group of intracellular replicating bacteria, which includes mycobacteria, *Listeria, Legionella, Brucella, Coxiella* and *Rickettsia* and some *E. coli* strains [12]. These bacteria have similar LPS composition, thus, the ELISA method based on *F. tularensis* LPS can lead to false-positive results caused by cross-reactions especially with antibodies against *Brucella* spp. and *Yersinia enterocolitica* [13, 14]. To overcome these cross-reactions and the dependence on the cultivation of *F. tularensis* to extract the LPS under certain biosafety aspects the aim was to develop an indirect ELISA based on recombinant proteins [2,15, 16]. Therefore, we expressed recombinant proteins, namely 2-oxoglutarate dehydrogenase component E2, succinyltransferase dihydrolipoamide (FTT0077/FTTSucB; 52 kDa), chaperone 60 (FTT1696/FTTGroEL; 57 kDa), and a conserved hypothetical protein FTT0975 (26 kDa). All three proteins have been shown to be immunoreactive [10]. In a preliminary study we could show that these proteins were recognized by sera of patients which had contact to *Francisella tularensis* and therefore, were potential candidates for an indirect enzyme-linked immunosorbent assay.

Materials and Methods

Human and Animal sera

Positive control swine serum was generated in the study [4]. Pre-immunisation sera of these animals served as negative controls. Positive control human serum was a pool of 5 sera samples (of the 2005 tularemia outbreak in Kosovo which were positively checked for *Francisella tularensis* LPS specific antibodies. Human normal serum (Merck Millipore) was used as negative control. All sera were tested in *Francisella tularensis* LPS specific ELISA and Immunoblot before usage [15, 16].

PCR primer design, PCR, and subcloning

PCR primers for the ORFs FTT1696, FTT0077 and FTT0975 in *F. tularensis holarctica* strain LVS were designed with reference to the database, incorporating appropriate restriction sites for cloning in pASG103 and pASG105 (Iba Lifesciences, Germany). All PCRs were performed with AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA). Complete coding sequences were amplified using the following protocol. The final volume of the reaction mixture was 25 µl and comprised of AmpliTaq Gold DNA Polymerase 1.25 U/50 µl, PE-Buffer 1X, dNTP mix 0.2 mM, MgCl2 1.5 mM, primer F 10 pM, primer R 10 pM, with 5 µl of purified template DNA (*F. tularensis holarctica* LVS). The primer sequences and cycling regimes are shown in Table 1 and Table 2, respectively. Before cloning, the PCR products were run on an 1.5% agarose gel to check whether one or more products have been amplified. If only one band was present with the appropriate size, the PCR product was directly purified using the MiniElute PCR Purification Kit (Qiagen, USA) according to the manufacturer's instructions. If several bands were visible, the band with the appropriate size was cut from the agarose gel and subsequently purified using the MiniElute PCR Purification Kit (Qiagen, USA) according to the manufacturer's instructions as well.

Table 1. **Primer systems used for amplifying the genes of recombinant proteins**

Name of primer	Primer sequence	Amplicon length, bp.
FTT1696 F	AGCGCGTCTCCAATGGCTGCAAAACAAGTTTTATTTTCAGATG	1632
FTT1696 R	AGCGCGTCTCCTCCCCATCATGCCAGGCATACCGC	
FTT0975 F	AGCGCGTCTCCAATGAAAAAGAGTATTATCCCAATTACTTTAATAAGT	687
FTT0975 R	AGCGCGTCTCCTCCCTTTCTCCATAAATGTAACATTTGCT	
FTT0077 F	AGCGCGTCTCCAATGGTTGAATTAAAAGTACCTATGTTCC	1467
FTT0077 R	AGCGCGTCTCCTCCCTACTTGTAGAAGAATTCTATTTGGATCTTC	

Notes. Recognition site for Esp3I are indicated in bold letters.

Table 2. **The thermocycling regimes for FTT1696, FTT0975 and FTT0077 primer systems**

Primer systems		Cycling regimes				Final elongation
		Preheating	Denaturation	Annealing	Elongation	
FTT1696 F/R		98 °C — 30 s	98 °C — 10 s	72 —30 s	72 °C –70 s	72 °C — 4 min
		1 cycle	35 cycles			1 cycle
FTT0975 F/R		98 °C — 30 s	98 °C — 10 s	58 °C — 20 s	72 °C — 30 s.	72 °C — 2 min
		1 cycle	35 cycles			1 cycle
FTT0077 F/R	1	98 °C — 30 s	98 °C — 30 s	56 °C — 15 s	72 °C — 50 s.	—
		1 cycle	10 cycles			—
	2	—	98 °C — 10 s	72 °C — 65 s	–	72 °C — 2 min
		—	20 cycles			1 cycle

