Tuberculosis 93 (2013) 381–388

Contents lists available at SciVerse ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

REVIEW

Tuberculosis diagnosis using immunodominant, secreted antigens of *Mycobacterium tuberculosis*

Aliya Bekmurzayeva, Marzhan Sypabekova, Damira Kanayeva*

Nazarbayev University Research and Innovation System, Nazarbayev University, 53 Kabanbay Batyr Avenue, Astana 010000, Kazakhstan

ARTICLE INFO

Article history:
Received 18 October 2012
Received in revised form
11 March 2013
Accepted 23 March 2013

Keywords:
Tuberculosis diagnosis
Mpt64
Esat6
Cfp10
Ag85
Biosensor

SUMMARY

Tuberculosis (TB) remains a major public health concern in most low-income countries. Hence, rapid and sensitive TB diagnostics play an important role in detecting and preventing the disease. In addition to established diagnostic methods, several new approaches have been reported. Some techniques are simple but time-consuming, while others require complex instrumentation. One prominent and readily available approach is to detect proteins that *Mycobacterium tuberculosis* secretes, such as Mpt64, the 6-kDa early secreted antigenic target (Esat6), the 10-kDa culture filtrate protein (Cfp10), and the antigen 85 (Ag85) complex. Although their functions are not fully understood, a growing body of molecular evidence implicates them in *M. tuberculosis* virulence. Currently these biomarkers are either being used or investigated for use in skin patch tests, biosensor analyses, and immunochromatographic, immunohistochemical, polymerase chain reaction-based, and enzyme-linked immunosorbent assays. This review provides a comprehensive discussion of the roles these immunodominant antigens play in *M. tuberculosis* pathogenesis and compares diagnostic methods based on the detection of these proteins with more established tests for TB.

1. The current use of immunodominant, secreted antigens in TB diagnosis

Although the World Health Organization (WHO) has recommended wider use of liquid cultures in countries with a high tuberculosis (TB) burden, traditional culture tests are prone to a high rate of false positives due to cross-reactivity with non-tuberculous mycobacteria. Thus, it is important to rapidly identify *Mycobacterium tuberculosis* (MTB) in positive liquid cultures to confirm TB infection.[1] Traditionally, MTB has been differentiated from other mycobacterial species on the basis of growth characteristics and biochemical reactions.[2] Unfortunately, biochemical tests are slow, while nucleic acid amplification tests (NAATs) are cumbersome in low resource settings.[1] Nowadays, these methods have been replaced by rapid but expensive molecular tests. Currently, there are also a number of commercially available serological tests for the diagnosis of TB.

Although many other diseases can be diagnosed reliably via the detection of antibodies or antigens in blood, diagnosing TB serologically remains a challenge. The most commonly used serological tests are rapid immunochromatographic assays (ICAs), based on lateral flow or flow-through analysis, or microwell enzyme-linked immunosorbent assay (ELISA) tests.[3] The advantages of serological methods are convenience and simplicity of use, a high negative predictive value, and cost-effectiveness. However, at present serological tests do not provide enough sensitivity and specificity to be used as a first-line screening tool. Detection of antibodies can lead to false positive results, since antibodies against environmental mycobacteria are often present; thus, serology cannot be used to distinguish between infection by MTB and infection by mycobacteria other than tuberculosis (MOTT). Moreover, serological methods cannot reliably distinguish between active and latent TB. For these reasons, despite their simplicity of use, serological tests are not yet recommended by the international TB community, and their primary use is in the private sector, where diagnostic regulatory bodies have less authority.[4–6] Nevertheless, new protein targets are being investigated, different combinations of antigens are being proposed, and the number of commercially available serological tests is increasing due to their simplicity. Targets for antigen detection must be highly immunogenic and abundantly secreted in culture medium.[7] Various culture filtrate proteins that could be used for the detection of disease have been proposed. Among them, the predominant immunodominant, secreted proteins are Mpt64, the 6-kDa early secreted antigenic target (Esat6), the 10-kDa culture filtrate protein (Cfp10) and the antigen 85 complex (Ag85).[2] These proteins have been used for TB diagnosis in

* Corresponding author. Tel.: +7 7172 70 64 78; fax: +7 7172 70 63 20.
E-mail address: dkanayeva@nu.edu.kz (D. Kanayeva).

1472-9792/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tube.2013.03.003

skin patch tests, various biosensing systems, and immunohistochemical, immunochromatography, ELISA-based, and polymerase chain reaction (PCR)-based assays. Here, we review what is known about each of these secreted proteins.

1.1. The Mpt64 protein

Mpt64 (also known as Mpb64 and Rv1980c) is a secreted, immunogenic protein of MTB.[8,9] It is highly specific for the *M. tuberculosis* Complex (MTBC), including MTB, *Mycobacterium africanum*, virulent *Mycobacterium bovis*, and some substrains of the *M. bovis* bacillus Calmette–Guérin (BCG). Most importantly, it is not found in MOTT.[10–12] Mpt64 falls within 1 of the 11 regions of difference (RDs) absent in certain BCG strains.[13–16] Loss of RD2 is associated with a decrease in virulence in animal infection models and attenuated vaccine lesions in humans.[17] Various molecular weights for Mpt64 have been reported in the literature: 22.3 kDa[18]; 23 kDa[10]; 23.5 kDa[19]; 24 kDa[20]; and 28 kDa.[21] Accounting for about 8% of the total protein found in culture filtrate,[22] it is one of the predominant proteins among more than 33 actively secreted during MTBC growth and is expressed early, when the bacterium is actively dividing.[10,20,23] The proteins secreted by *Mycobacteria* are important antigens recognized early in the host response to infection; they stimulate T-cell proliferation and interferon-gamma (IFN-γ) release from peripheral blood mononuclear cells (PBMCs).[11] In patients with early MTB infections, T-cells recognize several Mpt64 epitopes. For the reasons described above, Mpt64 has been studied extensively and used in the diagnosis of TB. Mpt64-based identification methods differ from each other in terms of specificity, sensitivity, detection time, availability, and price.

Mpt64 is being investigated as a candidate vaccine, both alone[24] and as a fusion protein with Esat6, a potent T-cell antigen,[25,26] and ubiquitin.[27] In guinea pigs, Mpt64 induces a strong, TB-specific, delayed-type hypersensitivity (DTH) response. Characterizing the proteins recognized by human T-cells is important for developing more effective vaccines and more specific diagnostic methods. The DTH activity of Mpt64 is due to a region named the T-cell reactive epitope CE15 that lies between amino acid residues G173 and A187.[14] In PBMCs from TB patients and cattle infected with *M. bovis*, Mpt64 is not very effective at eliciting IFN-γ release compared to TB antigens such as Esat6 and Cfp10.[28] Mpt64 is thought to have an anti-apoptotic effect[16] in MTB granulomas and to promote the persistence of *Mycobacterium*-infected macrophages.[29]

1.2. The Ag85 complex

The *Mycobacterium* Ag85 complex is involved in biological processes such as cell wall biosynthesis and immunogenic responses.[30–32] This complex compromises three variant Ag85 proteins, A, B and C, which are encoded by separate genes. They are generally expressed in a 3:2:1 ratio of B:A:C, but this ratio varies in response to changes in the environment. Although B has the greatest expression level, C is 8 times more biologically active than B. All three variants, in combination, are necessary for evading the host immune response.[33]

