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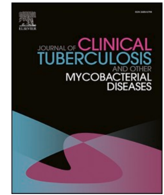

Cracking the antigenic code of mycobacteria: CFP-10/ESAT-6 tuberculosis skin test and misleading results

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ABSTRACT

There are different tuberculosis diagnostic tools available that detect an antigen-specific immune response. The present study aims to evaluate the potential of cross-reactive responses of a CFP-10 and ESAT-6 antigen-based TB test using bioinformatics tools. The study found that the presence of the sequences coding for the CFP-10 and ESAT-6 antigens in mycobacterial genomes is not associated with their pathogenicity, and not even consistent within a single species among its strains, which can lead to either false positive or false negative test results. The data that was analyzed included genome assemblies of all available mycobacterial strains obtained from the NCBI Genome database, while the standalone BLAST and tblastn programs were utilized to detect the presence of the CFP-10 and ESAT-6 sequences. The findings revealed that a number of non-pathogenic mycobacteria contained the aforementioned sequences, while some pathogenic mycobacteria did not, indicating that a standard tuberculin skin test should be more preferable for detecting various pathogenic mycobacteria compared to antigen-specific tests. In the *Mycobacterium tuberculosis* complex (MTBC), the proportion of positive strains varied within individual species, indicating a complex relationship. Among non-tuberculous mycobacteria (NTMB), more than half of the analyzed species did not contain these sequences which is consistent with their non-pathogenicity. Further research is necessary to fully comprehend the relationship between MTBC pathogenicity and the CFP-10 and ESAT-6 sequences. This could lead to a conclusion that a standard tuberculin skin test, although non-specific due to the undefined antigen content, may be able to detect various pathogenic mycobacteria in a more reliable manner than antigen-specific tests.

1. Introduction

Currently used tuberculosis (TB) diagnostic tools are aimed either at (a) identifying the pathogen in the body (e.g., bacterioscopy, liquid or solid medium cultivation, PCR), (b) detecting the immune response (tuberculin skin test, indirect hemagglutination test for the detection of antibodies, ELISA, IGRA, Diaskintest®), or (c) detecting any signs of developing infection (clinical signs, X-ray analysis, computed tomography). The accuracy of these tools is crucial in preventing false-positive or false-negative results which can lead to inappropriate prescription of antibiotics or delayed diagnosis and treatment [1].

One such diagnostic tool is the tuberculin skin test (TST), which detects immune response – the allergic skin reaction – caused by the mycobacteria-derived PPD antigen [2]. However, due to the undefined antigen composition of PPD, false-positive reactions may occur in individuals with non-TB mycobacteria or those who have been immunized with the BCG vaccine that contains a live attenuated *M. tuberculosis* variant *bovis* strain [3,4,5,6].

To address these limitations, interferon T-cell gamma release assays (IGRAs) have been developed as a potential replacement for TST. IGRAs detect interferons that are released from T-cells in response to exposure to specific antigens. IGRAs use either the RD1 (Region of Difference) antigens or the PPD antigens [2,7]. RD1-based IGRAs are more specific and reliable, discriminating TB-infected individuals from the BCG-immunized individuals [8–14] since the RD1 genomic region is absent in all tested BCG strains that are used for immunization [15]. The ESAT-

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6 (Early Secreted Antigenic Target 6) and CFP-10 (Culture Filtrate Protein 10) antigens, located in RD1, share the same messenger RNA transcript, suggesting that their combined use may be effective in TB diagnostics [16]. However, certain non-TB strains may also contain these antigens, leading to potential false-positive IGRA results [17].

In contrast to IGRAs, a convenient TB diagnostic tool must meet several requirements, such as simplicity of the set-up and use, reproducibility, use of inexpensive equipment and reagents, and the ability to obtain highly informative and easily interpretable results. TST satisfies these requirements but suffers from the disadvantage of capturing unspecific reactions and, therefore, producing false positives. To address this problem, specific efforts were undertaken to develop appropriate replacements for TST [18].