Cloning

The target proteins were cloned into vectors suitable for the Strep-Tactin®XT Superflow® chromatographic purification system (IBA, Germany). These vectors decode for a Twin-Strep-tag® affinity tail consisting of the following amino acids: WSHPQFEK-GGGSGGGSGG-SA-WSHPQFEK. PCR products were cloned into the pASG103 and pASG105 expression vector. A restriction and ligation reaction was performed for each protein using restriction enzyme Esp3I and DNA ligase by next protocol: 5 ng of *Strep*-tag pASG103 vector, 25 nM of the obtained amplicon, 2.5 µl of restriction enzyme buffer, 1 µl of DTT/ATP mixture, 1U of T4 DNA ligase and 5U of restriction enzyme were added to the mixture and filled up with deionized water to final volume of 25 µl. The mixture was incubated in a solid state thermostat at 30 °C for 1 hour.

Transformation

Resulting vector constructs were used to transform competent *Escherichia coli* TOP10 cells (Invitrogen). The transformation was performed using 50 µl of *E. coli* competent cells and 10 µl of the former described reaction mixture. The mixture was cooled for 30 min on ice, followed by an incubation step of 30 s at 42 °C in a water bath and cooled again on ice for 5 min. After the heat shock, cells were filled with 500 µl SOC liquid medium and incubated for 1 h at 37 °C and 200 rpm to allow recovery and expression of the antibiotic resistance marker. Subsequently transformed cells were plated on selective Luria-Bertani (LB) medium containing 100 mg/l of carbenicillin and 50 mg/l of X-Gal for blue-white screening and incubated at 37 °C overnight. Potential positive transformants were screened by colony PCR to determine the presence of inserted

DNA. Positive clones were cultivated in LB medium containing carbenicillin (100 mg/l) at 37 °C overnight. Plasmid purification was performed using a Plasmid Miniprep Kit (Qiagen, USA) according to the manufacturer's instructions verification of the inserted DNA was done by Sanger sequencing (Eurofins, Germany).

Protein induction

Verified plasmids were transformed into expression host *E. coli* Lemo21 (DE3) (NEB, UK). Overnight cultures of transformed *E. coli* Lemo21 (DE3) grown in selective medium were diluted 1:25 in 500 ml fresh LB medium containing carbenicillin and cultured for 3–6 hours at 37 °C to an OD of 0.4–0.6 that was spectrophotometrically measured against LB as blank (Eppendorf BioPhotometer D30, Germany). Expression was induced for 3–4 h with anhydrotetracycline (200 ng/ml). Cells were harvested by centrifuging at 4 500 x g for 10 min, resuspended in 20 ml BufferW (100 mM Tris-HCL, pH 8.0, 150 mM NaCl, 1 mM EDTA) and disrupted by 40 cycles of ultra-sonication (30 s "On", 30 s "Off") for purification of native protein. The suspension was centrifuged at 7 800× g for 5 min and passed through a 0.45 µm filter.

Purification

All solutions were degassed before chromatographic purification on ÄKTA pure (GE healthcare). FTT1696, FTT0077 and FTT0975 were purified as soluble proteins on Strep-Tactin XT columns (IBA Lifesciences, Germany) as described by the manufacturer. In brief, sample was loaded at a rate of 0.2–0.5 ml/min onto a 1 ml Strep Tactin XT column equilibrated with Buffer W. Column was washed at 1 ml/min until absorbance at 280 nm was stable and eluted with 10 column volumes (CV) Buffer BXT (100mM Tris-HCL, pH 8.0, 150 mM NaCl, 50mM biotin) at 0.5 ml/min. The eluted fractions were collected and stored at + 4 °C.

The small batch purification was performed according to the manufacturer's protocol Strep-Tactin®XT Spin column.

Concentration of purified proteins was determined using Qubit™ Protein Assay Kit (Thermo Fisher Scientific, USA). Sample purity was determined by SDS-PAGE analysis and InstantBlue™ Safe Coomassie protein stain. The purified proteins were analysed by Western blotting with a secondary anti-*Strep*-tag antibody according to the manufacturer's protocol of *Strep*-tag detection in Western blots (Iba Lifesciences, Germany).

