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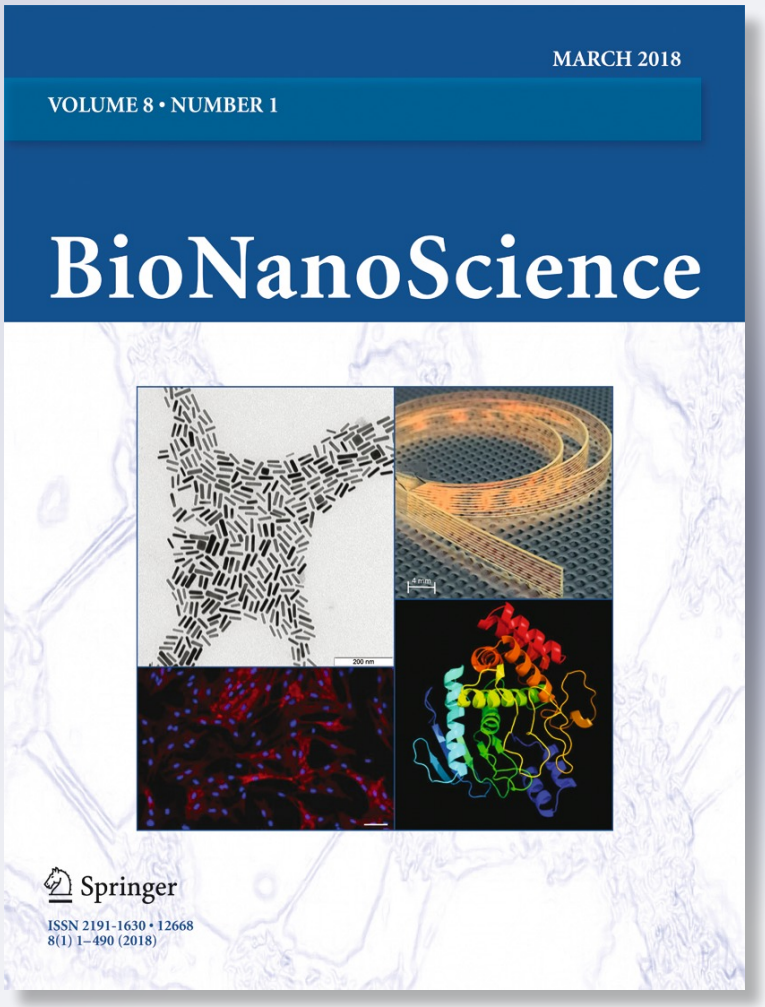

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Extraction and Serological Properties of Mycobacterium Cell Surface and Excreted Proteins

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Abstract

Modern medicine still faces the task of distinguishing active and latent tuberculosis cases at the early stage of the disease. Serological approaches have their advantages for their use in diagnostics. However, the progress of these approaches is ongoing but further progress is needed to meet the needs for this disease. Here, we extracted *Mycobacterium tuberculosis* H37Rv proteins from culture medium or from the cell surface and studied their reactivity with anti-*M. tuberculosis* serum in both ELISA and immunoblots. We found that *M. tuberculosis* surface proteins, extracted using dimethyl sulfoxide, with molecular weights in the range of 6.5–200 kDa, showed strong specific reactivity with anti-*M. tuberculosis* positive serum. While excreted proteins in the molecular weight range of 32–45 kDa had the highest reactivity. The latter was confirmed serologically when very weak signal was detected from the filtrate fractions compared to stronger activity from the Vivaspin 50 kDa MWCO retentates. Moreover, *Mycobacterium bovis* and *tuberculosis* proteins from the filter retentates had clear specific serum reactivity, which suggests that this approach can be used for differential diagnosis of two infections.