Diaskintest® (Generium Pharmaceuticals, Russia) is a novel diagnostic tool for tuberculosis (TB) that utilizes the recombinant CFP-10/ESAT-6 antigen, similar to the RD1-based IGRA. This test is administered subcutaneously and elicits an immune response in the form of an allergic skin reaction [19]. While the ESAT-6/CFP-10-based IGRA has been criticized for its lack of specificity in discriminating between TB and non-TB infections, the same criticism could apply to Diaskintest® which demonstrates similar test positivity rates [20]. However, Diaskintest® was developed as the first TB diagnostic tool administered subcutaneously and based on recombinant antigens, enabling differential TB diagnosis, assessment of infection progression, and treatment efficacy evaluation [17]. The manufacturer of this test claimed that non-pathogenic mycobacteria in the environment do not interfere with the diagnostic process, with potential difficulties only arising when it comes to *M. leprae*, *M. kansasii*, and *M. marinum*.

There are, nevertheless, other skin tests based on recombinant antigens for detecting TB. One example is the C-Tb skin test, developed by the Statens Serum Institute, Copenhagen, Denmark, which uses recombinant CFP10 and ESAT6 proteins [21]. C-Tb skin test works in a similar manner compared to conventional tuberculin skin tests by measuring delayed-type hypersensitivity reactions in response to the specific antigens present in *M. tuberculosis*. The only difference from Diaskintest® is that it uses the CFP10 and ESAT6 proteins as separate antigens, rather than as a single fusion protein with two immunogenic domains. Another example of a similar TB skin test is a test where the antigenic components in the fusion protein are arranged in a reverse order compared to Diaskintest® [22].

The advent of genome sequencing of *Mycobacterium tuberculosis* has enabled identification of TB-specific genomic regions, leading to the development of recombinant TB antigens for diagnostic purposes [23]. One such region is RD1, which is present in *M. tuberculosis* variant *bovis* and *M. tuberculosis*, but absent from *M. tuberculosis* variant *bovis* BCG vaccine strains [24]. The deletion of RD1 in BCG has been linked to attenuated phenotype [25,26]. Diagnostic tools based on RD1, such as Diaskintest®, have been shown to differentiate between BCG-immunized individuals and those infected with *M. tuberculosis* variant *bovis* or *M. tuberculosis* via immune response to the ESAT-6 and CFP-10 antigens. However, the potential of cross-reactivity with non-TB mycobacteria, such as *M. smegmatis* or BCG-mycobacteria, remains a concern. This issue can be addressed by comparing the genomes of different mycobacterial strains for the presence of the ESAT-6 and CFP-10 sequences.

2. Materials and methods

This study used data from the Genome section of GenBank as of August 2022 [27] to investigate the presence of the RD1 region in various mycobacterial species. The selected species were obtained from the NCBI Genome database using a specific search query "Mycobacterium [organism]". Genome assemblies of all currently available strains were included in the analysis. Hits that were labeled as "group of uncharacterized isolates" were left out from the total number of selected species. The same was done for mycolicibacteria, previously belonging to the *Mycobacterium* genus, using the "Mycolicibacterium [organism]" search query. The dataset for the analysis comprised genome assemblies of all available strains of the selected species, assigned either to *Mycobacterium* or *Mycolicibacterium* genera.

Standalone BLAST and tblastn programs [28] were used to detect the RD1 region, using the amino acid sequences of *M. tuberculosis* H37Rv strain for the CFP-10 and ESAT-6 proteins as input queries (GenBank accession numbers ACH88465.1 and P9WNK7.1, respectively) with e10 as a threshold e-value while all the other parameters were set as default. Genomic assemblies were classified as representing the same strain if the strain names coincided in their annotations. The results were manually curated and grouped into four mycobacterial strain clusters based on pathogenicity/ecological features: MTBC (*M. tuberculosis* complex), opportunistic NTMB (non-tuberculous mycobacteria), saprophytic (non-pathogenic) NTMB, and pathogenic NTMB (non-tuberculosis mycobacteria). All curated results are fully presented in Supplementary Table 1 in columns representing the total number of assemblies available, the number of strains sequenced, and the number of strains containing that RD1 region. The availability of corresponding genomic sequences in the RefSeq database was also manually checked based on assembly annotations. Supplementary Table 1 provides a comprehensive representation of all curated results. Some of the mycobacterial species changed their taxonomic status from *Mycobacterium* to *Mycolicibacterium* and were indicated in the table accordingly.