Immunoblotting

2 µl of each purified protein (FTT0975 — 273 µg/ml; FTT0077 — 274 µg/ml; FTT1696 — 208 µg/ml) was fractionated by SDS-PAGE and transferred to a Novex Nitrocellulose Pre-cut Blotting Membrane ("Invitrogen", USA) using a semi-dry transfer apparatus according to the manufacturer's protocols. After blocking with 5% skimmed milk in PBS (4 mM KH2PO4, pH 7.4, 16 mM Na_2HPO_4, 115 mM NaCl) for 1 h, the membrane was incubated with 1:200 diluted swine serum 5% skimmed milk in PBS-T (PBS with 0,05% Tween 20) with 2% rabbit serum or with 1:500 diluted human serum in 5% skimmed milk PBST with 2% goat serum for 1h on a rocking plate. The membrane was rinsed twice with PBS-T, washed once for 10 min and incubated with a 1:2 000 dilution of Rabbit-anti-Pig IgG Secondary HRP-coupled antibodies (Invitrogen, USA) and 1% rabbit serum for 45 min on a rocking plate. In case of human serum, membrane was incubated with a 1:10 000 dilution of Goat-anti-Human IgG Secondary HRP-coupled antibodies (Invitrogen, USA) and 1% goat serum. The membrane was briefly rinsed twice with PBS-T and finally washed with PBS for 10 min. The membrane was incubated with TMB SeramunBlau precipitate solution (Seramun Diagnostica, Germany) for 10 min in dark. The reaction was stopped by washing the membrane 3 times for 5 min with H_2O. Subsequently the membrane was dried and a picture was taken.

ELISA

Nunc MaxiSorp flat-bottom plates (Thermo Scientific, USA) were coated overnight at +4 °C with serial dilutions of recombinant proteins in carbonate buffer pH 9.5 starting from 1:25 to 1:800. After coating, the plates were blocked with 200 µl 10% skimmed milk (Millipore Corporation, Germany) in PBS at 24 °C for 1 h. After blocking step, the plates were turned upside down to discard the blocking buffer. The serum, serially diluted from 1:50 to 1:400 in dilution buffer (PBS-T with 10% skimmed milk and 1% rabbit serum) was applied into the wells (50 µl per well) and incubated at 24 °C for 2 h. After incubation, the plates were washed 4x with washing buffer, 200 µl per well. 50 µl of secondary antibodies (Rabbit-anti-Pig IgG Secondary HRP-coupled antibodies (Invitrogen, USA) diluted 1:2 000 in dilution buffer) was applied to each well and incubated for 1 hour at 24 °C. Subsequently, the plates were washed 4x with washing buffer, 200 µl per well. For detection, 50 µl

of TMB SeramunBlau slow solution substrate (Seramun Diagnostica, Germany) was added and incubated for 10 min in dark at 24 °C. The reaction stopped by adding 50 µl of 0.25 M H_2SO_4. Absorbance at 450 nm was measured using Multiskan™ FC Microplate Photometer with the reference wavelength of 620 nm, (Thermoscientific).

To determine the right coating concentrations, we performed a chessboard titration. The antigens were diluted 1:25, 1:50, 1:100, 1:200, 1:400 and 1:800 horizontally and the swine sera were diluted 1:50, 1:100, 1:200 and 1:400 times vertically. To find the optimal ELISA assay conditions for detecting low background signal and high positive signal, we compared the results using the ElisaMax and Maxisorp Nuncplates (Thermo Scientific, USA) and using different Tween20 concentration in the washing buffer (0,05%, 0,1% and 0,2%).

Results and Discussion

The protein selection was based on the *F. tularensis* proteomic microarray provided by Nakajima and co-authors (2016). These proteins were checked as antigens in microagglutination test and showed the highest signal over a background. Especially, the most sensitive was FTT1696 protein, with the specificity of 94% [10]. Also, based on the known cross reactivity with bacteria families *Yersiniaceae*, *Brucellaceae* and *Francisellaceae* we performed an additional BLAST analysis of the amino acid sequence to estimate potential cross reactivity. Table 3 shows the BLAST results for all three protein sequences in comparison with the potential homolog proteins from the *Brucellaceae* and *Yersiniaceae* clade, one *Francisella* species and three *F. tularensis* subspecies. For the selected proteins only low or even no similarity was predicted to the homolog proteins from two clades or *F. philomiragia* (Table 3).

Based on this sequence comparison, the protein FTT0975 had no significant similarity with Brucellaceae and Yersiniaceae and only little with *F. philomiragia*.

Cloning

The vectors pASG103 and pASG105 contain a tetracycline promoter for the induction of protein expression, ampicillin resistance gene and a Twin-Strep-tag® sequence decoding either a C-terminal or N-terminal tag, respectively. The pPSG103 and pPSG105 vectors contain an ampicillin resistance gene and decode a Twin-Strep-tag® either C- or N-terminally, respectively, as well. The only difference is that the protein expression is controlled via a T7 bacteriophage promoter. For cloning of these three genes into the vectors, we used the type 3 restriction enzyme Esp3I and DNA ligase in one reaction. The advantage of using type 3 restriction enzymes, which have different recognition and cutting site, is that specific sticky ends are created at the ends of the gene of interests (GOI) and within the vector sequence. By this, the recognition sites are deleted and the ligation of the GOIs into the vectors

Table 3. **The protein BLAST results of FTT1696, FTT0077, FTT0975 *F. tularensis* subsp. *holarctica* proteins with homological proteins of related bacteria.**

