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REVIEW ARTICLE

Role of alpha-crystallin, early-secreted antigenic target 6-kDa protein and culture filtrate protein 10 as novel diagnostic markers in osteoarticular tuberculosis

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Summary Osteoarticular tuberculosis constitutes about 3% of all tuberculosis cases. Early and accurate diagnosis of tuberculosis is a challenging problem especially in the case of osteoarticular tuberculosis owing to the lower number of bacilli. However, an accurate and timely diagnosis of the disease results in an improved efficacy of the given treatment. Besides the limitations of conventional methods, nowadays molecular diagnostic techniques have emerged as a major breakthrough for the early diagnosis of tuberculosis with high sensitivity and specificity. Alpha-crystallin is a dominantly expressed protein responsible for the long viability of the pathogen during the latent phase under certain stress conditions such as hypoxia and nitric oxide stress. Two other proteins—early secreted antigenic target-6 and culture filtrate protein-10—show high expression in the active infective phase of *Mycobacterium tuberculosis*. In this article, we focus on the different proteins expressed dominantly in latent/active tuberculosis, and which may be further used as prognostic biomarkers for diagnosing tuberculosis, both in latent and active phases.

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Introduction of osteoarticular tuberculosis

According to World Health Organization (WHO) statistics for 2013, India has the highest burden of tuberculosis (TB), with an estimated occurrence of ~2.1 million TB cases out of the global total of 9 million cases. The approximate figure for TB widespread for 2013 is given as 2.6 million [1], of which 1–3% have osteoarticular involvement (Figure 1). The incidence of TB is increasing, and skeletal or osteoarticular TB accounts for 10–20% of all extrapulmonary cases [2]. When TB affects a patient's skeleton, it means that the joints are involved, too. Although osteoarticular TB most commonly occurs in the vertebral column, other less frequently affected sites include the hip, knee, and sacroiliac joints [3]. This review focuses on the accurate diagnosis of TB by analysing the protein expression that is dominant in the different phases of *Mycobacterium tuberculosis* infection. With the help of this approach, we can easily distinguish between the active and latent phases of TB infection in an individual.

Aetiopathogenesis

The major causes of the spread of EPTB are poverty, overcrowding, malnutrition, repeated pregnancies in women and HIV infection. More than 40 years after the commencement of effective chemotherapy, TB sadly remains the single largest infectious disease that causes mortality, resulting in about 5 deaths/min [4]. To date, the disease is not completely eradicated, because of its complex pathogenesis. Primary infection with *M. tuberculosis* rarely leads to disease. However, the infection is instead typically controlled by the host's immune system and most of the viable bacilli are wiped out. Nevertheless, some are still able to survive in the host body and remain inactive for decades prior to reactivating to cause clinical disease again (Figure 2) [5]. Approximately one-third of the entire population may be latently infected with dormant bacilli [6]. Latent TB means a patient is infected with *M. tuberculosis*, but the patient does not have active TB. Active TB can be transmissible whereas latent TB is not, and it is therefore not possible to acquire TB from a person who is latently infected with *M. tuberculosis*.

The main risk is that probably 10% of patients infected with latent TB (5% in the first 2 years after the infection and 0.1% per year thereafter) will be more prone to develop active TB. The human tubercular bacillus (*M. tuberculosis*) is a causative agent for almost all types of osteoarticular TB. Osteoarticular TB can affect the spine, hip and knee joint, foot, elbow, wrist, hand, shoulder, and diaphysial foci. The most common method of proliferation is through the haematogenous route. It commonly proliferates to the vertebral column through Batson's prevertebral venous plexus. The spread of infection is through the haematogenous route (Figure 3).

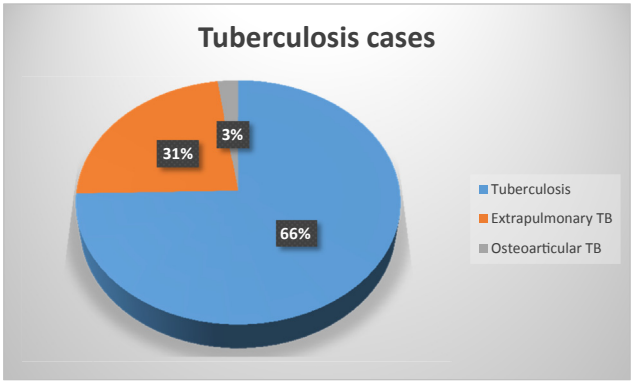

Figure 1 Worldwide prevalence of tuberculosis cases.

