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Microbiological and immunological diagnosis of tuberculous spondylodiscitis

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Abstract. – *Background:* **Tuberculous spondylodiscitis is one the many manifestations of active tuberculosis (TB) and can result following primary infection or, more frequently, from reactivation of active TB in subjects with latent TB. Definitive diagnosis of tuberculous spondylodiscitis requires the identification of *Mycobacterium tuberculosis* in the biological sample following microbiological analysis.**

Aim: **To summarize the recent advancement in the diagnosis of TB, focusing on classical and molecular microbiological procedures, providing an overview on the recent advancements in the understanding of TB pathogenesis and their implications for the immunological diagnosis**

Materials and Methods: **Isolation in culture of the bacilli and detection using molecular tools are the gold standards, though sensitivity of these assays is significantly lower compared to what observed for pulmonary TB, making diagnosis of spinal TB challenging.**

Results: **The use of the interferon-γ release assays (IGRAs) for the immunological diagnosis of TB infection could be of help and shall precede the invasive techniques, such as biopsy or surgery, required to obtain the biological sample. IGRAs measure the presence of effector T cells in the blood that can readily respond to an antigenic stimuli by secreting cytokines, and that are an indication of the presence of the bacilli *in vivo*. IGRAs are more sensitive and specific than the intradermic reaction of Mantoux, though both these immunological tests cannot distinguish between latent TB infection and active TB.**

Conclusions: **A modern diagnosis of TB spondylodiscitis should rely on the use of microbiological and immunological assays and the latter could potentially be of great help in monitoring therapy effectiveness.**

Key words:

Tuberculosis, Spinal TB, Pott's Disease.

Introduction

TB is one of the ancient human scourge that has afflicted mankind since millennia and is still one of the deadliest infectious disease agents, causing 1,7 million deaths and with 8,4 million new cases annually[1]. Most of the TB cases occur in developing countries where TB poses a major health problem, with relevant social and economic implications.

Mycobacterium (M.) tuberculosis is a human pathogen that belongs to the species *Mycobacterium tuberculosis* (MTB) complex, which comprises *M. africanum*, which causes TB in humans but is restricted to Africa, *M. bovis*, which is the etiologic agent of TB in animals and *M. microti* which infects voles and does not infect humans[2]. The complete sequencing of the *M. tuberculosis* genome has provided a new understanding on the biology of the tubercle bacillus, leading to a better understanding of the mechanism of pathogenesis and the definition of the genetic determinants of drug resistance, opening new avenues to combat the disease through more effective prophylactic and therapeutic measures[3-5]. The 80 years old live and attenuated vaccine strain Bacillus of Calmette and Guerin (BCG), while ineffective in preventing TB in adults, remains the only tool available to reduce the risk of fatal and systemic TB disease in children. There is an urgent need for a new and improved vaccine against TB and many efforts are underway using traditional and highly innovative strategies[6,7]. The same is true for the therapeutic regimens that still rely on more than forty years old drugs such as isoniazid, rifampin, streptomycin, pyrazinamide, ethambutol and few other second line drugs[8].

The new interest in TB research, that coincided with the spread of the HIV pandemic and the re-emergence of TB in western countries, had an impact primarily on the diagnosis of TB, with the introduction of more sensitive, specific and rapid methods that have contributed significantly to the control of TB in the last ten years.

Microbiological diagnosis of TB is always a challenging task, particularly when non-pulmonary TB is suspected, as in the case of spondylodiscitis[9,10], since the biological samples

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available to detect *M. tuberculosis* are difficult to obtain. At the same time, several causative agents of infectious spondylodiscitis have been identified, which require different antimicrobial therapies. For these reasons, it is recommended that the start of the antimicrobial therapy in case of infectious spondylodiscitis should be delayed until a microbial diagnosis is established[10].

In this review, we will summarize the recent advancement in the diagnosis of TB, focusing on classical and molecular microbiological procedures, providing an overview on the recent advancements in the understanding of TB pathogenesis and their implications for the immunological diagnosis and usefulness that these new tests may have in guiding the diagnostic query.

