Review Article

Mycobacteria-derived biomarkers for tuberculosis diagnosis

Magdalena Druszczynska[1], Sebastian Wawrocki[1], Rafal Szewczyk[2] & Wieslawa Rudnicka[1]

Departments of [1]Immunology & Infectious Biology & [2]Industrial Microbiology & Biotechnology,
Faculty of Biology & Environmental Protection, Institute of Microbiology, Biotechnology & Immunology,
University of Lodz, Lodz, Poland

Tuberculosis (TB) remains an escalating problem worldwide. The current diagnostic methods do not always guarantee reliable diagnosis. TB treatment is a time-consuming process that requires the use of several chemotherapeutics, to which mycobacteria are becoming increasingly resistant. This article focuses on the potential utility of biomarkers of mycobacterial origin with potential implications for TB diagnosis. Properly standardized indicators could become new diagnostic tools, improving and streamlining the identification of *Mycobacterium tuberculosis* infection and the implementation of appropriate therapy. These markers can also potentially provide a quick confirmation of effectiveness of new anti-mycobacterial drugs and TB vaccines, leading to a possible application in practice.

Key words Antigen 85 complex - biomarker - diagnosis - lipoarabinomannan - mycolic acids - transrenal DNA - tuberculosis - volatile metabolites

Introduction

Efforts to control the burden of tuberculosis (TB) throughout the past decade have lowered the incidence of TB worldwide but have not managed to combat the disease. Every year, almost nine million people suffer from TB and about two million die, despite the implementation of obligatory bacillus Calmette–Guerin (BCG) vaccinations and directly observed treatment, short-course (DOTS) programmes in over 180 countries[1]. The epidemic of human immunodeficiency virus (HIV) and the emergence of drug-resistant strains of *Mycobacterium tuberculosis* have hindered TB control. Early diagnosis of the disease is essential for the efficient treatment and prevention of the spread of TB bacilli. However,

conventional diagnostic assays, such as acid-fast staining and time-consuming culture (6-8 wk) of *M. tuberculosis*, fail in many patients with active lung and particularly extrapulmonary TB. Polymerase chain reaction-based molecular techniques have a variable specificity. These techniques are suitable for detecting *M. tuberculosis* in clinical samples in only some well-equipped microbiological laboratories. Chest lesions identified by radiography may be helpful in detecting lung TB, but these cannot identify the causal agent of pathology. In the light of these facts, improved, next-generation diagnostic tests with greater sensitivity and specificity for TB infection would significantly improve the effectiveness of TB control. New diagnostic techniques should be easy, inexpensive

and applicable to all TB cases, including patients from whom clinical specimens for culture are not easily obtained, such as those with extrapulmonary TB and children. In addition, these techniques should provide new tools to supervise TB response to therapy as well as potentially decrease the emergence of drug resistance. In recent years there has been an escalation of studies directed to the identification of new biomarkers for TB diagnosis. According to the definition established in 2001, a biomarker is a specific feature that can be objectively assessed and used for the evaluation of physiological and pathological processes or responses to therapeutic interventions[2]. The *M. tuberculosis*-host interactions as well as the responses to therapeutic intervention are very complex and dynamic processes, which remain only partially understood. The responses to TB bacilli are complex and multifaceted involving cascade reactions occurring in mycobacteria and in host cells and tissues. Taking into account the vast complexity of mycobacteria-host interactions, biomarkers can serve as surrogates for confirmation of TB infection, correlates of host protection against active TB, predictors of anti-TB treatment success and reactivation risk in healthy controls with latent TB infection. Many large-scale studies have been aimed at discovering mycobacteria and host components that can potentially serve as biomarkers useful in TB diagnosis, prognosis and surveillance. This review discusses the utility of some biomarkers of mycobacterial origin with important implications for improved diagnostics of TB.