Clinical manifestation

The most common form of osteoarticular TB is spinal TB. It accounts for almost 50% of all osteoarticular TB cases [7]. In most cases, the infection initially progresses gradually, and only in rare cases is there an acute manifestation. The common symptoms are weight loss, night sweats, fatigue, and rise in temperature in the evening. Whenever the infection occurs for a longer duration, occurrence of cold abscess is noticed in the soft tissues, following its path through the intermuscular planes. The distribution of spinal TB is as follows: 42% thoracic involvement, 12% thoracolumbar, 26% lumbar, 12% cervical, 5% cervicodorsal, and 3% lumbosacral. It is the second most common skeletal lesion occurring in any age group, but is frequently seen in children. Initially, it appears with a painful hip limp. In later stages, when destruction has been continuous, the limb becomes tilted, abducted, and rotated internally, with a noticeable shortening of the limb [8]. The different forms of osteoarticular TB according to the site of lesions are as follows: TB of the knee joint, ankle joint, foot, upper extremity, wrist, elbow, tuberculous osteomyelitis, TB of small bones, TB of tendons, sheath, and bursae. Despite ongoing efforts, effective global TB control is hampered by the need for long-term chemotherapy, which leads to poor patient compliance [9].

Disadvantage of current diagnostic methods of osteoarticular TB

The diagnosis as well as treatment of extrapulmonary tuberculosis (EPTB) remains a major problem until today. In active pulmonary tuberculosis (PTB), clinical symptoms confirmed by a laboratory test may give a relatively clear result, whereas the diagnosis can be problematic in patients with EPTB, children, elderly, and immunosuppressed individuals [10]. Radiographic analysis (X-rays) in EPTB is often not conclusive, and the tuberculin purified protein derivative skin test is considered unreliable by many clinicians. Bacteria in EPTB cases can be present in low numbers at inaccessible sites. As a result, invasive procedures are usually necessary to confirm the infection [10]. Furthermore, the HIV epidemic has changed the proportion of EPTB among TB cases, pushing the number of new EPTB cases to account for $>$ 15% of the total TB cases [11].

Testing for active TB disease through antibodies or antigens is found to be extremely difficult in blood. Patients can have different antibody responses, which could suggest that they have active TB even when they do not. Antibodies may also develop against other organisms, which again could be a