	FTT1696	FTT0077	FTT0975
Brucellaceae	Query 97%, Ident. 66%	Query 78%, Ident. 52%	No significant similarity
Yersiniaceae	Query 96%, Ident. 74%	Query 99%, Ident. 51%	No significant similarity
F. philomiragia	Query 97%, Ident. 92%	Query 100%, Ident. 85%	Query 99%, Ident. 68%
F. tularensis subsp. *novicida*	Query 100%, Ident. 99%	Query 100%, Ident. 98%	Query 100%, Ident. 99%
F. tularensis subsp. *tularensis*	Query 97%, Ident. 92%	Query 100%, Ident. 85%	Query 100%, Ident. 99%
F. tularensis subsp. *notautensis*	Query 97% Ident. 92%	Query 100% Ident. 85%	Query 100% Ident. 99%

Query: Percentage of sequence length that was considered in the analysis.
Ident.: Identity between the *F. tularensis* subsp. *holarctica* and the potential homology

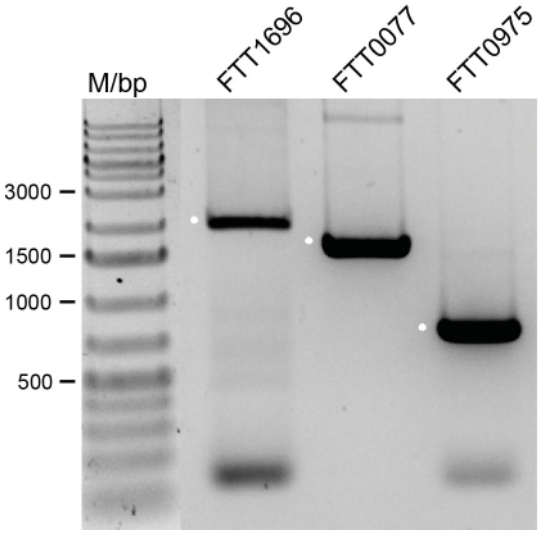

Fig. 1. **The PCR products of amplified protein sequences (FTT0975, FTT0077, FTT1696):**
M/bp: O×Gene Ruler™ 1kb Plus DNa ladder;
* — indicate expected pCR product band based on the calculated amplicon length (FTT1696: 1632 bp; FTT0077: 1467 bp; FTT0975:687)

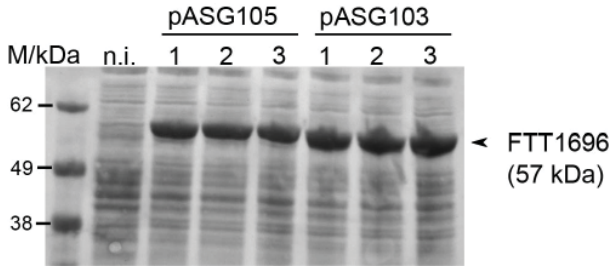

Fig. 2. **Result of the induction test of pASG103-FTT1696 and pASG105-FTT1696, for comparison reason in all lanes the same volume was loaded**
(M: SeeBlue Plus2 Prestained length marker (Thremo Fisher Scientific, USA); n.i.: non induced; 1–3: colony number)

are only possible to the direction of the GOI-vector product since the recognition site for Eps3I gets lost. Due to this, the final yield of vectors containing the GOI is increased.

The result of the amplification PCR for adding the restriction sites is shown in Fig. 1. The amplification of all three genes result in a prominent single band with expected sizes. Two small bands in the lanes of FTT1696 and FTT0975 are primer dimers based on the size. Therefore, it was possible to use these PCR products directly for further restriction and ligation reaction without previous gel purification. Due to the unspecific PCR product in the lane of FTT0077, this PCR was purified via gel extraction to avoid unwanted ligation products. After creating the expression vectors via the Esp3I restriction and ligation reaction, the sequence correctness of the GOIs and whether the Strep-tag® was in frame with the GOIs was verified via Sanger sequencing before starting the protein expression.

The protein FTT0975 was cloned into all vectors and subsequently used for initial expression and purification tests. To evaluate the expression performance based on the different expression vectors small scale protein purifications were performed with Strep-Tacin Superflow columns (IBA, Germany). Based on this initial test FTT0077 and FTT1696 were only cloned into the vectors pASG103 and pASG105 since *E. coli* transformed with the vectors pPSG103/105 showed already in the non-induced state a slight protein expression (data not shown).

Next, we compared the expression performance of *E. coli* transformed with either pPASG103 or pPASG105. For this, we compared three induced *E. coli* cultures for each gene and vector combination with the un-induced culture. In Fig. 2 the result for this induction experiment can be seen for FTT1696 as an example.

The protein FTT1696 was successfully induced in all three cultures. Furthermore, the induction of the protein expression was even stronger in *E. coli* cultures transformed with pASG103.

