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IMPROVEMENT AND OPTIMIZATION OF ANTIGENIC COMPOSITION FOR SERODIAGNOSIS OF TUBERCULOSIS

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There is a pressing of needs for improvement existing methods of tuberculosis (TB) diagnosis and screening. New methods should be characterized by high specificity, sensitivity, reliability of results; easiness of implementation; precision interpretation of the results. Regarding the TB, the ELISA test-systems have many advantages over traditional methods of disease diagnostics. However, the most difficult objective of creating such test-systems is the selection of optimal antigenic substance which would characterized by the high antigenicity, and high specificity from the other hand. The aim of the research was to improve previously created antigenic composition using full-size highly immunogenic proteins of *Mycobacterium tuberculosis* MPT63 and MPT83.

The genetic construction pET28a-MPT83(full)-MPT63 was obtain, with further isolation and purification of target protein and compared to its predecessor which based on FLD of MPT83(115–220 aa). Both fusion proteins were tested to the culture medium of hybridomas, which were obtained from mice immunized with a mixture of antigens from mycobacteria, including cell membrane, associated protein MPT83 closest homologue — MPT70. In addition, sera samples from infected *M. bovis* and healthy cattle were tested on several variants of *Mycobacterium fusion* proteins.

These data suggest the need of N-terminal amino acids for protein folding for better antibody recognition. The new antigenic substance gives the best results for serology-based diagnostics of tuberculosis. Using of specific monoclonal antibodies to new obtained antigen as a positive control for the test system can reduce the cost of it and avoid using genuine infected cattle or human serum among ELISA kit reagents.

Keywords: *Mycobacterium tuberculosis, Mycobacterium bovis*, antigenic substance, MPT63, MPT83, chimeric protein, serology-based diagnostics, tuberculosis

Introduction. Mycobacterioses (including TB, leprosy, atypical mycobacterioses, paratuberculosis) are the widespread infectious diseases of human being and animals (Mencarini et al., 2016). TB is a major reason of high levels of morbidity and mortality in the developing countries of the World (Banerjee et al., 2003) and causes a significant damage for livestock (Maia et al., 2014). Current tests most widely used for the detection of tuberculosis both in cattle and humans include measurement of delayed type hypersensitivity (i.e., skin tuberculin testing) to purified protein derivatives (PPD) and *in vitro* assay for gamma interferon (IFN-γ) produced in response to mycobacterial antigen stimulation (Waters et al., 2011). However, these methods are not enough specific, or quite expensive, either requires much time for diagnosis occurrence. Owing to the very high infectious power of pathogenic *Mycobacteria*, early diagnosis is essential to prevent spreading of the disease among the herd or people population.

Serodiagnosis by ELISA has been widely used in the diagnosis of different infectious disease, including TB. The major problem for this method is the optimal choice of highly immunogenic and pure substance for determination of antibodies against the pathogen. The aim of our work is reconstruction unique primary structure of a protein of *M. tuberculosis* MPT83 consisting of the chimeric protein MPT83 (full)-MPT63. Test systems based on specific recombinant antigens of mycobacteria, for example on the basis of two homologous proteins of *M. tuberculosis* MPT70 and MPT83 (Waters et al., 2011), existing in overseas markets. However, the using of proteins with a high degree of homology (Wiker, 2009) is not appropriate to meet the parameters of sensitivity and specificity of serology-based diagnostics. We suggest that the use of fundamentally different antigens (Manca et al., 1997; Chambers et al., 2010) as a parts of fusion protein with spacer link between protein's components can increase exposure of serologically important epitopes, for early diagnosis of the disease in animals and humans.

It was shown dramatically increased yield of MPT83 (full)-MPT63 antigen and it purity in comparison with shorter analogue based on FLD MPT83 (115–220 aa). In addition, used sera from natural infected and relatively healthy cattle have been shown serological value of new obtained antigenic substance. Based on data obtained

previously and these data, ELISA serves as superb method for screening of animals from the herd and potentially people, because antigens of *M. tuberculosis* MPT63 and MPT83 and *M. bovis* MPB63 and MPB83 identical by the amino acids composition in both representatives (Redchuk et al., 2010; Redchuk et al., 2010).