3. Results

The presence of the CFP-10 and ESAT-6 sequences in some mycobacteria, which are normally recognized as markers of pathogenicity, should imply that the mycobacteria in question are pathogenic, which is the reason why these antigens are used in Diaskintest®. Nonetheless, the presence of the CFP-10 and ESAT-6 sequences does not consistently imply pathogenicity, as some pathogenic species may not contain these sequences. On the other hand, some non-pathogenic environmental species may contain these sequences and lead to false positive results on an ESAT-6 and CFP-10 TB-based tuberculosis skin test.

Briefly, according to the summarized data presented in Table 1, two out of nine pathogenic MTBC species did not contain the ESAT-6 and CFP-10 sequences. The data also demonstrate that, on the contrary, opportunistic NTMB contained these sequences. Similarly, the majority of saprophytic NTMB was detected with the ESAT-6/CFP-10 present in the genome.

Once again, the presence of these sequences within a single species of the same mycobacterial cluster was not uniform. In the MTBC cluster (Table 1), all *M. tuberculosis* variant *africanum*, *M. tuberculosis* variant *caprae*, and *M. orygis* strains contained the ESAT-6 and CFP-10 sequences, whereas some strains of the *M. tuberculosis*, *M. canettii*, and *M. tuberculosis* variant *bovis* strains did not. The deletion of the ESAT-6/CFP-10 fragment in *M. tuberculosis* variant *bovis* BCG strain that is used for the vaccine is confirmed by the present data, as previously demonstrated in other studies [24]. This underscores the significant advantage of Diaskintest® which is to exclude the TB-negative BCG-immunized individuals from the positive test results in the context of mass BCG vaccination [29]. Moreover, the deletion of the RD1 fragment containing these sequences in *M. tuberculosis* variant *bovis* BCG strain is believed to cause an attenuated phenotype in this strain [25].

The MTBC cluster showed no straightforward explanation for the connection between the MTBC (pathogenic properties) and the ESAT-6 and CFP-10 antigens (their sequences). Most of the species that had these sequences did so in all their strains. Within this cluster, there was only one exception – *M. tuberculosis* variant *bovis*. In this species, most of the strains were ESAT-6/CFP-10-positive but not all (3 % of the strains lacked these sequences).

In the NTMB cluster (Table 1), majority of the analyzed species had the ESAT-6 and CFP-10 sequences although they are, by definition, considered non-pathogenic. As with the MTBC complex, there were

Table 1
The presence of the CFP-10 and ESAT-6 sequences in the genomes the pathogenic MTBC (*Mycobacterium tuberculosis* complex), opportunistic NTMB (non-tuberculous mycobacteria), and saprophytic (non-pathogenic NTMB). The fields showing the absence of CFP-10 and ESAT-6 in pathogenic species and their presence in non-pathogenic species are highlighted to emphasize the inconsistency of occurrence of these genes depending on pathogenicity and ecological characteristics of mycobacteria. The data was obtained from the NCBI Genome database.

group	species	# of strains	# of strains with CFP-10 and ESAT-6	source
MTBC (*Mycobacterium tuberculosis* complex)	*M. tuberculosis*	6911*	6899*	hum
	M. tuberculosis variant *africanum*	34	34	hum
	M. canetti	31	31	hum
	M. tuberculosis variant *bovis*	102	99	anim, hum
	M. bovis (BCG)**	37	0	national strain collection
	M. pinnipedii	3	3	anim
	M. tuberculosis variant *microti*	8	0	anim
	M. tuberculosis variant *caprae*	4	4	anim
	*M. orygis****	3	3	hum
opportunistic NTMB complex	*M. celatum*	3	3	env, hum
	M. kyorinense	4	4	env, hum
	M. haemophilum	5	5	env, hum
	M. bohemicum	2	0	env, hum
	M. arupense	4	0	env, hum
	Mycolicibacterium cosmeticum (M. oesypum)	3	1	env, hum
	M. xenopi	6	0	env, hum
saprophytic (non-pathogenic) NTMB complex	*Mycolicibacterium aromaticivorans*	3	0	env (soil)
	Mycolicibacterium aurum	2	2	env (soil)
	Mycolicibacterium chlorophenolicum	3	0	env (soil)
	Mycolicibacterium chubuense	3	2	env (soil)
	Mycolicibacterium gilvum	2	2	env (soil)
	M. vanbaalenii	3	3	env (soil)
	Mycolicibacterium neoaurum	11	9	env (soil)
	M. phlei	8	8	env (soil)
	Mycolicibacterium tusciae	2	2	env (soil)
	Mycolicibacterium smegmatis	60	60	env (soil)
	Mycolicibacterium thermoresistibile	3	3	env (soil)