The Ag85 proteins are secreted and can be found in the phagosome, the external layer of the bacterium's cell wall, and the blood.[33,34] In blood, the binding of the Ag85 complex to plasma fibronectin, an extracellular matrix glycoprotein, or immunoglobulin G alters the host's immune response. This interaction appears to reduce phagocytosis of MTB, thereby promoting infection.[35,36] Ag85 expression is essential for the intracellular survival of MTB within the macrophage.[37] and the protein complex possess mycolyltransferase activity. It also catalyzes the synthesis of trehalose 6,6-dimycolate, the most abundant glycolipid in the mycobacterial cell wall.[38]

1.3. Cfp10/Esat6 family proteins

Cfp10 and Esat6 are early secreted proteins with molecular weights of 10- and 6-kDa, respectively. In culture, they are some of the most abundant MTB antigens. They are not found in *M. bovis* BCG or most MOTT7 but are present in *M. kansasii*, *M. szulgai*, *M. marinum*, and *M. riyadhense*.[39] These proteins fall within RD1, which plays a role in virulence and are missing in BCG. Cfp10 and Esat6 are members of a protein family encoded by genes aligned in pairs within the *Mycobacteria* genome, and their secretion is an active process involving a specialized transport system, itself the product of several flanking genes.[40,41] The genes encoding these proteins are co-transcribed and form a tight 1:1 complex. The C-terminus of Cfp10 forms a long flexible arm, which plays an important role in cell surface attachment.[40] These proteins are important stimulators of T-cells and are used in interferon gamma releasing assays (IGRAs). BCG complemented with the esat6 gene exhibits increased virulence in mice infected with a low-dose aerosol.[42]

Approximately one-third of all TB patients are seronegative for any single antigen.[43] That is why combinations of proteins, either separately or fused, have been studied. Ag85B-Esat-6, Ag85B-Tb10.4, and Mtb10.4-HspX are currently vaccine candidates. Ag85B-Esat-6 and Ag85B-Tb10.4 combine early secreted proteins, while the Mtb10.4-HspX combination is based on both dormant and early secreted antigens.[44,45] Members of the Cfp family other than Cfp10, namely Cfp25, Cfp20.5 and Cfp3, are highly immunogenic as well. They, along with the Ag85A and B proteins, have been selected as candidates for a multicomponent subunit vaccine.[46]

2. Diagnostic assays that use proteins secreted by MTB

Many TB diagnostic assays exploit the immunogenicity of the MTBC secreted proteins discussed thus far, and more tests that use them are being developed (Figure 1). Such assays include new twists on established methods, such as the skin patch test, as well as novel tests that make use of highly sensitive biosensors. In the sections that follow, each of these assays will be described in detail.

2.1. Skin patch test

In the tuberculin skin test (TST), a very small amount of a substance called tuberculin purified protein derivative (PPD), a mixture of antigens derived from heat-killed MTB, is injected just under the top layer of the skin to see whether a hypersensitivity reaction occurs. A positive result (in the form of erythema and/or vesiculation) results when an individual has active TB, was recently vaccinated with BCG, or has been cured of TB. A positive reaction usually appears 3–4 days after PPD is administered. The TST cannot distinguish latent from active TB, and a positive result can occur even if an individual is infected with another *Mycobacteria* species.[47] Since PPD consists of a cocktail of MTB proteins, some of which may cross-react with environmental mycobacteria or BCG, alternatives to the traditional test have been investigated.

Mpt64 is one antigen capable of eliciting a distinctive response in people with active TB. A Mpt64-based TB patch test developed by Sequella has been shown repeatedly to yield negative results in patients with prior BCG vaccination, those who are infected with other *Mycobacteria*, and those who have been cured of TB.[48] In studies carried out in Japan and the Philippines, the Mpt64 skin patch test had 98% sensitivity and 100% specificity. One advantage of this test is its simplicity. There is no need for laboratory

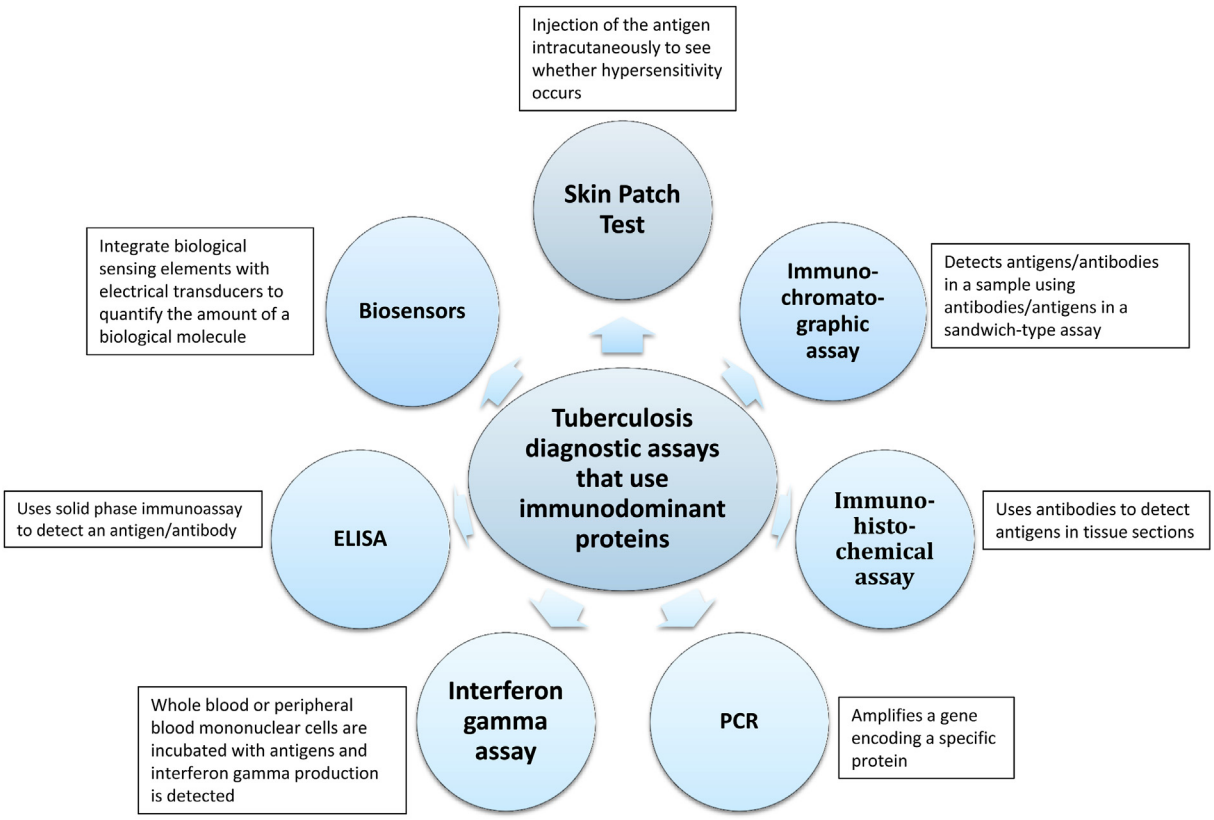

Figure 1. Types of assays based on immunodominant proteins of *Mycobacterium tuberculosis* for diagnosis of tuberculosis.

equipment or highly skilled professionals (Table 1).[49] This test has the potential to make an impact in countries with a heavy TB-burden, especially if it proves accurate, is capable of distinguishing between latent and active forms of TB, and is unaffected by anergy.[50]

Esat6 and Cfp10 are also being studied as alternatives to PPD. A test based on recombinant Esat6 and Cfp10 has been developed for testing for DHR; it has shown high sensitivity in the diagnosis of active and new TB infections. Furthermore, no false positives in BCG-vaccinated people have been observed.[51] Another study, this one in guinea pigs, has found that the DHR elicited by seven recombinant TB proteins actively secreted in culture (Esat-6, Cfp10, Tb10.3, Tb10.4, Mtsp11, Mpt70, and Mpt83) is stronger than that resulting from the standard PPD.[52] Finally, an intradermal diagnostic test containing recombinant Esat6, the 16-kDa heatshock protein (HspX), PhoS/PstS1, Mpb70, Mpb64, Mpb83, Ag85, and the SahH proteins has been developed for diagnosing TB in cattle. This method was designed for TB diagnosis in non-human mammals and uses recombinant proteins from *M. bovis*.[53] The researchers involved assert that the combination of many proteins in one test will enhance its diagnostic accuracy.