Wild animals such as badgers, deer could be natural foci of TB infection (Miller, Farnsworth, and Malmberg, 2013). Whereas, tuberculosis in humans may result from exposure to any one of the tubercle bacilli included within the *Mycobacterium tuberculosis complex*, including *M. bovis* (Waters et al., 2011). This implies, to abort the One Health TB triad (human-livestock-wildlife) (Wadhwa et al., 2014), there is an urgent need to isolate animals in the early stages to protect human life.

Materials and methods. *Obtaining of genetic construction of the chimeric protein MPT83(full-sized)-MPT63.* The gene encoded full-length soluble protein MPT83 amplified from plasmid DNA p*ET*24*a-mpt83* using pair of oligos which being finished by restriction endonucleases sites *Eco*RI and *Bam*HI — mpt83-SP TGGATCCAGCACCAAACCCGTGTCGCA and mpt83-ASP TAGAATTCTGTGCCGGGGGC. The product of amplification was insert in p*ET*28*a(+)* ('Novagen', Germany) plasmid DNA at the appropriate sites for restriction endonucleases. MPT63 gene was amplified from plasmid DNA p*ET*24*a-mp63* using a pair of primers mpt63-ASP TCAGCTCGAGCGGCTCCCAAATCAGCAGA and mpt63-SPACAAGCTTTTGCTCACCACAATGATC AAGACGGC. The obtained PCR product was nest in plasmid DNA p*UC*-19 ('Novagen', Germany) by blunt-ends cloning. After restriction analysis *mpt63* correct sequences and obtained vector p*ET*28*a-mpt83* were treated with restriction enzyme *Xho*I and *Hind*III. The resulting fragments of p*ET*28*a-mpt83* vector and DNA sequence *mpt63* mixed in a molar ratio of 1:3 and were crosslinked using T4 DNA ligase ('Thermo Scientific', Lithuania). The obtained construct was used to transform *E. coli* Rosetta (DE3) host cells ('Novagen', Germany).

Obtaining and purification of recombinant fusion protein MPT83(full-sized)-MPT63 in procaryotic expression system. Expressed MPT83(full)-MPT63 *E. coli* cells accumulated biomass in LB-Medium ('Carl Roth', Germany) with 50 μg/ml kanamycin and 1% glucose at 37°C and active mixing (250 rpm) to optical density A_{600} 0.3–0.5. The expression of obtained gene construct was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) ('Thermo Scientific', Lithuania). Target protein was obtained by the method described in (Siromolot et al., 2016) conjunction with use of protease inhibitors cocktail ('Thermo Scientific', Lithuania).

SDS-PAAG electrophoresis. Protein fraction separation was performed in 10% polyacrylamide gels (PAGE), at a voltage of 10 V/cm at the denaturing condition.

Indirect enzyme-linked immunosorbent assay (ELISA). The antigens were sorbed to the 96-well plate at final concentration 5 μg/ml per well in phosphate buffered saline (PBS) (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) and left for 1 hour at 37°C. As a blocking agent was used non-fat 1% milk in PBS. The incubation with monoclonal antibodies against MPT70 and MPT83 of *M. tuberculosis* in PBS-T (adding Tween-20 to a final concentration of 0.04%) was performed 1 hour. Cattle serum samples were diluted in ratio 1:10. Anti-mouse-HRP conjugate (1:12,000) for mAb and anti-bovine-HRP conjugate for cattle serum Ab detection were used. TMB (3,3',5,5'-tetramethylbenzidine) was used as chromogen substrate. The color reaction was quantified by measuring the absorbency at 490 nm. Sera samples were provided by National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine).