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1 Introduction

Tuberculosis (TB) is a widely spread chronic infectious disease caused by *Mycobacterium tuberculosis* [1, 2]. The emergence of multidrug-resistant tuberculosis makes treatment and TB remains a leading cause of death among HIV-infected patients [3–5]. Immune deficiency in HIV-infected patients along with the low sensitivity of the present diagnostic tests substantially increases the tuberculosis mortality rate due to TB because early diagnosis and treatment are the main components of successful anti-tuberculosis approaches [6]. The diagnosis of TB is usually based on several methods, such as chest X-ray, sputum microscopy, bacteriological method, polymerase chain reaction, the Mantoux test and immune cell response to mycobacterial antigens [7]. The immune approaches demonstrate the best results when a mix or cocktail of different *M. tuberculosis* antigens is used [8]. The direct detection of *M. tuberculosis* specific antigens, e.g., lipoarabinomannan (LAM), is one of the new promising diagnostic tests being investigated [9]. Combination of the LAM test with a cartridge-based nucleic acid amplification test Xpert MTB/RIF may further improve the diagnosis of active tuberculosis even in HIV-infected patients [10]. The majority of described approaches is time consuming and requires expensive equipment, which increases the need for the development of more specific, effective, rapid, and easy-to-use methods for diagnosis of TB. Serological approaches can potentially offer many advantages to help overcome many of these challenges.

The humoral immune response to the *M. tuberculosis* antigen spectrum depends on the stage and form of the disease [11]. This suggests that the right choice of antigens can be made on the basis of successful serological test that can allow detecting the events associated with the transition of mycobacterial infection into its active form. Cell surface and excreted proteins are among the proteins most exposed to the immune system and are known to be promising agents as both therapeutic and detection targets [12, 13].

The common extracellular bacterial proteins in general have high solubility in dimethyl sulfoxide (DMSO), which can be used as the reaction medium for protein separations [14]. DMSO extraction of *Brucella abortus* lipopolysaccharide and proteins has shown antigen properties for diagnosis of brucellosis [15]. It was previously proven that the multiple treatment of a bacterial mass of *M. tuberculosis* using aqueous solutions of DMSO with increasing concentrations from 5 to 20% makes it possible to obtain proteins with an extended spectrum of seropositive fractions in the immunoblot reaction [16].

In this study, we have developed methods for the isolation of serologically active mycobacterium cell surface and excreted proteins that can be used for the perspective diagnostic tests.

2 Materials and Methods

2.1 Mycobacteria Cultures

M. tuberculosis H37Rv TBC #1/47, strain 7004031, and *Mycobacterium bovis* Bovinus-8, strain 700201, were obtained from the Scientific Center for Expert Evaluation of Medicinal Products of the Ministry of Health of the Russian Federation. Cells were cultivated on the solid Lowenstein-Jensen medium for 28–30 days at 37 °C or in liquid modified Soton medium for 5 months. Before the experiments, the strains were adapted by culturing in the Lowenstein-Jensen medium for 3 months to shorten the passage time from 60 to 28–30 days. The culture media were prepared in the Federal Center for Toxicological, Radiation and Biological Safety, Kazan, Russia (FCTRBS).

2.2 Dimethyl Sulfoxide-Extraction of *M. tuberculosis* Surface Structures

The mycobacteria were removed from the Lowenstein-Jensen medium surface with a scraper. To purify the bacteria, they were washed three times with 0.1M PBS (Sigma-Aldrich), pH 7.2–7.4. Diluted cells were homogenized in a glass Round Body Homogenizer, with a grinding clearance for the pestle and mortar of 0.152–0.254 mm (GPE Scientific Ltd) for 5–10 min. The homogenate was washed with distilled water (+ 8–10°C) and re-homogenized four additional times.

Extraction of the cell surface structures from the purified homogenate was performed by incubation of the cells with 10% DMSO (Sigma-Aldrich) in PBS as described previously [16]. Cells were separated by centrifugation (7500 g, 20 min). The proteins from the obtained supernatant were precipitated by adding the equal volume of saturated (5.73 M) solution of ammonium sulfates at 20 °C for 24 h. The supernatant was dialyzed against 0.125 M Tris-HCl, pH 7.2–7.4.