2.2. Immunochromatographic assays

ICAs are rapid tests that detect one or more antigens or the antibodies against them. Test samples (blood/serum or bacterial culture) flow laterally in a simple device, and positive reactions are represented by colored strips. ICAs, along with ELISAs, are the most commonly used rapid tests for diagnosing TB. Commercially available ICAs detect IgG and IgM antibodies against TB antigens, although many manufacturers do not specify which antigens are immobilized by their assays. Available tests include the One-Step TB Test Kit (Health-Chem Diagnostics, USA), the Tell Me Fast Mycobacterium Tuberculosis IgG/IgM Test Device (BioCan Diagnostics, Canada), the SD TB IgG/IgM (SD Bioline, South Korea) and others.

Three ICAs based on the detection of Mpt64 have been developed: the Capilia TB rapid diagnostic test (Tauns Laboratories Inc., South Korea), the SD Bioline TB AG Mpt64 rapid test (Standard Diagnostics, Yongin, South Korea), and the BD MGIT TBc Identification test (BD Diagnostics, Sparks, MD, USA).[20,54] Each test differs slightly in terms of performance, sensitivity, and specificity. The WHO has endorsed the Capilia TB rapid diagnostic test, which is now being marketed as the Tibilia TB rapid test (Genesis, China),[2] for confirmation of TB in positive liquid or solid cultures. However, it is not as widely available as the other two tests.[9] While high TB-burden countries can obtain the Capilia test kit through the Foundation for Innovative New Diagnostics, middle and high income countries cannot. By contrast, the SD Bioline TB Ag Mpt64 Rapid test and the BD MGIT TBc Identification test can be obtained on the private market without any restrictions. When the Capilia and SD Bioline TB Ag MPT64 Rapid Test kits have been compared, they have yielded similar results.[10] Brent and colleagues performed a meta-analysis comparing the performance of the BD MGIT TBc test with that of line probe assays, which are based on reverse hybridization assays, and concluded that there was no significant difference in the accuracy of their detection of MTB isolates. Similarly, a meta-analysis comparing the three commercially available ICAs listed above demonstrated uniformly high sensitivities and specificities, as well as high positive and negative predictive values.[1] Many commercially available tests are very similar in performance. For example, the SD Bioline TB Ag Mpt64 ICA uses a mouse monoclonal anti-Mpt64 antibody. This assay can distinguish between MTB and MOTT and can be used with colony or

Table 1
Comparison of detection assays based on immunodominant secreted proteins of MTB and their genes. The table shows advantages and disadvantages of each assay.

Assay	Time until results	Advantages	Disadvantages
Skin patch test [6,48–50]	• Results are visible after 3–4 days	• No sample preparation/treatment required • Simple • No need for laboratory equipment or highly skilled professionals	• Follow-up visit required • Wide range of sensitivities and specificities depending on the population tested and reaction cut-off points • Results affected by infection with HIV, BCG, and other mycobacteria
Immunochromato-graphic assay[2,9,20,24,54,55,82]	• Sample growth* and pretreatment: 1–2 weeks • Pretreated sample detection: 15–20 min	• Can discriminate between MTB[a] and MOTT[b] • High sensitivity and specificity (97.9 and 98.6%, respectively) • Competitive cost (less than $5 USD for SD Bioline and MGIT tests)	• Must grow bacteria on liquid or solid media (detection limit 10^5 CFU/ml). Hence, special laboratory safety precautions and incubation time are required. • Mutation or deletion of the target gene can result in false negatives • Cannot distinguish between MTB species • Not designed for direct analysis of clinical samples • Cannot be used to characterize drug susceptibility
Immunohistochemical assay[58–60,62]	• Sample preparation and pretreatment: 1–3 weeks • Pretreated sample detection: 3–4 h	• High sensitivity (89–93%) and specificity (95– 98%). Can detect $\sim 5 \times 10^5$ to 1×10^6 organisms/g tissue • Can be used on lymph node aspirates, cerebrospinal fluid, and pleural effusions	• Biopsy must be formalin-fixed and embedded in paraffin • Due to cross-reactivity, specificity is low relative to other assays • Results must be validated using other diagnostic techniques such as PCR and AFB staining
ELISA[50,63]	• Sample preparation and pretreatment: 13 weeks • Pretreated sample detection: 2–3 h	• Sensitivity and specificity: 98.2% and 100%, respectively. Minimum detectable concentration of target protein: 0.01 mg/L. • Relatively inexpensive compared to nested PCR • Does not require sophisticated instrumentation	• Need to grow bacteria on liquid or solid media (detection limit is 10^5 CFU/ml). Hence, biosafety level-3 lab needed, and incubation time is required. • Not designed for direct use on clinical samples
PCR followed by automated sequencing[66,68–70]	• DNA digestion and extraction: 24–48 h • PCR reaction: 3–4 h • Automated sequencing: 4 h	• Delivers prompt results on size and sequence of gene • Does not distinguish between active and latent tuberculosis	• Requires sophisticated instrumentation and reagents • Time consuming Too expensive for routine use in low income countries
Interferon gamma release assay[6,83]	• 48 h	• More objective than tuberculin skin test • Response to multiple antigens is assessed • Can discriminate between MTB and environmental mycobacteria • No cross-reaction with BCG	• Requires fresh samples • Cannot discriminate between active and latent TB • Expensive
Biosensors[75–77,84,85]	• 1–3 h	• Offer rapid, sensitive, and specific detection • Reusable	• Too expensive for everyday use at present

MTB[a] – *Mycobacterium tuberculosis*; MOTT[b] – Mycobacteria other than tuberculosis.
* This time estimate is for the Bactect MGIT test.

condensation fluid from solid and liquid culture media. The test has high analytical performance, is user friendly, can be stored at ambient room temperature, has a detection limit of 10^5 CFU/mL, and can yield results in 15 min (Table 1). Nevertheless, studies have revealed that Mpt64-based immunochromotographic assays have limitations such as equipped facilities for bacterial growth, false negatives due to mutations in genes encoding tha protein this assay detects and others as shown in Table 1.