Based on these characteristics, the constructs pASG103-FTT0975, pASG103-FTT0077 and pASG103-FTT1696 have been selected for further protein expression and purification experiments.

Protein purification

The proteins have been purified via the C-terminally fused twin strep-tag (see materials and methods) using Strep-Tacin XT columns (Iba, Germany). The purification curve for FTT1696 protein is shown in Fig. 3 as an example for all three purification procedures. The graph in Fig. 3, *A* shows the absorbance at 280 nm of the liquid that was routed via the purification column and eluted from the column. The absorbance detection at 280 nm is an indirect measurement of the protein amount in the measured liquid. Fig. 3, *B* shows the close-up of the wash and elution step in Fig. 3, *A*. It can be seen that the main peak and therefore the main protein amount is present in the fraction E2.

The protein fractions purity after purification was evaluated via SDS-PAGE,

A Purification Chromatogram FTT 1696 *B* Elution FTT 1696

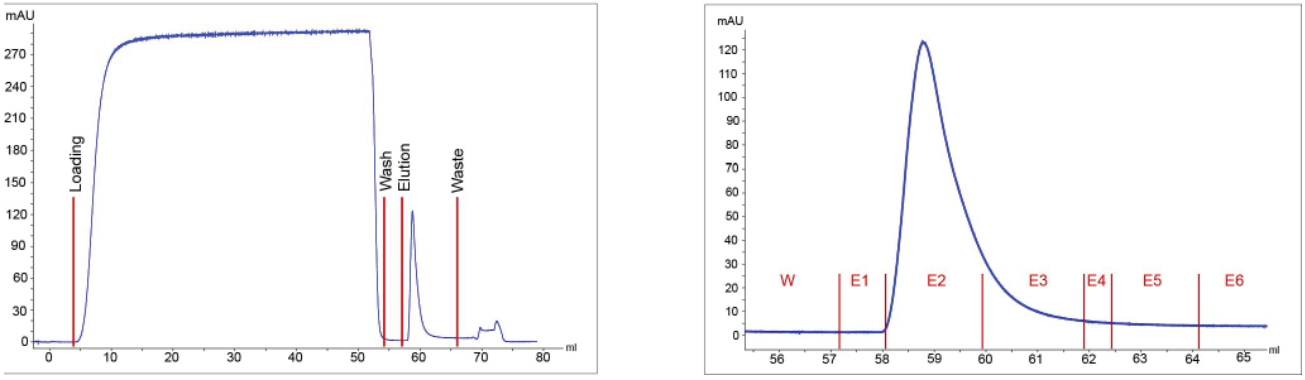

Fig. 3. A — **Whole purification chromatogram of FTT1696 (red lines indicting the different purification steps);** ***B*** **— Close-up of elution peak of FTT1696 protein (red lines indicating the different washing and elution fractions; W: Wash; E1-E6: Elution faction 1–6).** Blue curve shows the signal from UV 280 detector

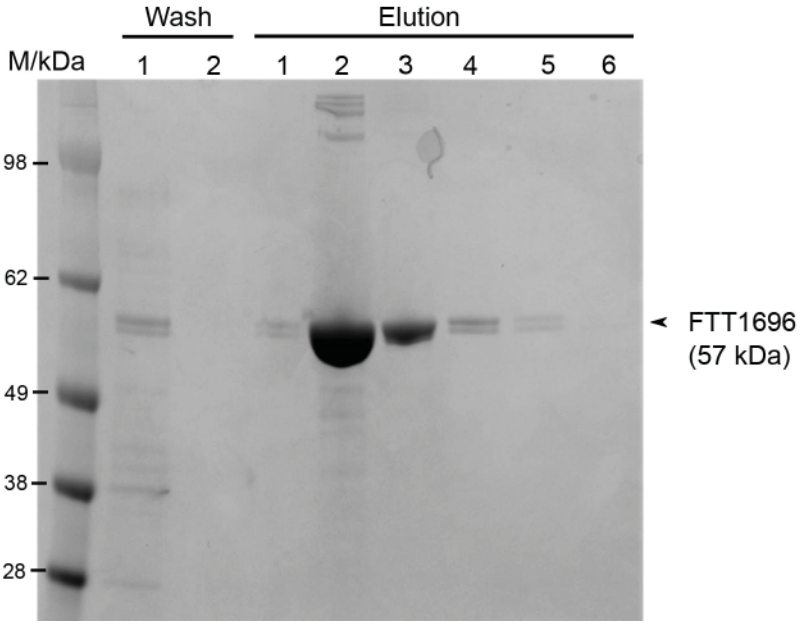

Fig. 4. **Coomassie stained SDS-PAGE of FTT1696 after purification**
For better comparison, the same sample volume was loaded in each lane. M: marker SeeBlue Plus2 Prestained; Wash 1 and 2: Fraction 1 and 2 of the washing step; Elution1—6: Fraction 1—6 of the elution step

shown in Fig. 4. The wash and elution fractions correspond with the indicated fractions in Fig. 3, *B*. As already expected based on the high absorbance at 280 nm in the E2 fraction (Fig. 3, *B*), the samples Elution 2 and 3 showed the highest protein amounts in the Coomassie stained SDS-PAGE with main bands at the expected protein size of FTT1696 of 57 kDa. In addition, only few further protein bands can be seen in the Elution 2.