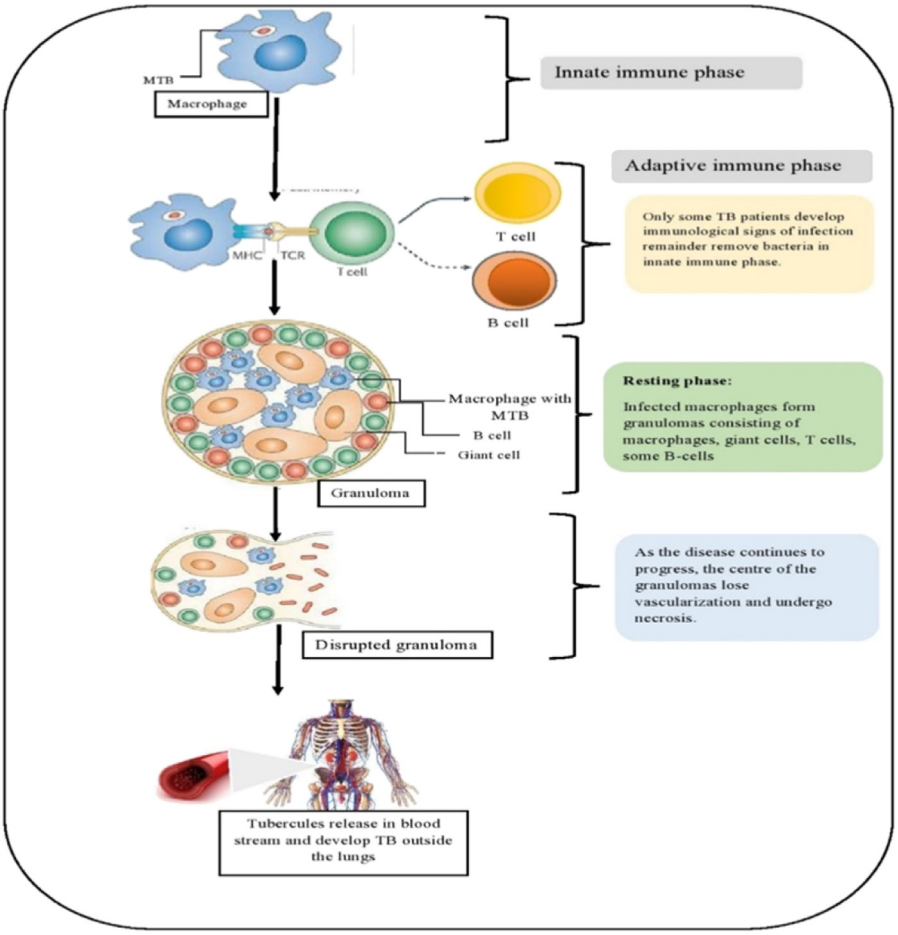

Figure 2 Basic mechanism of infection of *Mycobacterium tuberculosis*.

false indication of active TB. Besides this, different organisms share the same antigens, making test results unreliable. These factors can result in TB disease not being identified or wrongly diagnosed. The WHO [12] report suggests that in the evaluation of 94 studies—67 for PTB and 27 for EPTB. Strong evidence showed that the blood tests produced an unacceptable level of wrong results—false-positives or false-negatives—relative to the tests endorsed by WHO. The research revealed "low sensitivity" in commercial blood tests which leads to an unacceptably high number of patients being wrongly given the "all clear" (i.e., a false-negative when in reality they have active TB). This can result in the transmission of the disease or even death from untreated TB. It also revealed "low specificity" that led to an unfortunately high number of patients being wrongly diagnosed with TB (i.e., a false-positive when in reality they do not have active TB). However, patients' decision to undergo unnecessary treatment, while the real cause of their illness remains undiagnosed, may further lead to premature death. More than 1 million people worldwide are misdiagnosed with TB, although in reality they have an incurable disease with a similar outlook to many cancers. More than a million of these false diagnostic tests are carried out annually to diagnose active TB, often at a substantial cost to patients [12].

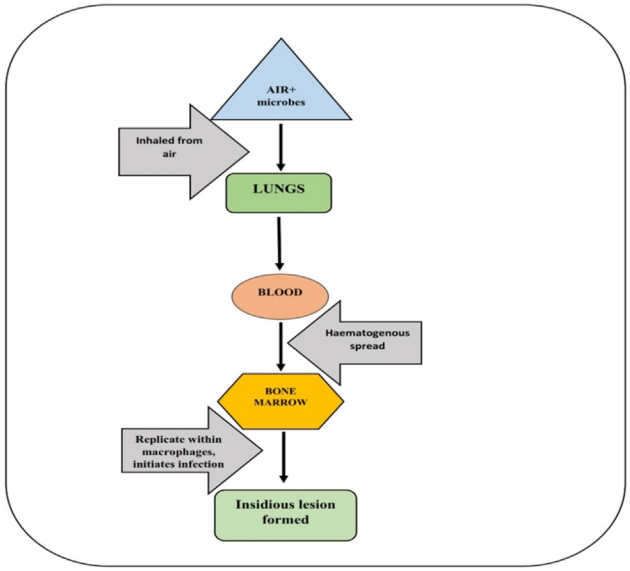

Figure 3 Diagrammatic representation of *Mycobacterium tuberculosis* infection in skeletal system spread.