Other assays use alternative TB-secreted proteins. An ICA that detects Esat6 and Cfp10 is reported to be less expensive than the Capilia TB test, but proteins similar to Esat6/Cfp10 are present in MOTT such as *M. kansasii*, *M. szulgai*, *and M. marinum*.[55] A more sophisticated version of ICA, the double antigen colloidal gold immunochromatographic assay, has been developed by Fan et al.[56] and detects an Esat6-16-38 fusion protein. In addition, a rapid membrane-based test system targeting the Ag85 complex has been developed to diagnose pulmonary TB. The overall specificity of this assay is 93%, and the positive predictive value is 95%.[57]

2.3. Immunohistochemistry

Immunohistochemistry (IHC) uses antibodies to detect antigens in tissue sections, and it has been used to diagnose various diseases. Using polyclonal and monoclonal antibodies (mAbs), IHC can be used to detect Mpt64 in biopsies fixed with formalin or embedded in paraffin (Figure 2).[58] In patients with abdominal TB or tuberculous lymphadenitis, this test has high sensitivity (89–93%) and specificity (95–98%). It can detect as few as 5×10^5 to 1×10^6 organisms per gram of tissue.[59] Other samples that have been successfully used for IHC are lymph node aspirates, cerebrospinal fluid, and pleural effusions.[59] In addition, Baba and colleagues have shown that using Mpt64 antibodies on formalin-fixed biopsies to diagnose pleural TB has a sensitivity of 81% and a specificity of 100%.[60] In an alternative approach, one group has stained formalin-fixed paraffin sections of lymph node biopsies with polyclonal antibodies against Esat6, HspX, Tb8.4, and the phospholipase C-encoding A (PlcA) protein. They report Esat6 antibodies are the most sensitive (88.6%), while Esat6 and PlcA antibodies are the most specific (100%). Overall results correlate well with Zeihl-Neelson staining, a "gold standard" in TB diagnostics.[61] The main drawback of IHC is that invasive tissue samples must be collected and treated, and this is time consuming (Table 1). Moreover, the specificity appears relatively low due to cross-reactivity with environmental mycobacteria. Therefore, at present IHC results should be validated using other diagnostic techniques, such as PCR and acid fast bacillus (AFB) staining.[62]

2.4. ELISA and PCR

ELISA and PCR have also been used as diagnostic tools and since many studies compare performance of these tests side-by-side they are discussed in one section. ELISA is based on a solid phase

Figure 2. Immunohistochemical staining by anti-MPT64 of granuloma in intestinal wall (abdominal tuberculosis). The area in square is magnified in subsequent sections (source: Ref. [59]).

immunoassay that detects the presence of antigens or antibodies. Liu and colleagues have developed a double antibody sandwich ELISA for the detection of Mpt64. The sensitivity of this assay is 97.3%, the specificity is 100%, and it can detect as little as 0.01 mg/L of the target protein. This compares to a sensitivity and specificity of 98.2% and 100%, respectively, for PCR amplification of the gene encoding Mpt64.[63] PCR is a molecular biology technique that amplifies one or more copies of a piece of DNA, generating thousands to millions of copies of a particular DNA sequence. Compared to nested PCR, the Mpt64-ELISA test system is relatively cheap and does not require sophisticated instrumentation. Hence, its use is feasible in the majority of high TB-burden countries[50] (Table 1).

ELISA is thought to be the gold standard for detecting antibodies or antigens in biological fluids. However, despite its simplicity, the sensitivity and specificity of this method can be problematic. The performance of tests based on the detection of the Ag85 complex illustrates this point. In one study, an Ag85-based ELISA was used to test body fluids from children with pulmonary and central nervous system TB ($n = 83$) and matched controls ($n = 32$). The results showed only 71.9% sensitivity. However, another comparison has shown that the sensitivity of an Ag85-based ELISA (51.9%) is significantly higher than that of the Lowenstein Jensen (LJ) medium culture method (19.3%), the BacT/Alert 3D system (24.1%), and ZN staining (16.9%).[64] It is important to note that tests based on detection of antibodies against the three different proteins of the Ag85 complex show different sensitivities, based on characteristics such as the abundance of the target antibody within a sample. One study has demonstrated that an ELISA targeting Ag85C antibodies shows good concordance with PCR positivity when used to diagnose childhood TB.[65]

As described above, PCR is also a commonly used diagnostic method. Real-time PCR amplification of the *mpt64* gene has been used to quantify the mycobacterial load in the vitreous humor of patients thought to have Eales disease.[66] This test is more sensitive than the conventionally used *IS6110* PCR, especially in patients with extra-pulmonary, smear-negative TB.[67] Another group has reported an increased diagnostic yield in the detection of osteoarticular TB by amplifying the *mpt64* and *IS6110* genes together, using multiplex PCR.[68] This type of test is especially important in countries such as India, where MTB strains lack the *IS6110* gene. In addition, multiplex, nested PCR amplification of these two genes along with *rrs* and *rpob* increases the test's specificity and sensitivity when diagnosing extrapulmonary TB.[69] Improved sensitivity has also been reported when an *mpt64*-based PCR test is used to diagnose central nervous system TB.[70]

The genes coding for the mycobacterial Ag 85 also can be amplified by different types of PCR. 12 patients with active disease (57 specimens), 12 patients with silent tuberculosis infection (23 specimens) and 27 patients without tuberculosis (77 specimens) were tested by amplification of Ag85 followed by hybridization of a probe specific for *M. tuberculosis* on the Southern blot of amplified DNA. The sensitivities of PCR then compared to the clinical diagnosis, and to other diagnostic techniques: 7.0% for acid fast staining, 22.8% for culture, and 24.6% for PCR/Ag85.[71] Authors were able to detect *M. tuberculosis* DNA in specimens containing a few microorganisms. PCR method is more sensitive than culture, and the results are available more quickly. However, during analysis some false positive results might occur that can lower specificity,[6] and in this case, culturing remains to be the gold standard to establish definitive diagnosis of primary tuberculosis infection.

2.5. Interferon gamma releasing assay

Interferon gamma (IFNg) releasing assay or IGRA is a relatively new type of *in-vitro* analysis based on cell mediated immune response. This assay became the first assay as an alternative for TST in detection of latent TB. However, it cannot discriminate latent TB from active disease. There are currently two commercially available assays: QuantiFERON-TB Gold In-Tube (QFT-GIT) assay (Cellestis Ltd, Carnegie, Australia) and the T-SPOT.TB assay (Oxford Immunotec, Oxford, UK). The first assay quantifies IFNg released when whole blood is incubated with peptides of CFP10, ESAT6 and TB7.7 antigens of TB in ELISA format. T-SPOT.TB is enzyme-linked immunospot (ELISPOT) assay that measures cells themselves releasing IFNg when treated with ESAT6 and CFP10.[72] There have been concerns about lack of sensitivity of IGRAs in detection of LTBI, these assays are shown to be unable to distinguish active TB from LTBI.[2,39] In order to overcome disadvantages of CFP10/ESAT-based ELISPOT, other proteins (Rv1978, NrdF1, Mpt64, CFP-21, Ppe57 and Ppe59) were expressed and used in ELISPOT. This study showed that they are specifically recognized by human PBMC in both active and latent TB patients and thus ELISPOT and probably other IGRAs can be further improved.[73] Also a more recent study states that IGRAs can be both used for active and latent TB infection.[74] However, authors of both papers recommend further large-scale and longitude studies.

2.6. Biosensors

The detection of disease-related proteins requires sensitive, specific, and low cost assays. Numerous biosensors have been developed for the detection of such molecules, and each differs in terms of specificity, composition, and price. In general, a biosensor is an analytical device that integrates a biological sensing element and an electrical transducer to quantify a biological event using electrical output.[75]

Since a biosensor can be used to detect more than one molecule, several groups have mentioned the potential of their proposed biosensing systems to detect Mpt64. One such group devised a system employing primary aptamer-functionalized silica

nanoparticles (NPs) and a fluorescein-labeled (cationic conjugated polymer) secondary aptamer to detect thrombin molecules in human serum.[76] The presence of thrombin induces the NPs and secondary aptamers to form sandwich structures, resulting in fluorescent NPs (detection limit: 1.06 nM). The system's creators state that this method could also be used to detect Mpt64, since Mpt64 contains two or more binding sites for aptamers. Another group has synthesized fluorescent ferritin nanoparticles via bacterial expression of a hybrid gene (combining the ferritin heavy chain, or *hFTN-H,* gene and the enhanced green fluorescent protein, or *egfp*, gene) for the detection of the platelet-derived growth factor b-chain homodimer in diluted human serum.[77] This complex uses a fluorescent reporter probe in an aptamer-based sandwich assay similar to the one described above. Using this methodology, the limit of detection has been lowered to 100 fM, and the same principle of detection could also be used to target Mpt4. To date, there is only one biosensor study that has specifically focused on Mpt64 detection.[78] The Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method has been used to generate aptamers specific to Mpt64. These aptamers are used in a sandwich-based assay, where they form a complex with Mpt64. This method is reported to have high specificity and sensitivity.