Pathogenesis of Tuberculosis

TB is an airborne transmitted disease, with the bacilli released by a patient with active pulmonary TB inhaled by a healthy person[11]. The bacilli once in the alveoli are ingested by macrophages which can kill the bacteria, though in 20-50% of the exposed subjects, *M. tuberculosis* resists to the innate immune response and actively multiply within the macrophages. Nearby cells are infected by the replicating bacilli, which cause a local inflammation that activates the secondary host immune response[12,13]. It is at these early times of the infectious process, when the host cannot yet contain bacterial multiplication, that *M. tuberculosis* spreads through the lymphatics, but most importantly can translocate to the blood stream and spread haematogenously to potentially reach any organ[14,15]. The mounting acquired cell mediated immune response then begins to contain the infection, starting at the site of primary infection when the cellular infiltrate organize in the typical granuloma. Only in ≅ 5% of cases, the host immune response fails to control primary infection which then progresses to overt pulmonary TB and it is the immune response itself that is responsible for the extensive tissue damage and necrosis which is the hallmark of active TB in immune-competent patients[11].

Conversely, it is estimated that in 90-95% of infected subjects, the host immune response can contain bacterial multiplication, leading to latent infection (*latent TB*). Latent TB is clinically silent, with no outward signs or symptoms of disease, and is characterized by the presence of a specific cell mediated immune response specific for *M. tuberculosis*, classically highlighted by the Mantoux test[16]. Latent TB can last for decades and people with latent TB have a ≅ 5% chance of developing active TB during lifetime, indicating that the host immune response cannot completely eradicate the bacteria *in vivo*. These observations have puzzled scientists since the early 20th century and only recently we are gaining a new understanding of the immunological processes associated with latent TB and on the metabolic status of the bacteria during this stage[17,18]. The old view that latent *M. tuberculosis* exists in classic TB lesions such as the primary complex has been contradicted by many findings, including the demonstration that bacilli can be found in non-professional phagocytic cells such as endothelial cells, pneumocytes and fibroblast that are scattered in the lung tissue[19]. *M. tuberculosis* also persists in adipose tissue where it can remain in a non-replicating state avoiding killing by antimicrobials and recognition by the effector immune cells[20]. The status of the bacilli during latent TB may vary greatly from a persistence non-replicating state, to dormancy, to active replication, and *M. tuberculosis* can either use aerobic and anaerobic metabolism[16-18]. What is remarkable is that *M. tuberculosis* can resist the attack from many immune cells, including CD4 and CD8 T cells, which provide an harsh and oxidizing environment for the bacteria[21]. In line with these findings, Fortune et al[22] have recently demonstrated that the mutation rate of *M. tuberculosis* during latent TB is similar to that observed during active TB, as a results of the active bacilli replication and of the oxidative damage to the *M. tuberculosis* DNA exerted by the antimicrobials produced by activated macrophages.

As a result of these findings, the traditional dichotomy of TB status (latent TB and active TB) is being revisited, with the introduction of the concept of TB spectrum, which identify a dynamic equilibrium between the host immune response and the bacilli[16,18]. The TB spectrum is seen as a gradient that may potentially vary from latent TB in an almost dormant state to overt active TB, and any event that can affect this equilibrium may lead to the progression of latent TB to overt disease.

Hence, tuberculous spondylodiscitis, also called Pott's disease, occurs when the bacteria that has reached the spine, starts replicating in the tissue forming a secondary lesion that progresses to cause the typical immunopathogenesis and tis-

sue damage that may evolve in complications such as vertebral collapse, paraplegia and paraspinal abscesses. Since haematogenous spread of the bacilli occurs readily following primary infection in the lung tissue, spinal TB can develop either soon after primary infections or many years or decades later following reactivation. Moreover, since during latent TB a dynamic equilibrium between the host immune response and actively replicating bacilli is maintained, and bacteria can be found in many tissues and cells, any event that can suppress the immune response can results in disease reactivation. For these reasons, tuberculous spondylodiscitis can occur in patients with concurrent infections such as HIV or undergoing biological therapy with anti-TNF drugs. Osteoarticular TB can also be associated with TB disease elsewhere in the body including pulmonary TB. In any case, proper diagnosis of spinal TB is a challenging task for the clinician that must identify the proper therapeutic regimen.