* - The absence of the CFP-10 and ESAT-6 sequences was demonstrated in the experiments on the construction of bacterial artificial chromosome in the *M. microti* OV254 strain [30], as well as in microarray and PCR experiments in ATCC strains 35782, 94/2272, 005004, and OV254 [31]. Independent evidence (from the complete genome sequencing data) on the presence of the ESAT-6 and CFP-10 sequences was provided by other authors [32–36]. ** - *M. bovis* (BCG) - vaccine strains from different countries and laboratories. *** - *M. orygis* was added to the MTBC group of pathogenic mycobacteria in 2014 [37]. Abbreviations for the source: env – environment, hum – humans, anim – animals.

Table 2
The presence of the CFP-10 and ESAT-6 sequences in the genomes of pathogenic mycobacterial species from the NTMB (non-tuberculosis mycobacteria) complex. The fields showing the absence of CFP-10 and ESAT-6 in pathogenic species are highlighted to emphasize the inconsistency of occurrence of these genes and the lack of clear association with pathogenicity. The data was obtained from the NCBI Genome database.

pathogenic NTMB (non-tuberculosis mycobacteria) complex: species	# of strains	# of strains with CFP-10 and ESAT-6	source
M. kansasii	39	39	hum
M. ulcerans	10	3	env, hum, anim
M. marinum	48	48	env, anim (*Danio sp.*)
M. liflandii	2	2	anim (*X. tropicalis*)
M. szulgai	1	1	env, anim, hums
M. leprae	6	6	hum
M. lepromatosis	2	2	hum
M. avium	273	0	anim, hum, env
M. intracellulare	54	1	env, hums
M. arosiense	1	0	env, hum
M. marseillense	6	0	env, hum
M. yongonense	6	0	env, hum
*M. vulneris**	1	0	env, hum
M. bouchedurhonense	1	0	e env, hum
M. timonense	2	0	en env, hum
M. colombiense	20	0	env, hum
M. intracellulare subsp. chimaera	25	0	env, hum
M. mantenii	6	0	env, hum
M. indicus pranii	3	0	env, anim
M. simiae	4	1	env, anim
M. parascrofulaceum	1	0	env, anim
M. genavense	1	0	env, anim
M. triplex	2	2	env, anim
M. europaeum	2	2	env, anim
M. lentiflavum	2	2	env, anim
Mycolicibacterium fortuitum	33	33	env, anim
Mycolicibacterium setense	4	4	env, anim

* - Two assemblies are available in GenBank for the same strain of *M. vulneries*. One of them contains both genes of the RD1 region, but it has been suppressed in the RefSeq database as “contaminated”. Abbreviations for the source: env – environment, hum – humans, anim – animals.

limited differences in the proportion of strains that were positive for the ESAT-6/CFP-10 sequences; the sequences were largely either present or absent within the species. The only exception in this cluster was *Mycolicibacterium cosmeticum*.

In the opportunistic NTMB cluster (Table 1), only four out of seven species contained the ESAT-6/CFP-10 sequences, contradicting initial expectations that the opportunistic mycobacteria would not contain these sequences. This observation may be explained by a limited number of sequenced genomes within this group. In the same manner, nine out of eleven saprophytic NTMB species had these sequences (Table 1), despite being non-pathogenic.

Similarly, contrary to what was expected, results for the pathogenic NTMB group demonstrate that many of the pathogenic strains do not contain the ESAT-6/CFP-10 sequences (Table 2). The loss of these sequences in *M. ulcerans* was proposed to have occurred due to genetic variations in RD1 and its proneness to mutations. The function of the loss was interpreted as helping mycobacteria bypass the host's immunological defense mechanisms. Therefore, this deletion may represent an ongoing adaptation [38]. Therefore, from all these results one general observation is evident. This observation is that the number of strains with the ESAT-6/CFP-10 sequences in a single species is either zero or all strains contain them (with very few exceptions).