Recently, a waveguide-based optical biosensor platform has been designed for the direct detection of three TB-specific biomarkers, namely liparabinomannan (LAM), Esat6 and Ag85.[79] The whole system is based on a sandwich immunoassay consisting of a biotinylated capture antibody, the TB antigen, and a fluorophore-labeled reporter antibody located on the sensor's surface. The generated evanescent field and a spectrometer are used to detect fluorescence in the proximity of the sensor surface. The proposed sensor has a detection limit of 0.5 pM for Esat6, 100 pM for Ag85, and 1 pM for LAM. However, poor antibody stability precludes the use of this assay on clinical samples at present.

Another group has developed an immunoassay based on surface plasmon resonance (SPR) spectroscopy that targets Cfp10.[7] Here, the sensing interface is constructed by directly immobilizing Cfp10 on the gold surface of an SPR chip, after which bare surfaces are blocked with cystamine. The limit of detection of this assay has been reported at 100 ng/mL, and a linear relationship between the SPR angle shift and Cfp10 concentrations has been observed from 0.1 to 1 μg/mL protein. This biosensor has been tested on urine samples from TB patients.[80] A total of 55 patients, classified by AFB stain stages from sputum samples, and a calibration curve obtained from studies with recombinant Cfp10 were used to investigate the correlation between SPR angle shift and AFB stage. The authors observed a significant difference in angle shift between healthy and infected patients' samples, as well as a linear relationship between Cfp10 concentration and the number of bacterial cells determined by AFB staining. Thus, this assay has the potential to be a rapid and label-free diagnostic tool for the early detection of TB as well as for the classification of disease severity.

3. Conclusions and future perspectives

Clearly, the prompt diagnosis of MTB is a crucial problem. A number of proteins secreted by MTB are both abundant in culture and absent in MOTT. Assays that exploit these features of secreted proteins could play an important role in detecting active TB.[6]

Nevertheless, there are limitations associated with assays based solely on the detection of secreted proteins or the genes that encode them. For instance, mutations and/or deletions in a protein's gene[1,20] or low protein concentration ($<10^5$ CFU/ml) in early or mixed cultures[1] can result in false negative results. In addition, protein-based methods such as ICAs cannot be used for drug susceptibility testing[1] and certainly cannot be used for differentiating between MTB species. Moreover, commercially available ICAs that detect antigens cannot be used directly on clinical samples. Prior to use, the kits must be decontaminated and mycobacteria must be grown in suitable conditions, i.e. BSL 3 laboratories. Finally, since the proteins targeted are secreted by actively dividing MTB, assays that rely on them can only be used to diagnose active TB. Assays based on immune response can result in varying results due to difference in immune response of the patients.

One potential solution to the limitations of these assays is to combine them with other, more conventional methods. For example, combining Mpt64-based assays with AFB smears and NAATs could potentially improve overall sensitivity.[68] Although commercially available kits are supposed to be performed on cultured samples, according to a patent by one manufacturer, Namba et al., ICAs can be carried out on non-cultured samples, e.g. sputum. Prior to testing, the MTB in samples can be inactivated, dispersed, or solubilized.[81]

Skin patch test system based on antigens very simple and no dedicated equipment is required for it. However, one must wait for several days to be able to see the result (Table 1), and also the procedure is invasive and unpleasant. PCR remains to be a gold standard method however reagents and equipment used are costly. ICA, IHC and ELISA are all based on the principle of antigen-antibody interaction. Generally speaking antibodies are not stable, slightly expensive, especially for poor resource countries, and they cannot be used repeatedly. In comparison to antibodies more stable newly emerged molecular detection tools - aptamers offer a great potential in disease diagnosis as they are much stable, can be reused and inexpensively produced. The usage of such aptamers in biosensor development seeds a hope in obtaining user friendly and cheap diagnostic assay which will be available to everyone. However, the development of such biosensors nowadays is in its infancy state and modifications made to them should improve its quality later. Overall, diagnosis of tuberculosis based on solely secreted immunodominant proteins is not feasible without losses in sensitivity and specificity. Their performance could be further improved with a right combination of antigens/antibodies, new insights into biology of tuberculosis and advances in detection techniques, so that they can be a good complement for gold standard methods or even become a recognized assay for TB detection.

Acknowledgments

The authors acknowledge funding support from Ministry of Education and Science of the Republic of Kazakhstan.

Funding

009 Targeted fund for Autonomous Organization of Education "Nazarbayev University" from Ministry of Education and Science of the Republic of Kazakhstan.

Competing interests

None declared.

Ethical approval

Not required.

References

1. Brent A, Mugo D, Musyimi R, Mutiso A, Morpeth S, Levin M, Scott J. Performance of the MGIT TBc identification test and meta-analysis of MPT64 assays