Osteoarticular TB accounts for about 1—3% of all TB cases and is one of the major causes of osteomyelitis [13]. Although any bone, joint, or bursa can be infected, the spine, hip, and knee are the preferred sites of infection, representing 70—80% of infections [14,15]. TB of the spine, if not diagnosed properly and treated adequately, may lead

to kyphosis and/or neurological complication(s) [16]. The accurate diagnosis of osteoarticular TB poses difficulty owing to deep inaccessible lesions and initiation of empirical antitubercular therapy (ATT) in the majority of cases [17]. For the most part, the diagnosis of osteoarticular TB is based on clinical suspicion and imaging findings, particularly in endemic regions [14]. Recently, Sharma et al [18] introduced a highly sensitive and specific multiplex polymerase chain reaction (PCR) targeting IS6110 and MPB-64 protein genes in the longitudinal evaluation of synovial fluid and pus samples from 80 cases of osteoarticular TB [18].

Clinical diagnostics

The traditional methods fail in early diagnosis of the disease (Table 1) [19—26]. Staining for acid-fast bacilli and culturing of *M. tuberculosis* have low sensitivity and specificity [27]. In imaging, by the time the disease becomes apparent on X-rays, the patient has already reached the advanced stage of the disease [28]. Magnetic resonance imaging is more sensitive than X-rays but lacks pathognomonic findings that differentiate TB from other spinal infections or possibly a neoplasm [23,29]. The evaluation of the 16-kDa antigen as a serodiagnostic tool in PTB and EPTB patients has been done earlier. Serodiagnostic tests for TB have always had drawbacks of suboptimal sensitivity and specificity. A previous study showed that the antigen used gave encouraging results in PTB only, whereas in EPTB, it had a limited impact in terms of sensitivity [30].

Molecular diagnosis

A major breakthrough in the diagnosis of EPTB is highly associated with the HIV—EPTB coinfection, detected by nucleic acid amplification tests such as PCR to detect nucleotide sequences unique to *M. tuberculosis* directly in extrapulmonary specimens, offering better accuracy than acid-fast bacilli smear microscopy and greater speed than culture [31—34].

The exposure of *M. tuberculosis* to nitric oxide donors accelerated the production of alpha-crystallin (acr), thus resembling the conditions induced by the immune response [35]. The presence of this protein was associated with increased thickness of bacterial cell wall, which is characteristic of bacteria under stressful conditions [36], and to other specific stressful conditions for *M. tuberculosis*, such as treatment with antibiotics (e.g., rifampicin or streptomycin) [37]. This increased production also leads to a higher expression of the sigma factor F (sigF), a σ-subunit of the RNA polymerase that is stimulated under stress. To understand the mechanisms associated with the metabolic changes that may lead to the induction of latent bacilli, the sigma factor proves to be a strong link [38]. With the real-time PCR system, expression of acr was studied *in vitro* unstirred culture, allowing a progressive limited O_2 concentration; the induction of stressed bacilli by decreasing pH and using two types of bacilli (i.e., from cultures in an exponential and a stationary phase), to establish a parallelism between bacilli in the latent and active phases in a murine model of TB was also studied. As a result, the expression of acr has been closely related to latency, and thus could be a valuable marker [37]. The expression of acr was maintained even when the levels of 16S rRNA were undetectable after a long-term chemotherapy. Therefore, this was the best value for detecting the expression of latent bacilli with the real-time PCR methodology used. In conclusion, data from experimental *in vitro* stressing process with progressive low oxygen levels and decreasing pH values have supported the search for a useful marker monitoring latent bacilli in a murine model of TB. Therefore, the expression of acr may be a new tool for monitoring this population and contribute to its eradication in the future [39]. On the contrary, the early secreted antigenic target-6 and culture filtrate 10 protein (CFP-10), which act as virulence factors in *M. tuberculosis*, are secreted by the ESX-1 system into the host cell and thereby contribute to pathogenicity. T cell responses to early-secreted antigenic target 6-kDa protein (ESAT-6) and the newly identified CFP-10, both of which are particularly expressed by *M. tuberculosis* but not by bacillus Calmette—Guérin (BCG) strains, were studied. In the study, most patients with active or treated TB responded to the specific antigens ESAT-6, CFP-10 or both. *In vitro* T cell responses to ESAT-6 and CFP-10 appear to be highly sensitive and specific for the

Table 1 Various diagnostic techniques for detecting *Mycobacterium tuberculosis*.