The result of protein purification shown in Fig. 4 confirms that the purification system based on Twin-Strep-tag® and Strep-Tactin®XT columns is an efficient system leading to a highly pure protein which can be used for further serological trials, including ELISA.

The proteins FTT0077 and FTT0975 have been purified and analyzed in the same way as described for FTT1696 showing similar elution curves and purity grades on a SDS-gel (data not shown). For the further serological analyses the elution fractions with the highest protein concentrations have been used.

Immunogenicity studies: Western Blot

As next step, the immunogenic properties of recombinant purified proteins were verified using Western blotting with positive serum

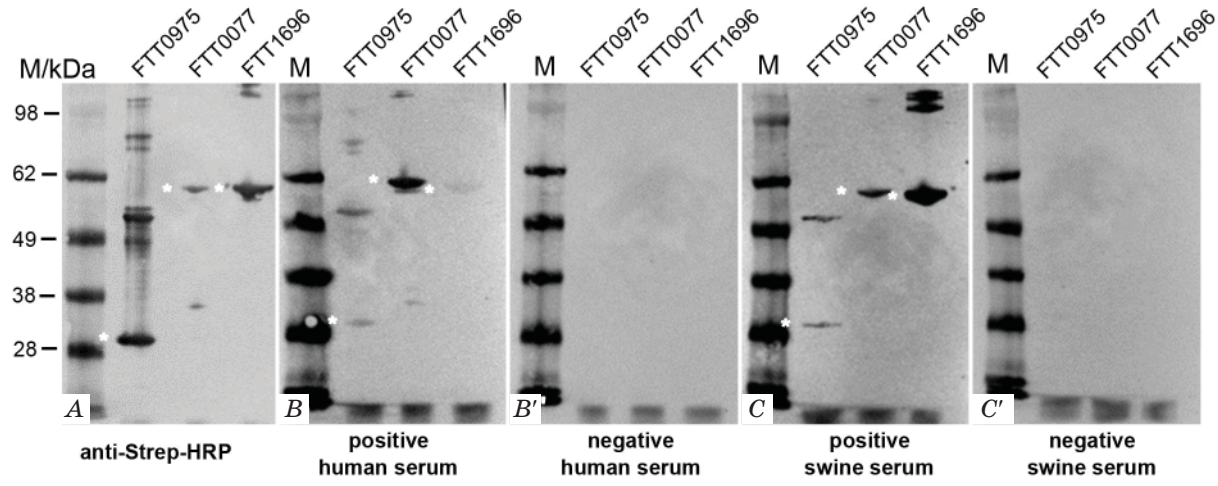

Fig. 5. **Western blotting of purified proteins:**
A — Detection of via anti-Strep antibody; *B*/*B*′ — Western blot using positive (*B*) and negative (*B*′) human sera and anti-human IgG-HRP as secondary antibody; *C*/*C*′ — Western blot using positive (*C*) and negative (*C*′) swine sera and anti-swine IgG-HRP as secondary antibody; M — SeeBlue Plus2 Prestained marker;
* — indicate expected protein band based on the calculated protein size (FTT0975: 26kDa; FTT0077: 52 kDa; FTT1696:57 kDA)