The findings presented in Table 1 and Table 2 of this review suggest that Diaskintest® may lack specificity not only for *M. leprae*, *M. kansasii*, and *M. marinum*, as reported by the manufacturer, but also for other non-tuberculosis species and strains. The presence or absence of the ESAT-6 and CFP-10 sequences in the genomes of pathogenic, opportunistic, or saprophytic mycobacteria is insufficient to distinguish between these groups and evaluate the efficacy of Diaskintest®. Furthermore, previous research has indicated that tests utilizing these antigens may be even less informative than the tuberculin skin test in some cases [18].

4. Discussion

TST is not recommended for the BCG-immunized individuals due to potential false positives and reduced ability to distinguish between allergic skin reactions caused by TB infection and BCG immunization. In other words, the TST test suffers from the lack of specificity which means it is unable to avoid false positives successfully. Therefore, RD1-specific tests are preferred for their HIV-status independence and reduced confounds associated with BCG immunization. Although Diaskintest® has been proposed as an alternative to TST for detecting active TB cases to avoid false positives and improve specificity in TB diagnostics [18], false negatives can occur as well. To address this, antigen dose adjustments may be necessary to improve the sensitivity of the test and increase its power to avoid false negatives [19]. Additionally, individuals with immunodeficiencies or a latent TB infection may test negative on Diaskintest®, which are also treated as false negatives [39]. Although Diaskintest® might be able to avoid false positives that are related to the BCG-immunization status, it still suffers from the lack of power to avoid false positives related to the immune response that was previously mounted against these antigens due to exposure to environmental, non-pathogenic mycobacteria. Therefore, although introduced to address false positives related to the BCG-immunization status, false positives are still its major limitation.

In the context of the sensitivity of antigen-based tests, recent investigations have highlighted the potential lack of ability of the tests utilizing the ESAT-6 and CFP-10 antigens to avoid false negatives, when the antigen dose is not in question. In a study involving 214 individuals diagnosed with non-TB mycobacteria (the NTMB) but without any previous indications of TB in their medical record, interferon T-cell response was assessed using the ESAT-6/CFP-10-specific QuantiFERON TB-2G (QFT-2G) test which is ESAT-6/CFP-10-specific [40]. The results are represented in Table 3. These results indicate that the use of ESAT-6/CFP-10-based assays for the detection of non-TB mycobacterial conditions is not recommended because of the possibility of yielding false negative results or non-detection of latent TB infection. Therefore, it is suggested that these tests should be used in combination with additional TB detection tools such as the tuberculin skin test. These findings have led researchers to conclude that caution should be exercised when using tests based on the ESAT-6 and CFP-10 antigens for TB diagnosis.

In another study, individuals with either TB or non-TB conditions were tested using 3 different TB diagnostic tests – two IGRAs (PPD and ESAT-6/CFP-10-based) and TST (Table 4) [41]. The results showed that even some non-TB positive individuals tested positive on TB. Based on this observation, the suggestion of expanding the application of the tests that are based on the ESAT-6 and CFP-10 antigens for the detection of non-TB mycobacterial conditions is not recommended, since only about half of these cases can be really detected (even with *M. kansasii*, where all genetically characterized strains have the ESAT-6 and CFP-10 sequences). In addition to the main disadvantage of the ESAT-6/CFP-10-based assays which is the possibility of yieling false negative results, non-detection of a latent TB infection is another disadvantage. These and other findings have led different researchers to a common conclusion that the tests based on the ESAT-6 and CFP-10 antigens should be used only in combination with additional TB detection tools such as the tuberculin skin test, for example [42,43].

The results of this study have important implications for TB detection. The mere presence or absence of the ESAT-6 and CFP-10 sequences cannot differentiate between pathogenic, opportunistic, or saprophytic mycobacteria. Therefore, the function of these antigens may not be entirely involved in pathogenicity. For example, the RD1 locus in *M. smegmatis*, as previously reported, regulates horizontal gene transfer [44,45].