2.3 Isolation of *M. tuberculosis* Excretion Products

Mycobacteria from the Soton liquid medium were centrifuged for 20 min at 7500 g. To purify the supernatant from the cell debris, it was filtered through the MF-Millipore Membrane Filter, mixed cellulose esters, 0.22 μm (#GSWP04700,

Merck). The culture medium filtrate (CMF) was incubated with saturated ammonium sulfate as described above for 24 h. The isolated by centrifugation precipitate was diluted in 0.05M Tris-HCl, pH 7.1–7.4. The equal volumes of the sample were passed through the Vivaspin 2 concentrators (Sartorius Stedim Biotech) with polyethersulfone (PES) membranes at 10, 30, and 50 kDa molecular weight cutoff (MWCO) using fixed angle centrifugal force at 4000 g with start volume 2 mL according to the manufacture's instruction.

2.4 Enzyme-Linked Immunosorbent Assay

Serological activity of the obtained material was performed as described earlier [17]. In brief, the ammonia sulfate precipitate of the DMSO extract was diluted 1:100 in 0.05M Tris-HCl buffer pH 7.2–7.4. CMF was diluted 1:4 in 0.05M Tris-HCl buffer, pH 6.8. Serial dilutions of the material were made in respective buffers and coated in wells of ELISA plates (Medpolimer, Russia) for sorption overnight at 4 °C. After washing three times with PBS, the plates were incubated for 1 h with 200 μl of PBS-BSA (5%) (Sigma-Aldrich) to reduce non-specific background binding. Hyper-immune *M. tuberculosis* H37Rv TBC #1/47 (K+) and control (K-) rabbit sera (serum collection of FCTRBS) diluted in 0.1M PBS with 0.05% Tween-20 (Bio-Rad) (PBST) were used to analyze antigenic properties of the plate absorbed material. Goat anti-rabbit IgG-peroxidase conjugate (Sigma-Aldrich) diluted 1:60,000 in PBST was used to detect binding serum antibodies. 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich) was used as a substrate. Independent triplicate experiments were performed several times.

2.5 Electrophoresis and Immunoblot

Electrophoresis was performed as described previously [18]. Cells were lysed in 0.0625 M Tris-HCl buffer (pH 6.8) containing 5% β-mercaptoethanol (Bio-Rad), 2.5% SDS (Roche Applied Science, Indianapolis, IN) at 100 °C for 5 min. Proteins were resolved by 12.5% sodium dodecylsulphate polyacrylamide gels electrophoresis (SDS-PAGE). Gels were stained with the standard coomassie brilliant blue and silver nitrate (Bio-Rad) staining.

For immunoblot, the material from the gel was transferred onto the nitrocellulose membrane (Bio-Rad) as described previously [19] and then blocked using bovine nonfat-dried milk (Sigma-Aldrich). To detect mycobacterial proteins, the hyper-immune rabbit serum was used followed by incubation with anti-rabbit IgG (whole molecule)-peroxidase conjugate (Sigma-Aldrich) and incubation with 3,3′-diaminobenzidine (DAB, Bio-Rad). Results were documented with Gel Doc XR+ System (Bio-Rad). To determine the molecular weight of proteins, a marker Unstained SDS-PAGE Standards, broad range (Bio-Rad) was used.

2.6 Statistical Analysis

Statistical analysis was performed using Student's t test. A p value ≤ 0.05 was considered to be significant.

3 Results

3.1 Antigenic Reactivity of *M. tuberculosis* Surface DMSO Extract

The DMSO extraction method for the bacterial surface structures was used to prepare an antigen-containing material from the *M. tuberculosis* cell surface. The serological activity and specificity of the obtained material absorbed to the plate were determined using ELISA with an anti-*M. tuberculosis* serum. Specific anti-bacterial signal was determined down to 1:12,800 dilution (Fig. 1) compared to the control rabbit serum used ($p < 0.01$). The spectrum of molecular weights for the extracted by DMSO proteins ranged between 6 and 200 kDa suggesting that this isolation method is applicable for wide range of proteins (Fig. 2).