Lipoarabinomannan (LAM)

TB diagnosis based on the direct detection of *M. tuberculosis* cell wall components represents one of the most challenging aspects of TB control. Many of these components are highly immunogenic and are believed to be key entities in the interaction of mycobacteria with the host immune system. The outer layer of the mycobacterial cell contains mainly polysaccharides and proteins with lipid components further inside. One of the major cell wall components - lipoarabinomannan (LAM) - is a 17.3-kDa phosphatidylinositol anchored glycolipid with diverse biological activities. It is a ubiquitous lipid of mycobacteria and is considered as one of the strongest non-protein antigens to modify the host immune response[3]. The structure of LAM is different in various mycobacterial species and determines the ability to influence the host immunity. Among three possible LAM variants, mannose-capped LAM (ManLAM), phospho-myoinositol-capped

LAM and non-capped LAM, ManLAM has been found to be a potent anti-inflammatory antigen and an important virulence factor. After binding to dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin and dectin-2 receptors on dendritic cells and macrophages, ManLAM impairs dendritic cell maturation and phagocytic activity, modulates cytokine release by dendritic cells and macrophages and causes the suppression of adaptive immune responses towards *M. tuberculosis*[4-7]. The immunogenicity and heat stability of LAM support the use of this antigen in TB diagnostics. During TB, LAM is released from both metabolically active and dying bacteria, serving as an immune-modulating virulence factor. Once produced at the inflammatory sites of TB, either pulmonary or extrapulmonary, the antigen circulates in the blood, easily traverses the basement membrane in the kidney glomeruli and can be detected in the urine[8-13]. Urine is an attractive and convenient diagnostic material that can be easily collected from all patients even those from whom obtaining sputum is not possible[14]. A urinary LAM-based test would be an important diagnostic aid, especially in paediatric TB, which, unlike TB in adults, often occurs with non-specific signs and symptoms[15]. None of the currently available microbiological, serologic or molecular tests designed for the diagnosis of active TB is good enough for clinical use in children[15,16]. A gas chromatography/mass spectrometry (GC/MS) analysis aimed at identification of D-arabinose and tuberculostearic acid as surrogates for mycobacterial LAM in the human urine substantiated the antigen as a powerful biomarker for active TB[17]. However, the concentration of excreted LAM depends on the clinical manifestation of TB and varies between 1 ng/ml and several 100 ng/ml[18-20]. It has been demonstrated that the sensitivity of urinary LAM tests strongly depends on the proper sample processing. Fast freezing of urine in liquid nitrogen immediately after the collection is the most efficient method for preserving the stability of the antigen[21]. Commercially available urinary LAM assays are characterized by overall good diagnostic specificity (87-99%) but suboptimal sensitivity (13-93%), which limit their use as general screening tests for TB[22-25]. In the study registered in Finland and Taiwan, the initial 100-fold concentration of the urine increased the sensitivity of the Clearview® TB ELISA assay from 7 to 57 per cent but decreased specificity from 97 to 89 per cent[26].

The LAM immunoassay has been more often positive in smear-positive than smear-negative

TB patients, and the level of LAM in urine was found to be strongly correlated with mycobacterial density in sputum samples and other markers of high mycobacterial burden[12,27,28]. On the other hand, the urinary LAM concentration did not always augment false reactivity[26]. Unlike conventional diagnostic methods, the tests showed increased sensitivity in HIV-TB co-infected patients, in particular in individuals with low levels of circulating CD4 T-cells and advanced form of HIV-induced immunosuppression, allowing the identification of TB and facilitating early initiation of anti-TB treatment[11,20]. However, the inability to detect low levels of LAM in HIV-uninfected patients makes these immunoassays unsuitable for use in settings with a low HIV prevalence. The combined rapid detection of active TB using the LAM assay and molecular GeneXpert MTB/RIF test detecting resistance to rifampicin has been proposed by Shah *et al*[29]. The sensitivity of urine LAM immunoassay and sputum GeneXpert technique used jointly was 85 per cent, which was superior to any of the tests alone. Further improvements in the sensitivity of urinary LAM assays based on the use of specific immunoassay platforms and high avidity monoclonal antibodies are necessary for extending their applicability to the general population of TB patients.