Results and discussion. *Obtaining of genetic construction pET28a-mpt83-mpt63 carrying a full-size gene nucleotide sequence of mpt83.* We have obtained fascyclin-like domain (FLD) of MPT83 (115–220 aa) in our lab previously, which was used for indirect ELISA-based test-system design. The value of a new developed chimeric protein with use recombinant full-length MPT83 consist in playback of a unique immunological properties of the original protein. New improved antigenic substance has been characterized with better antigenic feature that affect the sensitivity and specificity of analysis. The gene *mpt83* was amplified from pDNA construction p*ET*24*a-mpt83* by PCR. Cloning was carried out using *E. coli* Rosetta (DE3) ('Novagen', Germany) and vector p*ET*28*a* (+) (Fig. 1).

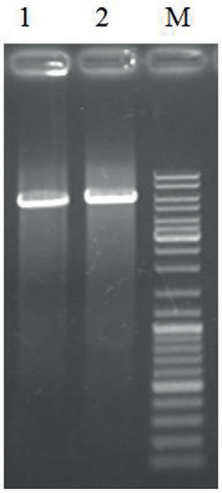

Figure 1. Electrophoregram of selected plasmid vector p*ET*28*a* (1) and created the first stage genetic construction p*ET*28*a*, which carries in itself a full-length gene insertion MPT83 (2)

To further fusion of the *mpt83* and *mpt63* genetic sequences it was decided to create genetic construction p*UC*-19-*mpt63*. Thus, we were extension nucleotide sequences before the restriction enzyme site *Hind*III. The gene *mpt63* cloning was performed by blunt-ends type. The anticipated consequence of cloning by blunt-ends type is a high probability of duplication of gene sequences in plasmid design construct (Fig. 2A). Due to such phenomenon, clone p*UC*-19-(*mpt63*)^2 has been selected in the processing of restriction enzyme *Hind*III on it we get not a single chain rupture with subsequent linearization of DNA but two separate products — *mpt63* and p*UC*-19-*mpt63* (Fig. 2B).

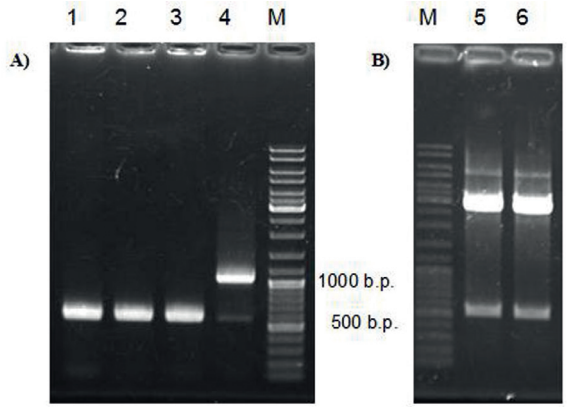

Figure 2. A) Electrophoregram of products of PCR-analysis using primers flanking vector polylinker area: 1–3 — PCR products corresponding to the nucleotide sequence of the gene *mpt63*; 4 — PCR product corresponding to the dimer of two nucleotide sequences of the gene $(mpt63)_2$. B) 5–6 — genetic construction p*UC*-19-$(mpt63)_2$ after treatment with restriction enzyme *Hind*III (circular and linearized form of plasmid DNA and gene *mpt63*)

Thus, resulting nucleotide sequence of the gene *mpt63* treated with endonuclease restriction enzyme *Xho*I to further fusion with previously obtained genetic construct p*ET*28*a*-*mpt83*. To determine the presence of the incorporated nucleotide sequence *mpt63*-*mpt83* consist into genetic construct was performed PCR analysis using primers flanking the gene *mpt63* on one side and the gene *mpt83* on the other. Results of PCR analysis confirmed the presence of the fusion nucleotide sequence *mpt63*-*mpt83* (the size of approximately 1,300 base pairs) in developed p*ET*28*a*-*mpt83*-*mpt63* construction (Fig. 3).