Diagnostic methods	Disadvantages	References
DNA-PCR	DNA-PCR is unable to differentiate between viable and nonviable organisms.	Rana et al [19]
CSP-Ag's ELISA	Could not recognise latent infection.	Tiwari et al [20]
Culturing of *M. tuberculosis*	Time-consuming; took nearly 6—8 wk to show growth, less sensitive.	Cheng et al [21]
MRI (Pott's spine)	There is no pathognomonic finding that reliably distinguishes tuberculosis from other infections.	Griffith et al [22]
Purified protein test	Because of its highly cross-reactive nature, it does not give reliable results in areas where there is high environmental load of nontuberculous mycobacteria.	Bass [23], Bates et al [24]
Acid-fast bacilli (AFB)	Low specific results.	Kramer and Rosenstein [25], Van der Spoel van Dijk et al [26]

ELISA = enzyme-linked immunosorbent assay; MRI = magnetic resonance imaging; PCR = polymerase chain reaction.

detection of infection with *M. tuberculosis* and to be significantly more discriminative than responses to the complex antigens, especially in BCG-vaccinated individuals. ESAT-6 and CFP-10 are therefore considered highly promising antigens which will be included in future studies aimed at specific immunodiagnosis of TB [40]. The DNA-PCR is unable to differentiate between viable and nonviable organisms, whereas bacterial mRNA with a mean half-life of 3—5 minutes is more prone to degradation than genomic DNA; thus, a positive mRNA signal would indicate the presence of viable organisms [19]. The mRNA-based reverse transcriptase-PCR (RT-PCR) is a rapid method used to differentiate viable from nonviable *M. tuberculosis* and has also been used for the diagnosis of EPTB as well as to monitor drug resistance [19,41].

Protein expressed in osteoarticular TB

In this review, we discuss a protein that is responsible for the long-term survival of *M. tuberculosis* in the stationary phase and those proteins that act as virulence factors of *M. tuberculosis*.

Some bacteria form spores when under stress. They have a genetic programme, triggered by hypoxia and respiratory poisons and controlled by the transcription factor DosR [42]. This transcription factor regulates the development of a latently growing form without morphological differentiation. This quiescent (latent) physiological state maintains viability for a longer period and, importantly, shows phenotypic (as opposed to genetic) drug resistance. Therefore, it is thought that this stage of the bacillus life cycle contributes to both key issues faced in the control of TB, the symptom-free latent state of TB infection and the persistence of active disease despite prolonged chemotherapy [43—45].

It now appears that hypervirulent mutants of *M. tuberculosis*, with higher growth rates *in vivo*, have mutations in both regulatory and structural genes. *DosR* (devR, Rv2031c) is a hypervirulent gene that activates the transcription of about 50 genes of *M. tuberculosis* in response to hypoxia and nitric oxide stress. The most significant activation (~80-fold) of the hspX (acr, Rv2031c) gene encodes a 16-kDa acr-like protein, a major antigen (Figure 4) [46]. Although the 16-kDa acr homologue of *M. tuberculosis* is the most dominant protein produced by stationary-phase cultures *in vitro*, it is unrecognisable in exponentially growing cultures [47]. Other proteins which we are going to study, ESAT-6 and CFP-10, have been mentioned as dominant antigens recognised by T cells and are considered virulence factors in *M. tuberculosis* [48].

Role of acr, ESAT-6, and CFP-10 for accurate diagnosis of osteoarticular TB

acr

Oxygen tension is one of the major factors frequently associated with the establishment and maintenance of latent TB [44]. *In vivo*, the bacilli number in a lesion commonly correlates well with the degree of oxygenation

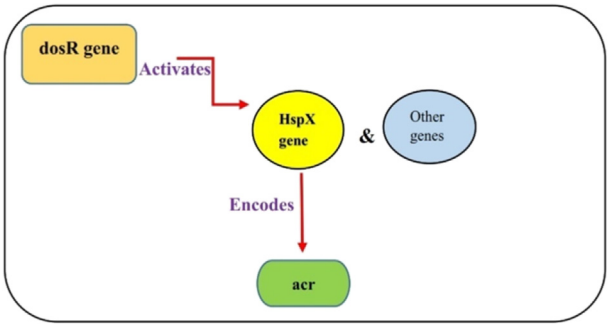

Figure 4 Hypervirulent gene dosR, which activates *hspX* with many other genes (activation of *hspX* ~80-fold). This *hspX* encodes alpha-crystallin (acr) protein, which is a major antigen. The acr is responsible for the long-term survival of the bacteria.