3.2 Protein Spectrum and Serological Activity of *M. tuberculosis* Excreted CMF Antigens

Proteins excreted by *M. tuberculosis* and collected in the CMF were also analyzed as a possible source of antigens for the diagnosis of tuberculosis by ELISA and immunoblot. To identify the most promising fraction, the CMF material was fractioned by molecular weights. The initial CMF material without cells and cell debris had the highest specificity compare to 10, 30, and 50 kDa filtrates (Fig. 3). Nevertheless, among the filtrate fractions, 50 kDa filtrate showed the strongest signal with anti-*M. tuberculosis* serum (Fig. 3d) compared to 10 and 30 kDa filtrates ($p < 0.05$).

To further investigate the CMF ELISA data, the protein fractions were analyzed by electrophoresis. The gel staining revealed that the filters inhibited the passage of proteins. Vivaspin 50 kDa MWCO membrane was the best for protein concentration. The retentates from the filter membranes contained high amount of proteins (Fig. 4a). These results suggest that the proteins in the culture medium can be aggregated and cannot pass through the Vivaspin filters. Further analysis of the material in immunoblot with anti-*M. tuberculosis* serum confirmed the gel staining results that *M. tuberculosis* antigens do not pass the filters (Fig. 4b). During electrophoresis, proteins were denatured by SDS. This, apparently, caused the destruction of polypeptide bonds, i.e., we received polypeptides. By our assumption, this led to a

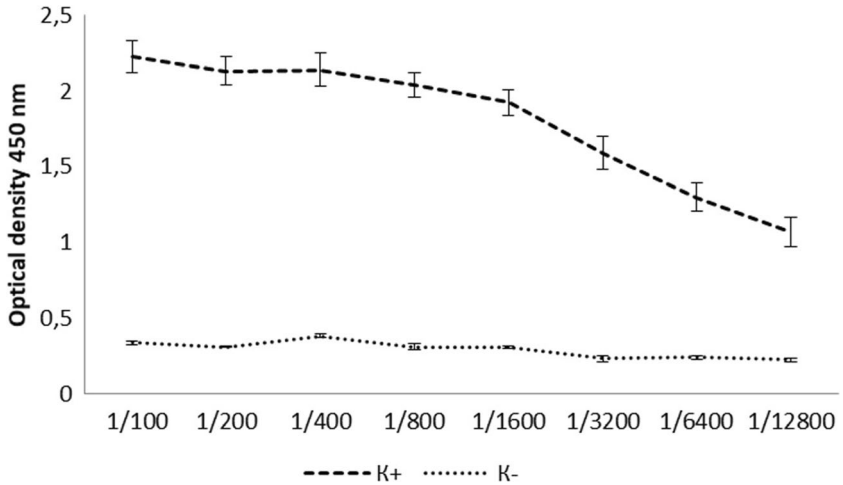

Fig. 1 Results of ELISA with positive (K+) and negative (K−) sera in serial dilutions from 1:100 to 1:12,800 using ammonium sulfate precipitate of *M. tuberculosis* DMSO extract as antigen at 1:100 dilution

loss of serological activity, which was observed in the ELISA, but absent in the immunoblot.

To confirm and investigate further sensitivity and applicability of the CMF approach to other mycobacteria, we repeated experiments with *M. bovis*.

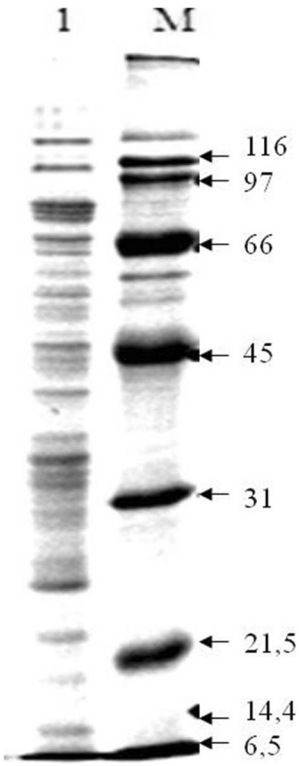

Fig. 2 Electrophoresis of DMSO extracts of 30-day *M. tuberculosis* culture grown on a Lowenstein-Jensen medium. Tracks: **1**—DMSO extract, **M***—set of proteins with known molecular weight *SDS-PAGE Standards, broad range (Bio-Rad) were used as a marker of molecular weight (M). The marker strips were stained using PonceauS (Sigma-Aldrich) 0.2% *w/v* in 3% *w/v* of trichloracetic acid within 30 min, then washed by 7% acetic acid solution and di H2O. The marker strips were placed beside immunoblot sample strips and then documented with Gel Doc XR+ System