Expression and comparative characteristics of obtained recombinant fusion protein MPT83(full length)-MPT63. The recombinant protein was obtained from 1 ml of *E. coli* culture, which were incubated 4 hours at active mixing at speed of 250 rpm and 30°C and optical density A_{600} 0.3–0.5 in the presence of the inductor 1mM IPTG. The precipitate cells were analyzed for the presence of protein in the soluble and insoluble fraction. Analysis factions of new fusion protein compared to chimeric protein-based FLD MPT83 (115–220 aa) (Fig. 4).

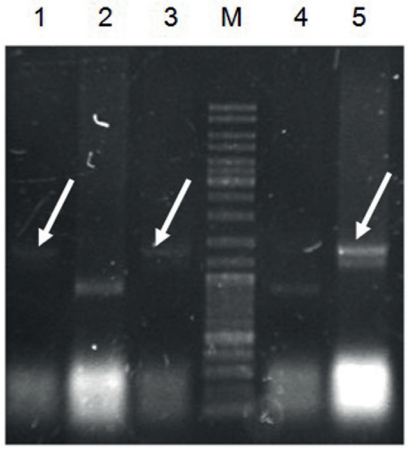

Figure 3. Electrophoregram of PCR-analysis of transformed *E. coli* clones with genetic construct p*ET*28*a*-*mpt83*-*mpt63*. Clones 1, 3 and 5 have the genetic structure of the combined nucleotide sequences of genes *mpt83* and *mp63* (indicated by arrows)

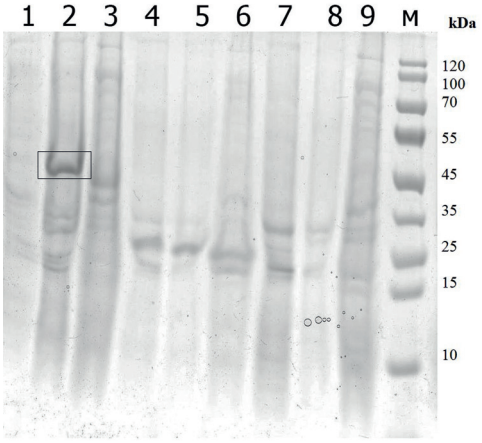

Figure 4. The results of electrophoretic separation of cell lysates of bacteria-producers during the analytic expression of target protein MPT83(full)-MPT63 (1–3), FLD MPT63-MPT83 (115–220 aa) (4–6) (1, 4 — soluble fraction; 2, 5 — insoluble fraction; 3, 6 — total cell lysate). The frame indicate protein product that meets the target protein molecular weight MPT83 (full)-MPT63

Immunological characterization of MPT83 (full)-MPT63. Thus, obtained genetic construction p*ET*24*a*-*mpt83* (full)-*mpt63* made it possible to expression of the functional target protein in the insoluble fraction (inclusion bodies). However, the main purpose of obtaining fusion protein was the creation of antigenic molecules consisting of two highly antigenic

components — full-size MPT83 and MPT63. In addition, the newly created protein characterized by the fact that both antigenic components not only full length, but also insert in plasmid design so that between them inserted spacer (in translational variant polypeptide chain length of 8 amino acid residues), which can affect their folding process biosynthesis. Instead that, our obtained previously fusion protein MPT63-MPT83 (115–220 aa) not contained in the structure spacer link polypeptide chain. Lack of those small sequences areas in the structure of MPT63-MPT83 (115–220 aa) can affect protein folding and, consequently, limit the exposure of diagnostically important epitopes in the structure of the whole molecule.

In order to conduct the comparative characteristics of antigenic properties of two kind fusion protein MPT63-MPT83 (115–220 aa) and MPT83 (full)-MPT63 we conducted indirect ELISA using monoclonal antibodies (mAb) to the N-terminal molecule sites of full-MPT83 of *M. tuberculosis.* The results of ELISA indicated the ability of mAb to the N-terminal molecule sites of full-MPT83 antigen specific recognition of the new recombinant chimeric MPT83 (full)-MPT63. Instead, the recombinant antigen based on the shortened form FLD MPT83 — MPT63-MPT83 (115–220 aa) not specified recognized by mAb (Fig. 5). So, the presence of additional N-terminal site in the structure of MPT83 (full)-MPT63 makes it more attractive and promising antigen composition for serology-based diagnostics.