[49]. Hypoxia is predicted to be a key host-induced stress limiting the growth of the pathogen *in vivo*. However, various studies *in vitro* and *in vivo* indicate that *M. tuberculosis* adapts to oxygen limitation by entering into a metabolically altered state while awaiting the opportunity to reactivate [50]. Replication of *M. tuberculosis* requires O_2, but bacteria have a phenomenal ability to survive for years without O_2 *in vitro* [49]. *M. tuberculosis* is maintained under anaerobic conditions *in vitro* and therefore loses its acid-fast characteristics [51,52]. Wayne and colleagues [53,54] have pioneered the use of hypoxic culture conditions to generate nonreplicating persistent bacilli *in vitro* as a model for latency. Variants of this model have been used to identify *M. tuberculosis* genes that are potentially important for the development or maintenance of the latent state [47,55]. One such gene is *acr* (also known as *hspX*, Rv2031), which encodes acr. The *M. tuberculosis* acr is a dominant antigen *in vivo*, recognised by most *M. tuberculosis* patient sera [56]. The acr is a member of the small heat shock protein family that forms high-molecular-weight aggregates and has chaperone activity *in vitro* [57]. Under hypoxic conditions, acr expression is rapidly increased significantly [47,58]. The 16-kDa protein, α-crystallin homologue [56] encoded by the *hspX* gene [59] is synthesised at a low level in logarithmic-phase cultures, but increases remarkably while in transition from the log phase to the stationary phase. This protein becomes one of the most abundant proteins in stationary-phase bacteria that is vital for the survival of stationary-phase bacteria [57].

Belay et al [60] reported on the dynamics of proinflammatory [interferon (IFN)-γ, tumour necrosis factor (TNF)-α)] and anti-inflammatory [interleukin (IL)-10] cytokines against Rv2031 (acr), using whole blood assay in human cohorts in a TB endemic setting. The levels of IFN-γ, TNF-α, and IL-10 against Rv2031 were found to be highest during latent TB infection; this may indicate their potential as markers of defence against TB. The findings of their study suggest the potential of IFN-γ, TNF-α, and IL-10 against Rv2031 (acr) as biomarkers of the host response to *M. tuberculosis* during convalescence from, and the absence of, active TB [60].

This protein was also investigated in some animal models; for example, adjunctive immunotherapy of mice

with a DNA vaccine expressing Rv2031(acr) was shown to significantly reduce bacillary load, shorten treatment duration, result in complete restoration of lung architecture and prevent reactivation [61]. Likewise, in guinea pigs, Rv2031-based vaccination was shown to impart protection against TB through enhanced production of IL-12, IL-10, and TGF-β [62].

ESAT-6 and CFP-10

ESAT-6 and CFP-10 have been mentioned as dominant antigens recognised by T cells and are considered virulence factors in *M. tuberculosis* [48]. Previous studies have shown that these two proteins can induce a strong T cell-mediated immune response, are apparently involved in membrane and/or host-cell lysis, and are key virulence factors secreted by *M. tuberculosis* [63].

ESAT-6 is a potent T-cell protein antigen synthesised by *M. tuberculosis* [64]. CFP-10 is a protein that is encoded by the *esxB* gene. CFP-10 is a 10-kDa secreted antigen from *M. tuberculosis*. It forms a 1:1 heterodimeric complex with ESAT-6. Both genes are expressed from the region of difference 1 (RD1) of the bacterial genome and play a key role in the virulence of the infection [65]. ESAT-6 or *esxA* and CFP-10 or *esxB* genes are located in RD1 (Figure 5). The ESAT-6/CFP-10 complex is secreted by the ESX-1 secretion system, also known as the RD1 region. In the RD1 locus, the *esx* genes are part of a conserved segment encoding members of five additional protein families [66]. These two have been described as dominant antigens recognised by T cells and are considered virulence factors in *M. tuberculosis* [67]. In the later stages of infection, these two proteins play a vital role in assisting in the translocation of *M. tuberculosis* from the phagosome into the cytoplasm of the host cell [68].