3.3 The Spectrum of Antigen Proteins in CMF of *M. tuberculosis* and *M. bovis* and Their Serological Activity

The comparison of the protein spectra obtained for *M. tuberculosis* (Fig. 4a) and *M. bovis* (Fig. 5a) demonstrated clear difference between these two bacteria.

The results of the comparison demonstrate that retentates from membranes contained specific antigens that are different for *M. bovis* and *M. tuberculosis*. The spectra of the secreted proteins varied substantially: in *M. tuberculosis*, the majority of proteins had molecular masses ranging from 32 to 45 kDa; in *M. bovis*, the protein spectrum was much wider.

The immunoblot of the material from *M. tuberculosis* (Fig. 4b) and *M. bovis* (Fig. 5b) retentate proteins demonstrated the bacterium species-specific patterns of reactivity with anti-*M. tuberculosis* serum, which has a potential for differential diagnosis of these mycobacteria.

4 Discussion

The antigen is a keystone of any diagnostic test based on immune reaction mediating its sensitivity and specificity. For ELISA-based tests, antigens should be sufficiently small, homogeneous, and capable of binding to the surface of the solid phase of the ELISA plate [20–22]. High temperature, ultrasound, and chemical treatments are routinely used to prepare different antigens; however, these approaches can change the structure of antigen. Therefore gentle processing methods like the extraction of surface cellular structures by salt solutions with different ionic strength using different mild detergents have become more and more popular [23–25]. Surface antigens from pathogens including pathogenic mycobacteria as well as the ones excreted by pathogens are the first targets of immune response [26–30]. At present, there are no unified diagnostic kits based on serological methods that would allow