However, tests based on these antigens, including Diaskintest®, can still be useful for TB detection. Subcutaneous administration of Diaskintest® is an inexpensive and convenient route of test antigen administration. Lastly, the concept of a specific antigen-based tuberculosis skin test merges the advantages of both the TST (subcutaneous administration) and the ESAT-6/CFP-10-based IGRA (antigen specificity). Additional diagnostic tools are required to confirm the obtained results, and the specificity of the assay depends on the antigens used and the accurate interpretation of the results. The more TB-specific (or even strain-specific) assay antigens used, the more accurate the detection. For example, QuantiFERON®-TB Gold uses an additional TB7.7(p4) antigen to increase the assay specificity in addition to the existing the ESAT-6 and CFP-10 antigens. In practice, the actual specificity of the assay would depend on the antigens used and the accurate interpretation of the results.

Table 3
IFN-γ T-cell secretion in the infections caused by different mycobacteria.

Infectious Agent	Positive Test Results (% of patients)
M. avium + *M. intracellulare* (163 patients)	2
M. kansasii (33 patients)	52
M. marinum (12 patients)	58
M. szulgai (3 patients)	33
M. abscessus (2 patients)	0
M. chelonei (1 patient)	0

Table 4
IFN-γ T-cell secretion test results in conditions caused by TB and non-TB mycobacteria.

Assay	TB (38 patients)	non-TB mycobacteria (40 patients)
QuantiFERON-TB Gold test (QFT-G) – PPD	89.5 % positive	34.3 % positive
QuantiFERON-TB test (QFT) – ESAT-6/CFP-10	86.8 % positive	35.3 % positive
Tuberculin skin test (TST)	70.6 % positive	47.5 % positive

5. Conclusions

This paper aimed to assess the potential of cross-reactive reactions of the CFP-10/ESAT-6 recombinant antigen-based test for tuberculosis diagnosis by analyzing genome assemblies of various pathogenic and non-pathogenic mycobacteria. The study reveals that the presence of the ESAT-6 and CFP-10 sequences in mycobacterial genomes is not consistently associated with pathogenicity or, in rare cases, even with all strains of a single species, leading to potential false negative or false positive test results. The findings suggest that the use of ESAT-6/CFP-10-based assays for TB detection should not be recommended without additional diagnostic tools, as certain MTBC strains may lead to false negatives, while certain non-pathogenic mycobacteria may lead to false positives. However, the assay can accurately exclude BCG-immunized individuals from false positives. The study highlights the need for caution when using tests based on the ESAT-6 and CFP-10 antigens for TB diagnosis and emphasizes the potential benefits of combining specific antigen-based TB skin tests with other TB detection tools to increase assay specificity and accuracy. Additionally, the inclusion of additional TB-specific antigens in these tests could further improve their reliability.

The study concludes that the use of Diaskintest® may lead to an untimely identification of individuals infected with pathogenic strains of mycobacteria that do not contain the ESAT-6 and CFP-10 sequences. Tuberculin, which contains almost all antigens including ESAT-6 and CFP-10, is suggested as a more reliable method for detecting TB infections, especially latent TB infections. The study also highlights the need for further research to determine whether the presence of the ESAT-6 and CFP-10 sequences implies pathogenicity or the capacity to gain pathogenic properties.

Taking into account the advantage of excluding the BCG-immunized individuals from the false positives (thus lowering the total number of false positives), Diaskintest® appears to be a convenient test in the setting of mass BCG immunization and limited resources to use IGRA as a TB diagnostic tool. Nevertheless, the assay does not have the capacity to clearly and reliably differentiate the conditions caused by the pathogenic from those caused by the non-pathogenic mycobacteria.

Additionally, there are some other factors that may play a crucial role in the performance of Diaskintest®. One significant factor to consider is the expression level of ESAT-6 and CFP-10 proteins following infection in different mycobacterial species. Variations in the expression levels of these proteins can impact the sensitivity and specificity of the test when the recombinant proteins are administered to an individual previously exposed to these antigens. Furthermore, this exposure of the host immune response to these antigens is another critical factor that can influence test performance taking into account the immune status, possible co-infections, or genetic predisposition. These variations can affect the accuracy of the test by affecting the production of antibodies or cellular responses that are detected by the test. In the end, this can all affect the ability of these test to accurately detect TB infection depending on the ability of the host to mount a measurable immune response that can be captured by the test.