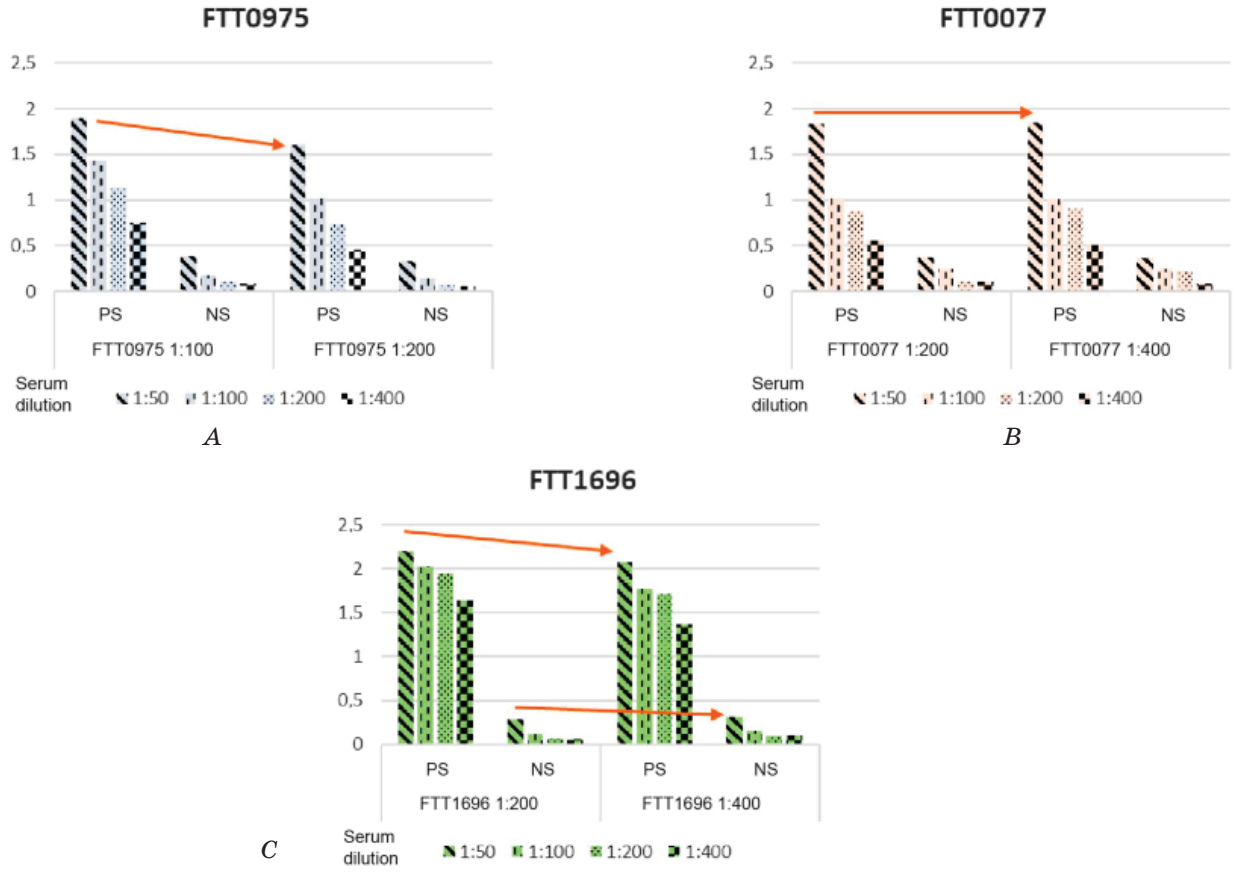

Fig. 6. **Pilot ELISA study of tittrated recombinant antigens and tittrated positive and negative swine sera:**
A—*C* show indirect ELISA results of the comparison of different antigen dilutions as coating material to gain the best signal-to-noise ratio between positive sera (PS) and negative sera (NS). Bars indicate the absorbance at 450 nm (OD). The arrows highlight the values which have to be compared for the decision which protein dilution should be used for further experiments

to *F. tularensis* subsp. *holarctica* from pigs (immunized with inactivated *Francisella tularensis* subsp. *holarctica* strain LVS) and humans (serum from patients after tularemia Kosovo outbreaks) and negative sera (commercial negative human sera and pre sera from immunized pigs) (Fig. 5, *B*–*C*′). In addition, proteins have been detected in Western blot by an anti-Strep-tag antibody (Fig. 5, *A*).

The Western blot analysis showed that all three tested proteins were immunogenic. These three proteins are not detected when tested with negative sera. Protein FTT0077 shows the highest reaction with human sera. In contrast, FTT1696 shows the highest reaction with swine sera and much weaker signal when using human sera. Interestingly, when using swine sera additional larger protein bands are visible at approx. 110 kDa in the FTT1696 lane. Due to the size and presence of bands at the same size in the anti-Strep western blot, these bands could be dimers of FTT1696. For FTT0975 two distinct bands are visible in the positive swine sera western blot as well. These bands and even a third band present in the FTT0975 lane in Fig. 5, *A* and 5, *B*. Based on the estimated size of the bands these could also be dimers and even trimers of FTT0975.

Due to the specific detection of these three proteins by positive swine sera, we decided to use the proteins for initial ELISA tests. For all further studies, the protein elution fractions have been used with the following concentrations: FTT0975 — 273 µg/ml, FTT0077 — 274 µg/ml, and FTT1696 — 208 µg/ml.

Pilot ELISA studies

We determined the assay conditions and found Maxisorp plates and Tween20 in a concentration of 0.1% in washing buffer as optimal. The concentration of the coating antigen was titrated and the following concentrations (FTT1696: 1.04 µg/ml; FTT0077: 1.37 µg/ml and FTT0975: 2.73 µg/ml) yielded the best signal-to-noise ratios. The results of the comparison of two different antigen concentrations under the optimized assay conditions can be seen in Fig. 6.