Direct diagnosis

Definitive diagnosis of TB requires the detection of *M. tuberculosis* from the biological sample by at least one of current microbiological techniques: microscopical analysis, isolation in culture or molecular methods. There are several major problems with the direct diagnosis of TB spondylodiscitis. First, the biological sample can be obtained only using invasive techniques, such as biopsy or tissue sampling during surgery. Second, TB spondylodiscitis is usually paucibacillary, which makes microscopical analysis following acid-fast staining not very reliable do to its low sensitivity. Moreover, microscopic analysis does not allows the proper identification of the mycobacterial species. Isolation in culture of *M. tuberculosis* from the specimen is considered the gold standard, since it demonstrate the presence of the etiologic agent and allows for the determination of the susceptibility testing to first and second line drugs. This is becoming of paramount importance at a time when *M. tuberculosis* strains resistant to two more drugs currently used in TB therapy are found responsible for TB disease, including spondylodiscitis[23]. Moreover, the isolation in culture is required to extract genomic DNA that could be used for the identification of the genetic determinants of drug resistance and the genotypic characterization and identification of the epidemiological features of the *M. tuberculosis* strain. Unfortunately, *M. tuberculosis* is a slow growing mycobacteria and isolation in culture may require up to 45 days, resulting in a delay of diagnosis and in the initiation of appropriate therapy.

A recent report by Kumar et al[24], where 51 patients with Pott's disease were evaluated, indicated that the sensitivity of the microscopic analysis was only of 33%, that of culture of 43% and the two combined of 59%. These results underscore the problems associated with the microbiological diagnosis of TB osteomyelitis.

The use of molecular diagnosis to detect and identify *M. tuberculosis* directly in the specimen is of great usefulness, given its high sensitivity and the possibility to obtain the results in one day. There are several commercially available assays to detect *M. tuberculosis* in biological specimens and though these tests have not yet been licensed for use in non-respiratory specimens, several reports clearly indicate that they should be considered highly reliable[25,26]. The specimens sent to the laboratory are directly subjected to amplification of specific *M. tuberculosis* DNA sequences using one of the many available techniques such as polymerase chain reaction (PCR), real-time PCR or strand displacement amplification. To broaden the search for the detection of other possible bacterial infectious agents, it is possible to amplify by PCR the DNA genomic fragment encoding the 16S RNA fragment, using universal primers that can amplify DNA from any bacteria. If an amplification is achieved, the DNA fragment is subjected to sequencing to identify the bacterial species detected in the specimen[27].

While it is widely accepted that molecular tools can greatly improve the diagnosis of tuberculous spondylodiscitis, more studies are required to carefully determine the sensitivity and specificity of these assays also in comparison to traditional microscopic and culture techniques.

Immunological diagnosis

The immunological diagnosis of TB has been historically performed by the Mantoux test or tuberculin skin test (TST), which consists in the intradermic inoculation of the purified protein derivative (PPD) and in measuring 48-72 hours later a delayed-type hypersensitivity which measure the cell mediated immune response against the