Mycolic acids

Lipids constitute about 60 per cent of the dry mass of the mycobacterial cell wall. These form a permeability barrier constituting the first line of defence against the host's immune system. Mycolic acids, long-chain fatty acids esterified to the arabinogalactan, are one of the basic lipid components of the mycobacterial cell wall[30,31]. These contribute to the structural integrity of the cell wall, protect mycobacteria from chemical damage or dehydration and allow them to grow safely inside macrophages. Although the basic structure of mycolic acids is conserved, there are substantial differences in the composition and length of their meromycolic chains. Mycobacterial cell walls contain various mycolic acid types that are used for bacterial taxonomy and identification. The most widely distributed form of mycolic acids in *M. tuberculosis* is alpha-mycolates, which contain no oxygen substitutions in their chains[30]. Keto- and methoxy-mycolates, characterized by *cis* and *trans* cyclopropane rings in the proximal positions, are significantly less common[31]. Interestingly, structural alterations and variations in the chemical composition of mycolates have a profound effect on the virulence and physiology of the bacilli. It was found that the deletion of cyclopropane molecule inhibits the growth of mycobacteria[32,33]. Many studies have confirmed the presence of mycobacterial mycolic acids in both modern and ancient TB, suggesting the use of mycolates as sensitive biomarkers of the disease[34,35]. Mycolic acids seem to be appropriate diagnostic indicators of mycobacterial infections for many reasons. First, the compounds are present in a high concentration in the bacterial cell wall regardless of growth conditions. Second, these are bacteria-specific and are not produced in the human body. Third, the chemical stability of mycolic acids and their relatively easy extraction allow the use of chemical analysis techniques for their studies. Although it has been demonstrated that mycolic acids are secreted during mycobacterial infection inducing the production of specific antibodies, no standardized immunoassays allowing the detection of such antibodies have been developed[36]. This is partly due to their hydrophobic properties and heterogeneous chemical structure. A surface plasmon resonance (SPR) inhibition biosensor test abbreviated MARTI (mycolic acid antibody real-time inhibition) has enabled the detection of serum antibodies to mycolic acids; however, the method has not been tested in any clinical trial[36-38]. One of the most recommended methods for the identification of mycobacterial mycolic acids is high-performance liquid chromatography (HPLC). However, the protocol suggested by the Centers for Disease Control and Prevention (CDC) does not provide complete information about the structure of mycolic acids[39-42]. A potentially fast method for the identification of *M. tuberculosis* lipids is the surface-enhanced Raman spectroscopy. However, despite its high diagnostic value in the differentiation of tuberculous and non-tuberculous mycobacteria, it is rarely used as the technique is expensive and sophisticated[43]. More accurate approaches of mycolic acids analysis involve the use of high-throughput MS techniques. The profiling of mycolates is thought to be a rapid and relatively low-cost method for the identification of mycobacterial species. MS/MS analysis of mycolic acids profiles in multiple reaction monitoring (MRM) mode is a successful method for identification of *M. tuberculosis*, as described earlier[42,44]. Our approach involving electrospray ionization tandem MS technique has been based on analysis of 10 most frequent and distinctive MRM pairs allowing the reliable identification of *M. tuberculosis* and other mycobacterial species. The sensitivity and specificity of the developed method surpassed the parameters of classic diagnostic methods and allowed

robust identification of TB-positive patients after two hours of direct sputum analysis[42]. MS combined with GC provides trustworthy techniques that distinguish *M. tuberculosis* from non-tuberculous mycobacteria and help in fast implementation of the appropriate treatment[45]. MS appears to be a sensitive technique for the analysis of *M. tuberculosis* in paleopathological remains[35,46]. The use of the LC-MS/MS method allowed the identification of *M. tuberculosis* infection in the neolithic skeleton with TB-suggestive morphological changes and suggested that MRM-based analysis has a potential to be a promising technique for the studies of ancient mycobacterial strains[35].