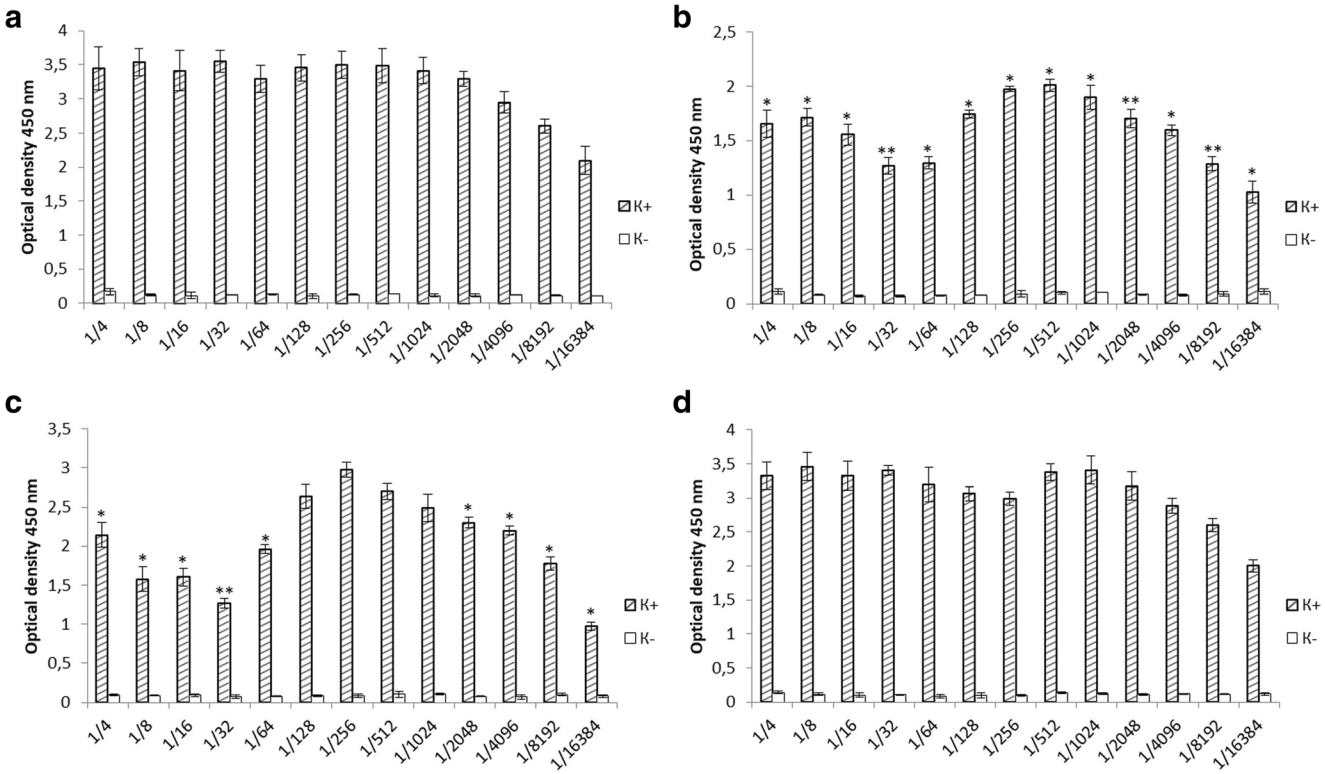

Fig. 3 Results of ELISA with CMF (**a**) and filtrates obtained using Vivaspin concentrators with pores for filtering proteins of 10 kDa (**b**), 30 kDa (**c**), and 50 kDa (**d**) molecular weight. The test material was immobilized in the wells of the plate at dilutions from 1:4 to 1:16,384. The test was performed with control positive (K+) and negative (K−) blood sera in a 1:100 dilution * $p < 0.05$, ** $p < 0.01$ compare to results of ELISA with CMF (**a**)

differentiation for the humoral response based on the antibody response to *M. tuberculosis*, *M. bovis*, BCG vaccine, and other mycobacteria, although work is in progress [31, 32]. In this paper, we show that the spectra of the secreted proteins and their serological properties depend on the mycobacterium species. The differences reported here in protein spectra and differences in serological activity create the prerequisites for further work on the isolation of unique differential antigens of these microorganisms.

In this work, we demonstrated that the antigen-containing material obtained by DMSO-extraction from *M. tuberculosis* had high activity and specificity when tested in an ELISA. However, a large number of fractions, as seen from the results of electrophoresis, show that the extract is an antigen with a large set of polypeptides that exhibit high-serological activity. This is apparently associated with antigenic complexes formed during the spontaneous association of polypeptides during storage in a liquid medium. Extraction of the components of the bacterial cell structure using a 10% solution of DMSO allowed obtaining an antigen-containing material with a wide range of protein with molecular weights from 6 to 200 kDa. The use of concentrators such as Vivaspin 2 allowed the isolation of preparative amounts of excreted *M. tuberculosis* and *M. bovis* proteins.

CMF material obtained with the initial preparation had the maximum specificity. At the same time, the signal was three times higher in the test with positive serum for all dilutions of the antigen. The obtained results indicate that the serologically active components of the filtrate are located in the molecular weight zone up to 50 kDa. Using electrophoresis, it was found that main fraction of antigens with a molecular weight of less than 50 kDa is not filtered through Vivaspin 50 kDa MWCO, which is probably due to the aggregation of proteins in the culture medium. Using immunoblot, it was shown that retentate from the membrane-contained antigens with high-serological activity, while the serological activity of the filtrates was practically absent.

The main purpose of using Vivaspin was to obtain native-secreted antigens. From the results of experiments, it becomes clear that native proteins most likely form high-molecular complexes that cannot pass through the pores of the membranes used. Experiments performed under denaturing conditions (using SDS) showed a significant amount of proteins that could potentially pass through this membrane. Reflecting this fact is the serological activity for the filtrate obtained using Vivaspin 50 kDa MWCO. Thus, the native material was above the capacity of the columns selected.