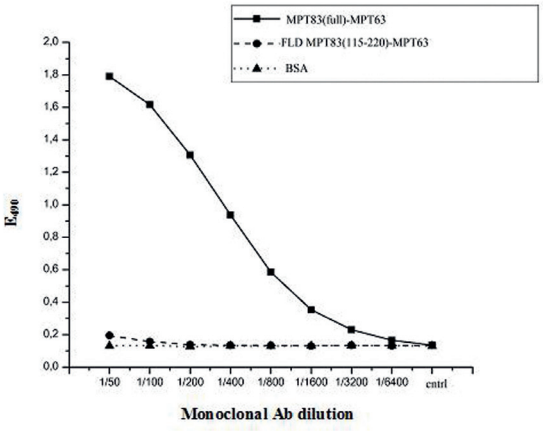

Figure 5. Recognition results of obtained recombinant protein MPT83(full)-MPT63 with monoclonal antibodies to the N-terminal area of MPT83 and MPT70 compared to the shortened form of recombinant antigen FLD MPT83(115-220 aa)-MPT63

Confirming our hypothesis has found in the testing of cattle serum samples of healthy and infected by *M. bovis* animals on fusion proteins consist of not only MPT63, MPT83 and FLD MPT83(115–220 aa) antigens, but also other kind of *Mycobacterium* proteins (Fig. 6).

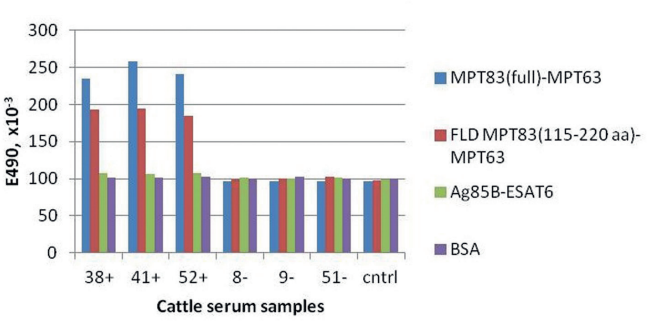

Figure 6. IgG level to the target and control antigens in the cattle serum samples

Screening results show that the improved variant of antigenic substances was able for better identification of sick animals in comparison with 3rd type of chimeric recombinant proteins that have been tested on cattle sera.

Thus, the data indicate that improved antigenic substance consisting of full-size antigens proved itself better for infected animals' detection. Use of mAb which were derived from mice immunized by wide range of *Mycobacterium* proteins, including homologous antigens MPT70 and MPT83 showed the effectiveness of the establishment of the substance based on the whole molecule. Noteworthy, that using a hybridomas culture fluid can replace positive control in the test sets instead a dangerous serum from infected animal or human.

We believe that chosen successful combination of antigens: MPT63 — secretory protein of *M. tuberculosis*, and MPT83 — associated with cell membrane antigen. Therefore, it should be noted that the antigens of *M. bovis* MPB63 and MPB83 and antigens of *M. tuberculosis* MPT63 and MPT83 do not distinguish in functionally product and primary protein structure. This could mean that these fusion proteins can be used to create test systems for the diagnosis of TB in humans.

Conclusions. Conserving of the unique primary protein structure can achieve the best indicators of sensitivity of antigenic substances in the development of test systems and diagnostics to identify markers of infectious diseases. The use of highly immunogenic antigens of *M. tuberculosis* MPT63 and MPT83 which are secretory and miristilated with cell membrane respectively achieves the best parameters of specificity too. It was shown dramatic significance of spatial organization of antigenic composition and its flexibility for exposure of serologically important epitopes.

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