Antigen 85 complex

The antigen 85 complex (Ag 85) represents a major mycobacterial secreted antigen although it has also been found in the mycobacterial cell wall. The complex comprises three different proteins - A, B and C that are encoded by three separate genes - *fbpA*, *fbpB* and *fbpC2*. The A, B and C proteins are generally expressed by all mycobacteria species regardless of their pathogenicity in a ratio 2:3:1; however, the ratio might depend on the environmental conditions[47,48]. The Ag 85C is much more biologically active than variant B with the greatest expression level[49]. The complex possesses a mycolyltransferase activity, catalyzing the synthesis of trehalose dimycolate, the characteristic lipid of the mycobacterial cell wall[50]. Furthermore, all three Ag 85 proteins have a capacity for binding of human fibronectin, an extracellular matrix glycoprotein that reduces the phagocytosis of *M. tuberculosis* by macrophages and facilitates the adherence and dissemination of the pathogen in tissues[50].

The Ag 85 complex has been detected in various biological samples such as blood, urine, sputum and cerebrospinal fluid derived from *M. tuberculosis*-infected patients[51,52]. Although the proteins are expressed by many mycobacterial species, their potential applicability as new TB biomarkers and vaccine candidates requires extensive research[53,54]. The serum detection of the Ag 85 complex yielded a sensitivity of 96 and 14 per cent among TB and non-TB patients, respectively[55]. Elevated levels of the Ag 85 were also observed in tuberculous meningitis, suggesting the possibility of using this protein as a biomarker for diagnosis of this TB form[51]. The monitoring of sputum Ag 85 may represent an important complement to the monitoring of antituberculous therapy as Ag 85 complex has been found in patient's sputum some days after the initiation of the treatment[52]. However, rapid Ag 85B RNA elimination from sputum in cured TB patients did not allow considering the antigen as a predictor of the relapse[56].

Volatile metabolites of mycobacteria

An alternative TB diagnostic strategy might be detection of *M. tuberculosis*-specific volatile metabolites in the exhaled breath[57,58]. Breath testing techniques are promising screening tools in populations with high TB exposure as these are non-invasive, rapid and safe. These attractive methods of diagnosis rely on the identification of specific organic compounds such as nicotinic acid, methyl nicotinate methyl phenylacetate or p-methyl anisate[57]. An increase in the levels of methyl nicotinate and terbium complexes in the presence of nicotinic acid observed in the breath condensates of TB patients confirmed the potential application of these compounds in clinical practice[59,60]. Scott-Thomas *et al*[61] detected methyl nicotinate, methyl phenylacetate and o-phenylanisole in approximately one per cent of healthy individuals and suggested that these markers were not sufficiently good indicators of *M. tuberculosis* infection. Analysis of TB patients' breath samples by Phillips *et al*[58] identified other volatile biomarkers such as cyclohexane, benzene, decane and heptane and their derivatives. These chemicals identified active TB with 84 per cent sensitivity, 64.7 per cent specificity and 85 per cent accuracy. Some non-tuberculous mycobacterial species - *M. avium*, *M. gordonae*, *M. gastri*, *M. kansasii* or *M. szulgai* were identified by the patterns of produced volatile metabolites such as long-chain fatty acids (C_{14}-C_{26}) and their derivatives[62,63]. Mycobacteria-specific volatile compounds can also be detected by tests using non-toxic substrates labelled with isotopes. Jassal *et al*[64] showed that one of the mycobacterial enzymes urease might serve as an attractive marker for diagnosis and therapeutic effects in pulmonary TB. The authors demonstrated the detection of $^{13}CO_2$ resulting from the conversion of ^{13}C urea in the breath of *M. tuberculosis*-infected rabbits within 15 min of bronchoscopic administration of the bacilli. The $^{13}CO_2/^{12}CO_2$ ratio was easily assessed by MS and demonstrated a significant $^{13}CO_2$ increase in the early phase of TB before the treatment with isoniazid. The urea breath test-TB test has been shown to detect volatile organic compounds in TB patients with 71.2 per cent sensitivity and 72 per cent specificity[64].