CRediT authorship contribution statement

Igor Krasilnikov: Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Conceptualization. **Tatyana Lehnherr-Ilyina:** Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Milana Djonovic:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Irena Artamonova:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Mikhail Nikitin:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Nikolay Kislichkin:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jctube.2024.100436.

References

[1] Koretskaya NM. Diaskintest is a new method for diagnosing tuberculosis infection. Siberian Medical Review 2013;2(80):94–7.

[2] Kunst H. Diagnosis of latent tuberculosis infection: the potential role of new technologies. Respir Med 2006;100(12):2098–106 [CrossRef].

[3] Fine PE, Bruce J, Ponnighaus JM, Nkhosa P, Harawa A, Vynnycky E. Tuberculin sensitivity: conversions and reversions in a rural african population. Int J Tuberc Lung Dis 1999;3(11):962–75.

[4] Floyd S, Pönnighaus JM, Bliss L, Nkhosa P, Sichali L, Msiska G, Fine PE. Kinetics of delayed-type hypersensitivity to tuberculin induced by bacille Calmette-Guérin vaccination in northern Malawi. J Infect Dis. 2002;186(6):807–14 [CrossRef].

[5] Huebner RE, Schein MF, Bass Jr JB. The tuberculin skin test. Clin Infect Dis. 1993; 17(6):968–75 [CrossRef].

[6] Kwamanga DO, Swai OB, Agwanda R, Githui W. Effect of non-tuberculous mycobacteria infection on tuberculin results among primary school children in Kenya. East Afr Med J 1995;72(4):222–7.

[7] Munk ME, Kern P, Kaufmann SH. Human CD30+ cells are induced by Mycobacterium tuberculosis and present in tuberculosis lesions. Int Immunol. 1997;9(5):713–20 [CrossRef].

[8] Lalvani A, Pathan AA, Durkan H, Wilkinson KA, Whelan A, Deeks JJ, et al. Enhanced contact tracing and spatial tracking of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. Lancet 2001;357(9273): 2017–21 [CrossRef].

[9] Ewer K, Deeks J, Alvarez L, Bryant G, Waller S, Andersen P, et al. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of *Mycobacterium tuberculosis* infection in a school tuberculosis outbreak. Lancet 2003;361(9364): 1168–73 [CrossRef].

[10] Richeldi L, Ewer K, Losi M, Bergamini BM, Roversi P, Deeks J, Fabbri LM, Lalvani A. T cell-based tracking of multidrug resistant tuberculosis infection after brief exposure. Am J Respir Crit Care Med. 2004;170(3):288–95 [CrossRef].

[11] Brock I, Weldingh K, Lillebaek T, Follmann F, Andersen P. Comparison of tuberculin skin test and new specific blood test in tuberculosis contacts. Am J Respir Crit Care Med. 2004;170(1):65–9 [CrossRef].

[12] Shams H, Weis SE, Klucar P, Lalvani A, Moonan PK, Pogoda JM, et al. Enzyme-linked immunospot and tuberculin skin testing to detect latent tuberculosis infection. Am J Respir Crit Care Med 2005;172(9):1161–8 [CrossRef].

[13] Kang YA, Lee HW, Yoon HI, Cho B, Han SK, Shim YS, Yim JJ. Discrepancy between the tuberculin skin test and the whole-blood interferon gamma assay for the diagnosis of latent tuberculosis infection in an intermediate tuberculosis-burden country. JAMA. 2005;293(22):2756–61.

[14] Mori T, Sakatani M, Yamagishi F, Takashima T, Kawabe Y, Nagao K, Shigeto E, Harada N, Mitarai S, Okada M, et al. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. Am J Respir Crit Care Med. 2004;170(1):59–64.

[15] Lalvani A, Nagvenkar P, Udwadia ZZ, Pathan AA, Wilkinson KA, Shastri JS, Ewer K, Hill AV, Mehta A, Rodrigues C. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent Mycobacterium tuberculosis infection in healthy urban Indians. J Infect Dis. 2001;183(3):469–77 [CrossRef].

[16] Berthet FX, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiol (Reading). 1998;144 (Pt11):3195–203 [CrossRef].