At all stages of the experiment, we used the same volume of the original material, which implies equal conditions. On all

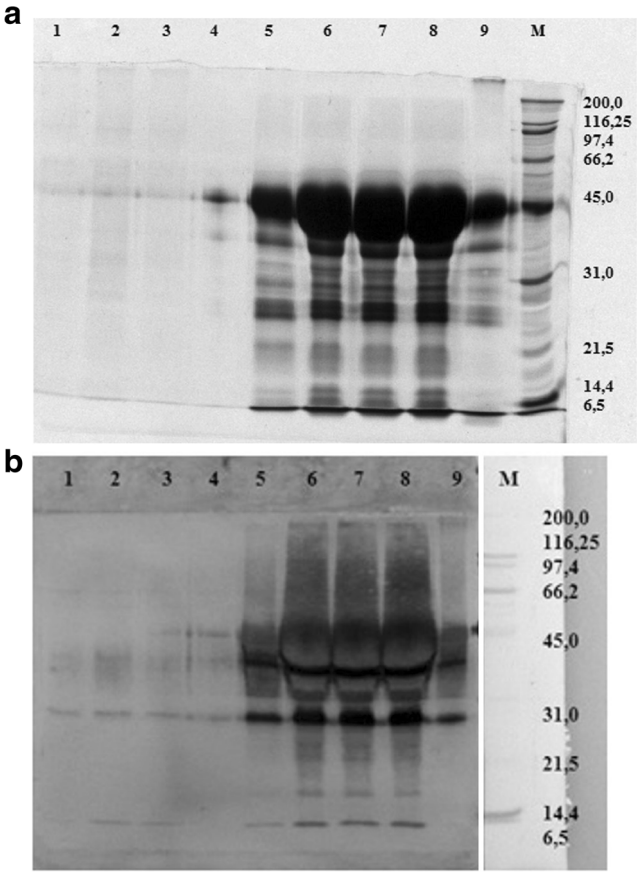

Fig. 4 Electrophoresis (**a**) and immunoblot (**b**) of antigen-containing preparations isolated from Soton medium during cultivation of *M. tuberculosis* using Vivaspin concentrators with pore sizes of 10, 30, and 50 kDa. Tracks: **1**—original CMF, **2**—10 kDa filtrate, **3**—30 kD filtrate, **4**—50 kD filtrate, **5**—ammonium sulfate precipitate of CMF, **6**—retentate from a 10 kDa concentrator, **7**—retentate from a 30 kDa concentrator, **8**—retentate from a 50 kDa concentrator, **9**—ethanol precipitate of CMF, **M***—set of proteins with known molecular weight *SDS-PAGE Standards, broadrange (Bio-Rad) were used as a marker of molecular weight (M). The marker strips were stained using PonceauS (Sigma-Aldrich) 0.2% *w/v* in 3% *w/v* of trichloracetic acid within 30 min, then washed by 7% acetic acid solution and di H_2O. The marker strips were placed beside immunoblot sample strips and then documented with Gel Doc XR+ System