tubercle bacilli. In the last few years, more sensitive and specific immunological assays designed for the immunological diagnosis of *M. tuberculosis* have been developed and are now widely used as surrogate of TST[28]. These are named interferon-γ release assays (IGRAs) and measure the interferon-γ cytokine released by T cells obtained from a blood sample following restimulation with *M. tuberculosis* antigens. These antigens are specific for MTB complex and few other mycobacteria such as *M. kansasii* and *M. szulgai* and are not found in atypical mycobacteria and in the vaccine strain BCG, which lacks the region of difference 1 (RD1), encoding in MTB complex for these antigens[29]. IGRAs measure the presence of effector T cells in the blood that are specific for *M. tuberculosis*. Effector T cells can readily respond to an antigenic stimuli by secreting cytokines, and are different from memory T cells that require more time (>24 hours). Effector T cells are present only when the immune system is currently exposed to the antigenic stimuli, which in this case is *M. tuberculosis*. A positive IGRAs gives therefore an indication of an *M. tuberculosis* infection, but cannot distinguish, similarly to TST, between an active TB from a latent infection. The Quantiferon TB Gold In Tube (QFT-IT) is an IGRAs that measure the amount of interferon-γ secreted following restimulation, and many laboratories provide to the clinicians the results in UI/ml. Many researchers have attempted to correlate the amount of interferon-γ secreted with TB status but so far unclear correlation has been found.

The possibility to identify an immunological correlate of TB disease would be of paramount importance in the case of non-pulmonary TB such as spondylodiscites, where the possibility to link a clinical suspicion with an immunological data strongly indicative of active TB would certainly help the implementation of an effective anti-TB therapy. To address this issue modified version of IGRAs have been developed, including the use of different mycobacterial antigens to restimulate T cells *in vitro*, the measurements of more and/or different cytokines such as TNF and IL-2 or chemokines such as IP-10[30]. So far, these attempts have been only partly successful and more studies are required to identify immunological correlates of TB disease or latent TB. One of the most promising evolution of these IGRAs involves the use of another *M. tuberculosis* antigen, named the heparin binding haemagglutinin (HBHA)[14]. It has been shown that T cells obtained from blood drawn from subjects with latent TB respond to the HBHA stimuli by secreting interferon-γ while patients with active TB are unable to deploy a similar response[31,32]. Interestingly, patients with active TB develop antibodies against HBHA while subjects with latent TB do not[31-33]. Recent advances in the purification protocol of HBHA[34] have made possible to use this protein in parallel with the QFT-IT assay and recent results have indeed demonstrated the usefulness of the HBHA-based assay to discriminate, among subjects infected with *M. tuberculosis*, those with active TB from those with latent TB[35]. It would be of great interest to investigate whether in patient with TB spondylodiscitis the immune response against HBHA is similar to what seen in patients with pulmonary TB, since this may open the possibility of using IGRA to guide in the diagnosis of spondylodiscitis.

In any case, the use of the IGRAs currently licensed provides more specific and sensitive results compared to TST for the diagnosis of TB infection and shall be used to inquiry whether a TB spondylodiscitis is suspected.

In a recent report, the sensitivity of these IGRAs in patient with Pott's disease was found at 84%, with a specificity of 95%, which was higher than the 30% sensitivity measured for the combined smear plus culture classical microbiological tests[24]. Interestingly, when the immunological assay such as IGRA where combined with the smear and culture, sensitivity reached 88%. These results, which certainly deserve to be confirmed in larger studies including more patients, underscore the usefulness of these immunological assay in the diagnosis of spinal TB.

Moreover, it has been demonstrated the disappearance of the interferon-γ responses in patients who received effective therapy and persistent response in patients who experienced failure of therapy[36]. Hence, IGRAs may also be useful in monitoring TB therapy in patients with spinal TB.

Conclusions

TB is still one of the most important infectious disease in the world, with a very high incidence in developing countries. The new migratory trends, with the arrival of workers from countries where TB is endemic to nations where TB was thought to be conquered, is determining the

reemergence of TB disease, including less common clinical manifestations such as Pott's disease. Diagnosis of tuberculous spondylodiscitis is a challenging task, and the clinician has to define a correct diagnostic protocol to maximize the chances to identify the etiologic agent. The introduction of the IGRAs for the immunological diagnosis of TB could be of great usefulness and shall be used before biopsy or surgery is performed. A definitive diagnosis requires the identification of the bacilli in the biological sample by at least one of the currently used methods (microscopy, culture or molecular). When TB is suspected, molecular diagnosis shall always be considered and can provide rapid and reliable results that could guide the adoption of proper therapeutic regimes.

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