The proteins show strong signal with positive sera, especially FTT1696 (the highest OD was 2.204) and low background with negative sera (the OD is less than 0.4 for all proteins with the highest protein concentration and the lowest sera dilution) (Fig. 6, *C*). For the FTT0975 coating concentration 1:100 (2.73 µg/ml; 5.23 pmol/well) was chosen, because the signal of positive sample became higher while the background signal did not change (Fig. 6, *A*). For the same reason the coating concentration 1:200 (1.04 µg/ml; 0.9 pmol/well) was chosen for FTT1696. Since the dilution of FTT0077 did not show any differences and to save protein material concentration 1:200 (1.37 µg/ml; 1.31 pmol/well) was chosen for further assays (Fig. 6, *B*). Thus, these recombinant proteins are perspective good candidates for the further iELISA development.

Conclusions

With the present study we could show that the recombinant expression and purification of the three antigens FTT1696, FTT0077 and FTT0975 by Strep-tag affinity chromatography leads to high protein purity.

Moreover, we could show, that all three recombinant proteins (FTT1696, FTT0077, FTT0975) display immunogenicity with swine sera positive for *F.tularensis* antibodies over background level which is in line with the results of Nakajima et al. [10]. We optimized the iELISA conditions so that the positive and negative sera show the best signal-to-noise ratio. However, further investigation with swine and especially wild boar sera will be necessary, since these animls are prone to have been in contact with potentially cross reactive antigens of related gram-negative bacteria. Nevertheless, with this study we set the basis for further testing a panel of swine sera, positive for different related bacteria with the final goal to validate the ELISA. Besides, in further studies these proteins can be checked in ELISA for detection of anti-tularemia antibodies in human sera as well.

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РОЗРОБЛЕННЯ І ПІЛОТНЕ ДОСЛІДЖЕННЯ ІЕА, ЗАСНОВАНОГО НА РЕКОМБІНАНТНИХ ПРОТЕЇНАХ, ДЛЯ ДЕТЕКЦІЇ АНТИТУЛЯРЕМІЙНИХ АНТИТІЛ У СИРОВАТКАХ ВІД СВИНЕЙ ТА ЛЮДИНИ

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На сьогоднідля встановлення туляремійної інфекції обов'язково використовують комбінацію з двох методів: імуноензимний аналіз (ІЕА) та вестерн-блотинг (ВБ) як підтвердження. Обидва методи базуються на використанні ліпополісахаридів (ЛПС) *Francisella tularensis* subsp. *holarctica* як антигену. Мета роботи — отримати та оцінити три рекомбінантні протеїнові антигени (FTT1696, FTT0077, FTT0975) для проведення непрямого ІЕА без обов'язкового підтвердження результатів у ВБ. У роботі докладно описано стратегію клонування у векторі pASG103, експресію у клітинах *E. coli* та очищення протеїнів з використанням системи Strep. Сироватки з підтвердженими титрами антитіл проти *F. tularensis* реагували з усіма трьома антигенами, що робить їх придатними для серологічного виявлення *F. tularensis* у сироватках крові від свиней та людини.

Ключові слова: ІЕА, рекомбінантні протеїни, туляремія, FTT0077, FTT0975, FTT1696.

РАЗРАБОТКА И ПИЛОТНОЕ ИССЛЕДОВАНИЕ ИЭА, ОСНОВАННОГО НА РЕКОМБИНАНТНЫХ ПРОТЕИНАХ, ДЛЯ ДЕТЕКЦИИ АНТИТУЛЯРЕМИЙНЫХ АНТИТЕЛ В СЫВОРОТКАХ ОТ СВИНЕЙ И ЧЕЛОВЕКА

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На сегодняшний день туляремийную инфекцию устанавливают с обязательным использованием комбинации двух методов — иммуноэнзимного анализа (ИЭА) и вестерн-блотинга (ВБ) в качестве подтверждения. Оба метода основаны на использовании липополисахаридов (ЛПС) *Francisella tularensis* subsp. *holarctica* в качестве антигена. Цель работы — получение и оценка трех рекомбинантных протеиновых антигенов (FTT1696, FTT0077, FTT0975) при проведении непрямого ИЭА без обязательного подтверждения результатов в ВБ. В работе подробно описана стратегия клонирования в векторе pASG103, экспрессия в клетках *E. coli* и очистка протеинов с использованием системы Strep. Сыворотки с подтвержденными титрами антител к *F. tularensis* реагировали со всеми тремя антигенами, что делает их пригодными для серологического выявления *F. tularensis* в сыворотках крови от свиней и человека.

Ключевые слова: ИЭА, рекомбинантные протеины, туляремия, FTT0077, FTT0975, FTT1696.