for identification of the *Mycobacterium tuberculosis* complex in liquid culture. *J Clin Microbiol* 2011;**49**:4343–6. http://dx.doi.org/10.1128/JCM.05995-11.
2. Ngeow YF, Wong YL, Ng KP, Ong CS, Aung WW. Rapid, cost-effective application of tibilia TB rapid test for culture confirmation of live and heat-killed *Mycobacterium tuberculosis*. *J Clin Microbiol* 2011;**49**:2776–7. http://dx.doi.org/10.1128/jcm.00711-11.
3. Singh S, Katoch VM. Commercial serological tests for the diagnosis of active tuberculosis in India: time for introspection. *Indian J Med Res* 2011;**134**:583–7.
4. Hong SC, Lee J, Shin H-C, Kim C-M, Park JY, Koh K, Kim H-J, Chang CL, Lee J. Clinical immunosensing of tuberculosis CFP-10 in patient urine by surface plasmon resonance spectroscopy. *Sens Actuators B Chem* 2011;**160**. http://dx.doi.org/10.1016/j.snb.2011.10.006.
5. Deng S, Yuan T, Xia J, Huang H, Cheng X, Chen M. Clinical utility of a combination of lipoarabinomannan, 38-kDa and 16-kDa antigens as a diagnosis tool for tuberculosis. *Diagn Microbiol Infect Dis* 2011;**71**:46–50. http://dx.doi.org/10.1016/j.diagmicrobio.2011.04.015.
6. FIND. *Diagnostics for tuberculosis global demand and market potential* 2006.
7. Hong SC, Chen H, Lee J, Park H-K, Kim YS, Shin H-C, Kim C-M, Park TJ, Lee SJ, Koh K, Kim H-J, Chang CL, Lee J. Ultrasensitive immunosensing of tuberculosis CFP-10 based on SPR spectroscopy. *Sens Actuators B Chem* 2011;**156**. http://dx.doi.org/10.1016/j.snb.2011.04.032.
8. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry 3rd CE, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* 1998;**393**:537–44. http://dx.doi.org/10.1038/31159.
9. Martin A, Bombeeck D, Fissette K, de Rijk P, Hernandez-Neuta I, Del Portillo P, Palomino JC. Evaluation of the BD MGIT TBc Identification Test (TBc ID), a rapid chromatographic immunoassay for the detection of Mycobacterium tuberculosis complex from liquid culture. *J Microbiol Methods* 2011;**84**:255–7. http://dx.doi.org/10.1016/j.mimet.2010.12.003.
10. Roche P, Winter N, Triccas J, Feng C, Britton W. Expression of Mycobacterium tuberculosis MPT64 in recombinant Myco-smegmatis: purification, immunogenicity and application to skin tests for tuberculosis. *Clin Exp Immunol* 1996;**103**:226–32. http://dx.doi.org/10.1046/j.1365-2249.1996.d01-613.x.
11. Chaves D, Sandoval A, Rodriguez L, Garcia JC, Restrepo S, Mercedes Zambrano M. Comparative analysis of six *Mycobacterium tuberculosis* complex genomes. *Biomedica* 2010;**30**:23–31.
12. Abe C, Hirano K, Tomiyama T. Simple and rapid identification of the *Mycobacterium tuberculosis* complex by immunochromatographic assay using anti-MPB64 monoclonal antibodies. *J Clin Microbiol* 1999;**37**:3693–7.
13. Behr MA. BCG – different strains, different vaccines? *Lancet Infect Dis* 2002;**2**:86–92. http://dx.doi.org/10.1016/s1473-3099(02)00182-2.
14. Wang Z, Potter BM, Gray AM, Sacksteder KA, Geisbrecht BV, Laity JH. The solution structure of antigen MPT64 from *Mycobacterium tuberculosis* defines a new family of beta-grasp proteins. *J Mol Biol* 2007;**366**:375–81. http://dx.doi.org/10.1016/j.jmb.2006.11.039.
15. Weldingh K, Andersen P. Immunological evaluation of novel *Mycobacterium tuberculosis* culture filtrate proteins. *FEMS Immunol Med Microbiol* 1999;**23**:159–64. http://dx.doi.org/10.1111/j.1574-695X.1999.tb01235.x.
16. Kozak R, Behr MA. Divergence of immunologic and protective responses of different BCG strains in a murine model. *Vaccine* 2011;**29**:1519–26. http://dx.doi.org/10.1016/j.vaccine.2010.12.012.
17. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, Small PM. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 1999;**284**:1520–3. http://dx.doi.org/10.1126/science.284.5419.1520.
18. Protein Data Bank R. 2HHI literature report; 2012.
19. Chu T-PJ, Yuann J- MP. Expression, purification, and characterization of protective MPT64 antigen protein and identification of its multimers isolated from nontoxic *Mycobacterium tuberculosis* H37Ra. *Biotechnol Appl Biochem* 2011;**58**:185–9. http://dx.doi.org/10.1002/bab.26.
20. Gaillard T, Fabre M, Martinaud C, Vong R, Brisou P, Soler C. Assessment of the SD Bioline Ag MPT64 Rapid (TM) and the MGIT (TM) TBc identification tests for the diagnosis of tuberculosis. *Diagn Microbiol Infect Dis* 2011;**70**:154–6. http://dx.doi.org/10.1016/j.diagmicrobio.2010.12.011.
21. Kumar VG, Urs TA, Ranganath RR. MPT 64 Antigen detection for Rapid confirmation of *M. tuberculosis* isolates. *BMC Res Notes* 2011;**4**:79.
22. Liu Z, Zhu C, Yang H, Hu H, Feng Y, Qin L, Cui Z, Bi A, Zheng R, Jin R, Fan L, Hu Z. Clinical value of ELISA-MPT64 for the diagnosis of tuberculous pleurisy. *Curr Microbiol* 2012;**65**:313–8.
23. Wang Z, Potter B, Gray A, Sacksteder K, Geisbrecht B, Laity J. The solution structure of antigen MPT64 from *Mycobacterium tuberculosis* defines a new family of beta-grasp proteins. *J Mol Biol* 2007;**366**:375–81. http://dx.doi.org/10.1016/j.jmb.2006.11.039.
24. Roche P, Feng C, Britton W. Human T-cell epitopes on the *Mycobacterium tuberculosis* secreted protein MPT64. *Scand J Immunol* 1996;**43**:662–70. http://dx.doi.org/10.1046/j.1365-3083.1996.d01-260.x.
25. Bai Y, Xue Y, Gao H, Wang L, Ding T, Bai W, Fan A, Zhang H, An Q, Xu Z. Expression and purification of *Mycobacterium tuberculosis* ESAT-6 and MPT64 fusion protein and its immunoprophylactic potential in mouse model. *Protein Expr Purif* 2008;**59**:189–96. http://dx.doi.org/10.1016/j.pep.2007.11.016.
26. Brodin P, Rosenkrands I, Andersen P, Cole ST, Brosch R. ESAT-6 proteins: protective antigens and virulence factors? *Trends Microbiol* 2004;**12**:500–8. http://dx.doi.org/10.1016/j.tim.2004.09.007.