CFP-10 and ESAT-6 are encoded in RD1. The importance of ESX-1 proteins for pathogenicity was shown via the reintroduction of the extended RD1 region into BCG [69], deletion of RD1 from *M. tuberculosis* [70], signature tagged and insertional mutagenesis [71,72] and deletions of targeted gene [73—75]. Several effects attributed to pathogenicity have occurred and are associated with the expression of ESX-1 in *M. tuberculosis* and/or *M. marinum*, a fish pathogen that harbours an ESX-1 system similar to that of *M. tuberculosis* [76]. These include suppression of proinflammatory responses [77], interaction with Toll-like receptor 2 [78] and/or syntenin [79], cytotoxicity and/or cytolysis [80], necrosis [81], phagosome maturation arrest [82], and formation of granuloma [83].

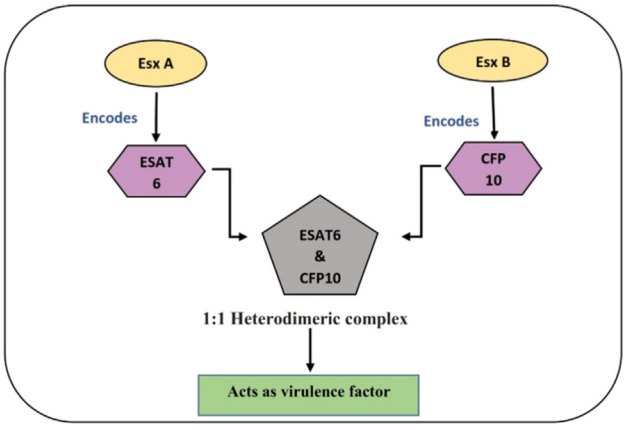

Figure 5 Diagrammatic representation of esxA and esxB genes, which encode ESAT6 and CFP-10, respectively. The latter forms a heterodimeric complex which acts as a virulence factor in *Mycobacterium tuberculosis* infection. CFP-10 = culture filtrate protein 10; ESAT6 = early-secreted antigenic target 6-kDa protein.

In the study conducted by Borgstrom et al [84], Th1 proinflammatory cytokines were expressed at high levels with both CFP-10 and ESAT-6 stimulation in samples from patients with microbiologically/histopathologically verified active TB, and the results correlated very well with proliferative responses [84].

Yuan et al [85] evaluated the value of ESAT-6/CFP-10 fusion protein using the enzyme-linked immunospot (ELlSPOT) assay for the diagnosis of spinal TB. As a result, with high sensitivity and specificity, the ELISPOT assay using CFP-10/ESAT6 fusion protein as antigen proved to be an effective technique for auxiliary diagnosis of spinal TB [85].

In another study performed by Belay et al [60], a positive and significant correlation between Rv2031 (acr) and ESAT-6 CFP-10 specific cytokine responses in each study group was reported. From previous studies on the three proteins (acr, ESAT-6, and CFP-10) of *M. tuberculosis*, we have come to the conclusion that acr is responsible for the bacterial growth survival when the microbe is in stationary/latent phase, whereas the other two proteins (i.e., ESAT-6 and CFP-10) act as virulence factors, which means the active phase of the disease.

Conclusion and perspectives

Misdiagnosis of TB extracts a huge toll on patients both in terms of health and wealth. For correct and sensitive diagnosis, we need to cope up with the drawbacks of the TB diagnostic techniques. For treatment of any disease, accurate diagnosis is essential; however, a wrongly diagnosed disease may cost the patient's life. Using the expression of α-crystallin, ESAT-6, and CFP-10 proteins of MTB to differentiate between latent and active MTB infection among different individuals may help overcome these problems. Moreover, target populations can be more specifically identified, which can help in wise decision making and logical prescription. Patient morbidity can also be reduced. Accurate diagnosis will also help prevent the development of antibiotic-resistant strains (which develop because of the empirical and generous use of these drugs viz., ATT) and consequent development of multidrug-resistant TB, which in itself represents a bigger challenge. Accurate diagnosis of active TB can help us formulate new specific treatment guidelines.

Conflicts of interest

The authors have no conflicts of interest to declare.

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