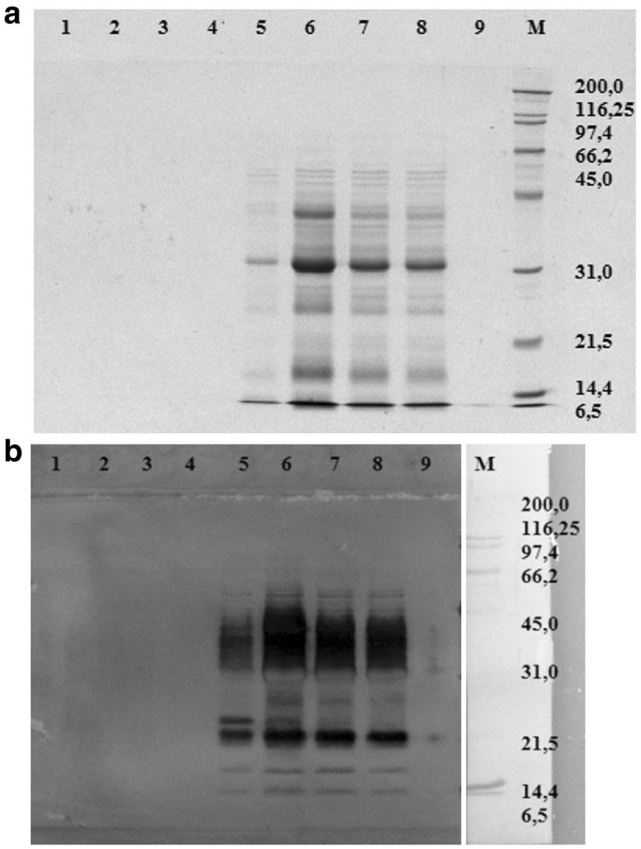

Fig. 5 Electrophoresis (**a**) and immunoblot (**b**) of antigen-containing preparations isolated from Soton medium during cultivation of *M. bovis* using Vivaspin concentrators with pore sizes for proteins of 10, 30, and 50 kDa. Tracks: **1**—original CMF, **2**—10 kDa filtrate, **3**—30 kD filtrate, **4**—50 kD filtrate, **5**—ammonium sulfate precipitate of CMF, **6**—retentate from a 10 kDa concentrator, **7**—retentate from a 30 kDa concentrator, **8**—retentate from a 50 kDa concentrator, **9**—ethanol precipitate of CMF, **M***—set of proteins with known molecular weight *SDS-PAGE Standards, broadrange (Bio-Rad) were used as a marker of molecular weight (M). The marker strips were stained using PonceauS (Sigma-Aldrich) 0.2% *w/v* in 3% *w/v* of trichloracetic acid within 30 min, then washed by 7% acetic acid solution and di H_2O. The marker strips were placed beside immunoblot sample strips and then documented with Gel Doc XR+ System

the columns, regardless of the pore size of the membrane, a tenfold concentration of the material was carried out. Thus, the absence of differences between the non-concentrated material (track 1, Figs. 4 and 5) and the filtered material (tracks 2, 3, 4, Figs. 4 and 5), both in electrophoresis and immunoblot, indicates a very low concentration of proteins in these samples. The differences that we observe between samples concentrated with ammonium sulfate and ethanol (tracks 5 and 9, Figs. 4 and 5) are associated with the different efficiencies of these techniques.

It was found that retentates from the membranes contain specific antigens, which can serologically differentiate *M. bovis* and *M. tuberculosis* infections. The spectra of excreted proteins of these microorganisms differ significantly: in *M. tuberculosis*, the major proteins had molecular weights ranging from 32 to 45 kDa and in *M. bovis*, the protein spectrum included five major fractions in the range of 6.5 to 43 kDa. The serological activity of the fractionated antigens in the immunoblot confirmed the possibility of using these proteins for the differential diagnosis of *M. bovis* and *M. tuberculosis*.

Further work is required to fully investigate the findings presented here; we expect that with further more specific enrichment of DMSO extracts, improvements in results over what is reported here could be possible. In this paper, we showed the potential of using serological methods for differential diagnosis of *M. bovis* and *M. tuberculosis* infections. However, further studies in which sera from well-characterized patients are used to confirm results are required.

5 Conclusion

Extraction of *M. tuberculosis* cell surface structures using a 10% solution of DMSO resulted in the preparation of an antigenic material in the molecular weight of 6.5 to 200 kDa which displayed high-serological activity. The series of experiments with CMF revealed that the most promising, in terms of serological detection, was the antigenic fraction prepared with the retentates from the Vivaspin 50 kDa MWCO filter membranes. The spectrum of serologically active expression products of *M. tuberculosis* was determined to be in the range 32 to 45 kDa. Based on this initial report, results indicate that further study of extracellular expression products could be useful for differential diagnosis of *M. bovis* and *M. tuberculosis* infections.

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Compliance with Ethical Standards Animals were not used in this study.

Conflict of Interest The authors declare that they have no conflict of interest.

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