27. Wang Q, Lei C, Wan H, Liu Q. Improved cellular immune response elicited by a ubiquitin-fused DNA vaccine against *Mycobacterium tuberculosis*. *DNA Cell Biol* 2012;**31**:489–95. http://dx.doi.org/10.1089/dna.2011.1309.
28. van Pinxteren LAH, Ravn P, Agger EM, Pollock J, Andersen P. Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. *Clin Diagn Lab Immunol* 2000;**7**:155–60.
29. Chen J-W, Faisal SM, Chandra S, McDonough SP, Moreira MAS, Scaria J, Chang C-F, Bannantine JP, Akey B, Chang Y- F. Immunogenicity and protective efficacy of the *Mycobacterium avium* subsp. paratuberculosis attenuated mutants against challenge in a mouse model**30**:3015–25. http://dx.doi.org/10.1016/j.vaccine.2011.11.029.
30. Tang X, Deng W, Xie J. Novel insights into Mycobacterium antigen Ag85 biology and implications in countermeasures for *M. tuberculosis*. *Crit Rev Eukaryot Gene Expr* 2012:179–87. United States.
31. Baldwin SL, D'Souza CD, Orme IM, Liu MA, Huygen K, Denis O, Tang A, Zhu L, Montgomery D, Ulmer JB. Immunogenicity and protective efficacy of DNA vaccines encoding secreted and non-secreted forms of *Mycobacterium tuberculosis* Ag85A. *Tuber Lung Dis* 1999:251–9. Scotland.
32. Langermans JA, Doherty TM, Vervenne RA, van der Laan T, Lyashchenko K, Greenwald R, Agger EM, Aagaard C, Weiler H, van Soolingen D, Dalemans W, Thomas AW, Andersen P. Protection of macaques against *Mycobacterium tuberculosis* infection by a subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. *Vaccine* 2005:2740–50. Netherlands.
33. Ronning DR, Klabunde T, Besra GS, Vissa VD, Belisle JT, Sacchettini JC. Crystal structure of the secreted form of antigen 85C reveals potential targets for mycobacterial drugs and vaccines. *Nat Struct Biol* 2000;**7**:141–6. http://dx.doi.org/10.1038/72413.
34. Anderson DH, Harth G, Horwitz MA, Eisenberg D. An interfacial mechanism and a class of inhibitors inferred from two crystal structures of the *Mycobacterium tuberculosis* 30 kDa major secretory protein (Antigen 85B), a mycolyl transferase. *J Mol Biol* 2001:671–81. Academic Press, England.
35. Bentley-Hibbert SI, Quan X, Newman T, Huygen K, Godfrey HP. Pathophysiology of antigen 85 in patients with active tuberculosis: antigen 85 circulates as complexes with fibronectin and immunoglobulin G. *Infect Immun* 1999;**67**:581–8.
36. Ronning DR, Vissa V, Besra GS, Belisle JT, Sacchettini JC. *Mycobacterium tuberculosis* antigen 85A and 85C structures confirm binding orientation and conserved substrate specificity. *J Biol Chem* 2004. http://dx.doi.org/10.1074/jbc.M400811200.
37. Armitige LY, Jagannath C, Wanger AR, Norris SJ. Disruption of the genes encoding antigen 85A and antigen 85B of *Mycobacterium tuberculosis* H37Rv: effect on growth in culture and in macrophages. *Infect Immun* 2000;**68**:767–78.
38. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, Besra GS. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* 1997;**276**:1420–2.
39. Nguyen YH, Ma X, Qin L. Rapid identification and drug susceptibility screening of ESAT-6 secreting Mycobacteria by a NanoELIwell assay. *Sci Rep* 2012;**2**. http://dx.doi.org/10.1038/srep00635.
40. Renshaw PS, Lightbody KL, Veverka V, Muskett FW, Kelly G, Frenkiel TA, Gordon SV, Hewinson RG, Burke B, Norman J, Williamson RA, Carr MD. Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. *EMBO J* 2005;**24**:2491–8. http://dx.doi.org/10.1038/sj.emboj.7600732.
41. Lightbody KL, Ilghari D, Waters LC, Carey G, Bailey MA, Williamson RA, Renshaw PS, Carr MD. Molecular features governing the stability and specificity of functional complex formation by *Mycobacterium tuberculosis* CFP-10/ESAT-6 family proteins. *J Biol Chem* 2008;**283**:17681–90. http://dx.doi.org/10.1074/jbc.M800123200.
42. Majlessi L, Brodin P, Brosch R, Rojas MJ, Khun H, Huerre M, Cole ST, Leclerc C. Influence of ESAT-6 secretion system 1 (RD1) of *Mycobacterium tuberculosis* on the interaction between mycobacteria and the host immune system. *J Immunol* 2005;**174**:3570–9.
43. Raja A, Ranganathan UD, Bethunaickan R. Improved diagnosis of pulmonary tuberculosis by detection of antibodies against multiple *Mycobacterium tuberculosis* antigens. *Diagn Microbiol Infect Dis* 2008;**60**:361–8. http://dx.doi.org/10.1016/j.diagmicrobio.2007.11.006.
44. Niu H, Hu L, Li Q, Da Z, Wang B, Tang K, Xin Q, Yu H, Zhang Y, Wang Y, Ma X, Zhu B. Construction and evaluation of a multistage *Mycobacterium tuberculosis* subunit vaccine candidate Mtb10.4-HspX. *Vaccine* 2011;**29**:9451–8. http://dx.doi.org/10.1016/j.vaccine.2011.10.032.
45. Parlane NA, Compton BJ, Hayman CM, Painter GF, Basaraba RJ, Heiser A, Buddle BM. Phosphatidylinositol di-mannoside and derivates modulate the immune response to and efficacy of a tuberculosis protein vaccine against *Mycobacterium bovis* infection. *Vaccine* 2012;**30**:580–8. http://dx.doi.org/10.1016/j.vaccine.2011.11.055.
46. Sable SB, Verma I, Khuller GK. Multicomponent antituberculous subunit vaccine based on immunodominant antigens of *Mycobacterium tuberculosis*. *Vaccine* 2005;**23**:4175–84. http://dx.doi.org/10.1016/j.vaccine.2005.03.040.
47. Lighter J, Rigaud M. Diagnosing childhood tuberculosis: traditional and innovative modalities. *Curr Probl Pediatr Adolesc Health Care* 2009;**39**:61–88.
48. FIND. *New laboratory diagnostic tools for tuberculosis control* 2012.
49. Chaudhary S, Gupta S, Khare S, Lal S. Diagnosis of tuberculosis in an era of HIV pandemic: a review of current status and future prospects**28**:281–9.