Transrenal DNA (trDNA)

One of the laboratory available diagnostic methods is the detection of *M. tuberculosis*-specific

DNA sequences in biological specimens. Although all commercially available molecular tools for the laboratory TB diagnosis are characterized by high accuracy in the identification of *M. tuberculosis*, their utility in routine clinical practice depends on their easy application, rapidity of sample preparation and results obtained as well as their expensiveness or infrastructure requirements. The GeneXpert MTB/RIF test is the only nucleic acid-based system endorsed and recommended by the World Health Organization for the identification of *M. tuberculosis* and its resistance to rifampicin[65]. The method enables a rapid diagnosis of TB after only two hour of sputum analysis. The sensitivity of the assay reaches 98 per cent in *M. tuberculosis* smear-positive and 75 per cent in smear-negative patients, but it was lower (50%) in some high HIV prevalence settings[65-67].

An interesting approach in TB diagnosis is the detection of transrenal DNA (Tr-DNA) sequences, which are released from dying *M. tuberculosis* cells to the circulation and appear in the urine[68,69]. The molecular urine-based diagnosis of TB seems to be an attractive and promising method since it allows detecting TB infection in any age group and in any location of TB lesions. Such an approach could improve complicated and unreliable diagnosis of TB in children, who belong to the group of most TB-susceptible individuals with a high likelihood of developing active disease shortly after *M. tuberculosis* infection[70]. Urine tests in this group of patients might be particularly useful as the sputum collection from children is extremely difficult. However, the studies assessing the diagnostic usefulness of Tr-DNA showed a great variation in sensitivity (from 7 to 79%) related to the length of the target size, amount of specific DNA sequences, collection and storage of urine specimens and chosen nucleic acid amplification technique[71-73]. Therefore, further studies should be focussed on the optimization of the Tr-DNA detection method that will be the suitable for laboratories in both developed and developing countries.

Concluding remarks

Despite the significant progress of research aimed to explain the pathogenic properties of *M. tuberculosis* bacteria, some major issues are still elusive. There is a need for cost-efficient but reliable techniques to help us understand the TB disease mechanisms and advance the discoveries of new diagnostic options. A prominent feature of *M. tuberculosis* infection is the formation of granulomas, driven by both mycobacterial components and host acquired immunity comprising macrophages and T-lymphocytes, in the lungs of infected individuals. The bacteria persist in granulomas, which are separated from the healthy tissues by extracellular matrix and may prevent dissemination of the pathogen. When the defence of the immune cells weakens, the active TB disease may develop. The bacteria begins to multiply; the granuloma structures become disorganized and caseous resulting in tissue damage and spread of TB bacteria in the body and to others[74]. This causes the current diagnostics of pulmonary TB rely on sputum samples, but the identification of *M. tuberculosis* in the respiratory secretions is possible only when there is caseous granuloma. This requirement causes a delay in the possible detection of bacteria in sputum of TB patients who suffered from TB disease for some time already. For this reason, there is an urgent need to develop highly sensitive and specific laboratory tests that can be performed on the easily obtainable non-sputum samples such as blood or urine[75,76].

It is evident that the detection of mycobacterial LAM, mycolic acids or Tr-DNA in non-sputum samples, can significantly enrich the range of rapid TB diagnostics, yet the methods available still need to be refined. Additional studies are required to improve the sensitivity of the tests which identify these biomarkers by changes in pre-analytical preparation of the samples, concentration and using serum in place of whole blood[26,76]. The genetic diversity of *M. tuberculosis* is another factor that must be taken into account; therefore, studies using the strains from different areas are needed when the accuracy of the assays is validated. Recent progress in the area of metabolomics has created the ability to detect hundreds to thousands of small molecules in clinical samples. There is an expectation that metabolic profiling of plasma or urine can make it possible to differentiate patients with active TB from those with latent *M. tuberculosis* infection as well as to monitor clinical response to TB therapy[77,78]. New rapid and reliable metabolomic TB assays should be developed for routine laboratories. The high cost of the necessary equipment and materials necessary to perform the tests may limit their application in developing countries with the highest burden of TB.

***Conflicts of Interest*:** None.

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