50. Pai M, Minion J, Steingart K, Ramsay A. New and improved tuberculosis diagnostics: evidence, policy, practice, and impact. *Curr Opin Pulm Med* 2010;**16**:271–84. http://dx.doi.org/10.1097/MCP.0b013e328338094f.
51. Kiselev VI, Baranovskii PM, Rudykh IV, Shuster AM, Mart'ianov VA, Mednikov BL, Demin AV, Aleksandrov AN, Mushkin AI, Levi DT, Slogotskaia LV, Ovsiankina ES, Medunitsin NV, Litvinov VI, Perel'man MI, Pal'tsev MA. Clinical trials of the new skin test Diaskintest for the diagnosis of tuberculosis. *Probl Tuberk Bolezn Legk* 2009:11–6.
52. Malaghini M, Thomaz-Soccol V, Probst CM, Krieger MA, Preti H, Kritski A, Soccol CR. Recombinant antigen production for assays of intradermoreaction for diagnosis and surveillance of tuberculosis. *J Biotechnol* 2011;**156**:56–8. http://dx.doi.org/10.1016/j.jbiotec.2011.07.015.
53. *Method and apparatus for relating datasets by using semantic vectors and keyword analyses* 2011. IP.com.
54. Parsons LM, Somoskoevi A, Gutierrez C, Lee E, Paramasivan CN, Al Abimiku, Spector S, Roscigno G, Nkengasong J. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. *Clin Microbiol Rev* 2011;**24**:314. http://dx.doi.org/10.1128/cmr.00059-10.
55. Shen G-H, Chiou C-S, Hu S-T, Wu K-M, Chen J-H. Rapid identification of the *Mycobacterium tuberculosis* complex by combining the ESAT-6/CFP-10 immunochromatographic assay and smear morphology. *J Clin Microbiol* 2011;**49**:902–7. http://dx.doi.org/10.1128/jcm.00592-10.
56. Fan C-M, Li W, Fan D-L, Zheng X-Y, Ding J-Z. A study of double antigen sandwich colloidal gold immunochromatography rapid detection for *Mycobacterium tuberculosis* antibody. *Chin J Tuberc Respir Dis* 2011;**34**:356–8.
57. Cole RA, Lu HM, Shi YZ, Wang J, De-Hua T, Zhou AT. Clinical evaluation of a rapid immunochromatographic assay based on the 38 kDa antigen of Mycobacterium tuberculosis on patients with pulmonary tuberculosis in China. *Tuber Lung Dis* 1996:363–8. Scotland.
58. Ramos-Vara JA. Technical aspects of immunohistochemistry. *Vet Pathol* 2005;**42**:405–26. http://dx.doi.org/10.1354/vp.42-4-405.
59. Purohit M, Mustafa T, Wiker H, Morkve O, Sviland L. Immunohistochemical diagnosis of abdominal and lymph node tuberculosis by detecting Mycobacterium tuberculosis complex specific antigen MPT64. *Diagn Pathol* 2007;**2**. http://dx.doi.org/10.1186/1746-1596-2-36.
60. Baba K, Dyrhol-Riise A, Sviland L, Langeland N, Hoosen A, Wiker H, Mustafa T. Rapid and specific diagnosis of tuberculous pleuritis with immunohistochemistry by detecting *Mycobacterium tuberculosis* complex specific antigen MPT64 in patients from a HIV endemic area. *Appl Immunohistochem Mol Morphol* 2008;**16**:554–61. http://dx.doi.org/10.1097/PAI.0b013e31816c3f79.
61. Sumi S, Radhakrishnan V. Evaluation of immunohistochemistry with a panel of antibodies against recombinant mycobacterial antigens for the diagnosis of tuberculous lymphadenitis. *Int J Med Med Sci* 2009;**1**:215–9.
62. Mustafa T, Wiker HG, Mfinanga SGM, Morkve O, Sviland L. Immunohistochemistry using a *Mycobacterium tuberculosis* complex specific antibody for improved diagnosis of tuberculous lymphadenitis. *Mod Pathol* 2006;**19**:1606–14. http://dx.doi.org/10.1038/modpathol.3800697.
63. Liu Z-H, Qin L-H, Feng Y-H, Bi A-X, Yang H, Ding Y-S, Cui Z-L, Jin R-L, Zheng R-J, Hu Z-Y. Identification of the *Mycobacterium tuberculosis* complex by detecting recombinant secretory protein MPT64. *Chin J Tuber Respir Dis* 2009;**32**:608–12.
64. Dayal R, Singh A, Katoch VM, Joshi B, Chauhan DS, Singh P, Kumar G, Sharma VD. Serological diagnosis of tuberculosis. *Indian J Pediatr* 2008;**75**:1219–21. http://dx.doi.org/10.1007/s12098-008-0222-3.
65. Kumar G, Dagur PK, Singh M, Yadav VS, Dayal R, Singh HB, Katoch VM, Sengupta U, Joshi B. Diagnostic potential of Ag85C in comparison to various secretory antigens for childhood tuberculosis. *Scand J Immunol* 2008:177–83. England.
66. Singh R, Toor P, Parchand S, Sharma K, Gupta V, Gupta A. Quantitative polymerase chain reaction for *Mycobacterium tuberculosis* in so-called Eales' disease. *Ocul Immunol Inflamm* 2012;**20**:153–7. http://dx.doi.org/10.3109/09273948.2012.658134.
67. Sethi S, Yadav R, Mewara A, Dhatwalia SK, Sharma M, Gupta D. Evaluation of in-house mpt64 real-time PCR for rapid detection of *Mycobacterium tuberculosis* in pulmonary and extra-pulmonary specimens. *Braz J Infect Dis* 2012;**16**:493–4. http://dx.doi.org/10.1016/j.bjid.2012.08.010.
68. Sharma K, Sharma A, Sharma SK, Sen RK, Dhillon MS, Sharma M. Does multiplex polymerase chain reaction increase the diagnostic percentage in osteoarticular tuberculosis? A prospective evaluation of 80 cases. *Int Orthopaed* 2012;**36**:255–9. http://dx.doi.org/10.1007/s00264-011-1241-7.
69. Vadwai V, Shetty A, Rodrigues C. Using likelihood ratios to estimate diagnostic accuracy of a novel multiplex nested PCR in extra-pulmonary tuberculosis. *Int J Tuberc Lung Dis* 2012;**16**:240–7. http://dx.doi.org/10.5588/ijtld.11.0322.
70. Dil A, Mir AW, Kirmani A, Shakeel ul R, Eachkoti R, Siddiqi MA. Improved diagnosis of central nervous system tuberculosis by MPB64-target PCR. *Braz J Microbiol* 2008;**39**:209–13. http://dx.doi.org/10.1590/s1517-83822008000200002.
71. Fauville-Dufaux M, Waelbroeck A, De Mol P, Vanfleteren B, Levy J, Debusschere P, Farber CM. Contribution of the polymerase chain reaction to the diagnosis of tuberculous infections in children. *Eur J Pediatr* 1996;**155**:106–11.
72. Pai M, Riley LW, Colford JM. Interferonn assays-gamma in the immunodiagnosis of tuberculosis: a systematic review. *Lancet Infect Dis* 2004;**4**:761–76. http://dx.doi.org/10.1016/s1473-3099(04)01206-x.
73. Chen J, Su X, Zhang Y, Wang S, Shao L, Wu J, Wang F, Zhang S, Wang J, Weng X, Wang H, Zhang W. Novel recombinant RD2-and RD11-encoded *Mycobacterium tuberculosis* antigens are potential candidates for diagnosis of tuberculosis infections in BCG-vaccinated individuals. *Microbes Infect* 2009;**11**:876–85. http://dx.doi.org/10.1016/j.micinf.2009.05.008.
74. Bautista J, Banaei N. Sensitivity of QuantiFERON-TB GOLD In-Tube for diagnosis of recent versus remote *M. tuberculosis* infection. *Diagn Microbiol Infect Dis* 2012;**73**:257–9. http://dx.doi.org/10.1016/j.diagmicrobio.2012.03.018.
75. Cooper JC, Hall EAH. The nature of biosensor technology. *J Biomed Eng* 1988;**10**:210–9. http://dx.doi.org/10.1016/0141-5425(88)90001-5.
76. Wang Y, Liu B. Conjugated polyelectrolyte-sensitized fluorescent detection of thrombin in blood serum using aptamer-immobilized silica nanoparticles as the platform. *Langmuir* 2009;**25**:12787–93. http://dx.doi.org/10.1021/la901703p.
77. Kim S-E, Ahn K-Y, Park J-S, Kim KR, Lee KE, Han S-S, Lee J. Fluorescent ferritin nanoparticles and application to the aptamer sensor. *Anal Chem* 2011;**83**:5834–43. http://dx.doi.org/10.1021/ac200657s.
78. Qin L, Zheng R, Ma Z, Feng Y, Liu Z, Yang H, Wang J, Jin R, Lu J, Ding Y, Hu Z. The selection and application of ssDNA aptamers against MPT64 protein in *Mycobacterium tuberculosis*. *Clin Chem Lab Med* 2009;**47**:405–11. http://dx.doi.org/10.1515/CCLM.2009.097.
79. Mukundan H, Kumar S, Price DN, Ray SM, Lee YJ, Min S, Eum S, Kubicek-Sutherland J, Resnick JM, Grace WK, Anderson AS, Hwang SH, Cho SN, Via LE, Barry 3rd C, Sakamuri R, Swanson BI. Rapid detection of *Mycobacterium tuberculosis* biomarkers in a sandwich immunoassay format using a waveguide-based optical biosensor. *Tuberculosis (Edinb)* 2012;**92**:407–16. http://dx.doi.org/10.1016/j.tube.2012.05.009.
80. Seong Cheol Hong JLV, Shin Hyun-Chul, Kim Cheol-Min, Park Jung Youn, Koh Kwangnak, Kim Hwa-Jung, Chang Chulhun L, Lee Jaebeom. Clinical immunosensing of tuberculosis CFP-10 in patient urine by surface plasmon resonance spectroscopy**160**:1434–8. http://dx.doi.org/10.1016/j.snb.2011.10.006.
81. Namba Y, Yasuharu NJP. Method for detecting *Mycobacterium tuberculosis*, involves performing immunoassay using antibody with respect to epitope of *Mycobacterium tuberculosis* specific secreted protein MPB64. Bl Co Ltd.
82. Brent AJ, Mugo D, Musyimi R, Mutiso A, Morpeth S, Levin M, Scott JAG. Performance of the MGIT TBc identification test and meta-analysis of MPT64 assays for identification of the *Mycobacterium tuberculosis* complex in liquid culture. *J Clin Microbiol* 2011;**49**:4343–6. http://dx.doi.org/10.1128/jcm.05995-11.
83. Andersen P, Doherty TM, Pai M, Weldingh K. The prognosis of latent tuberculosis: can disease be predicted. *Trends Mol Med* 2007;**13**:175–82. http://dx.doi.org/10.1016/j.molmed.2007.03.004.
84. Qin LH, Zheng RJ, Ma ZX, Feng YH, Liu ZH, Yang H, Wang J, Jin RL, Lu JM, Ding YS, Hu ZY. The selection and application of ssDNA aptamers against MPT64 protein in *Mycobacterium tuberculosis*. *Clin Chem Lab Med* 2009;**47**:405–11. http://dx.doi.org/10.1515/cclm.2009.097.
85. Zhu C, Liu J, Ling Y, Yang H, Liu Z, Zheng R, Qin L, Hu Z. Evaluation of the clinical value of ELISA based on MPT64 antibody aptamer for serological diagnosis of pulmonary tuberculosis. *BMC Infect Dis* 2012:96.