[17] Mednikov, B.L.; Slogotskaya, L.V. Skin test with Diaskintest® (recombinant tuberculosis 0.2 μg allergen in 0.1 ml solution for intradermal administration) to identify tuberculosis infection: a guide for physicians. Available at: http://do.telec linica.ru/override/newmed/cixfiles/2136886/kozhnaja_proba_s_preparatom_dia skintest.pdf. Accessed August 20, 2021.

[18] Miheeva IV, Burdova EY, Melnikova AA. Comparative evaluation of methods for allergic diagnosis of tuberculosis in children. Epidemiology and Vaccine Prophylaxis 2016;15.3(88):41–4.

[19] Levi DT, Nakonechnaya AV, Obuhov UI, Ruhamina ML, Aleksandrova NV. Tuberculosis allergens - drugs for the specific diagnosis of tuberculosis. BioDrugs*Prophylaxis, diagnostics, treatment* 2013;4(48):10–7.

[20] Nikitina IY, Karpina NL, Kasimceva OV, Gergert VY, Ergeshov A, Lyadova IV. Comparative performance of QuantiFERON-TB gold versus skin test with tuberculosis recombinant allergen (diaskintest) among patients with suspected pulmonary tuberculosis in Russia. Int J Infect Dis 2019;86:18–24.

[21] Ruhwald M, Aggerbeck H, Gallardo RV, Hoff ST, Villate JI, Borregaard B, Martinez JA, Kromann I, Penas A, Anibarro LL, de Souza-Galvão ML, Sánchez F, Rodrigo-Pendás JÁ, Noguera-Julian A, Martínez-Lacasa X, Tuñez MV, Fernández VL, Millet JP, Moreno A, Cobos N, Miró JM, Roldan L, Orcau A, Andersen P, Caylá JA. TESEC Working Group Safety and efficacy of the C-Tb skin test to diagnose Mycobacterium tuberculosis infection, compared with an interferon γ release assay and the tuberculin skin test: a phase 3, double-blind, randomised, controlled trial. Lancet. Respir Med. 2017;5(4):259–68 [CrossRef].

[22] Li F, Xu M, Zhou L, Xiong Y, Xia L, Fan X, Gu J, Pu J, Lu S, Wang G. Safety of recombinant fusion Protein ESAT6-CFP10 as a Skin test reagent for tuberculosis diagnosis: an open-label, randomized, single-center phase i clinical trial. Clin Vaccine Immunol. 2016;23(9):767–73 [CrossRef].

[23] Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J Bacteriol 1996;178(5):1274–82 [CrossRef].

[24] Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999;284(5419):1520–3 [CrossRef].

[25] Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, Behr MA, Sherman DR. Deletion of RD1 from Mycobacterium tuberculosis mimics bacille Calmette-Guérin attenuation. J Infect Dis. 2003;187(1):117–23 [CrossRef].

[26] Pym AS, Brodin P, Brosch R, Huerre M, Cole ST. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. Mol Microbiol. 2002;46(3):709–17 [CrossRef].

[27] Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Ostell J, Pruitt KD, Sayers EW. GenBank. Nucleic Acids Res. 2018;46(D1):D41–7 [CrossRef].

[28] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25(17):3389–402 [CrossRef].

[29] Slogotskaya L, Bogorodskaya E, Ivanova D, Sevostyanova T. Comparative sensitivity of the test with tuberculosis recombinant allergen, containing ESAT6-CFP10 antigen, and mantoux test with 2 TU PPD-L in newly diagnosed tuberculosis children and adolescents in Moscow. PLoS One 2018;13(12).

[30] Brodin P, Eiglmeier K, Marmiesse M, Billault A, Garnier T, Niemann S, Cole ST, Brosch R. Bacterial artificial chromosome-based comparative genomic analysis identifies Mycobacterium microti as a natural ESAT-6 deletion mutant. Infect Immun. 2002;70(10):5568–78 [CrossRef].

[31] Gordon SV, Brosch R, Billault A, Garnier T, Eiglmeier K, Cole ST. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Mol Microbiol. 1999;32(3):643–55 [CrossRef].

[32] Garcia-Pelayo MC, Caimi KC, Inwald JK, Hinds J, Bigi F, Romano MI, van Soolingen D, Hewinson RG, Cataldi A, Gordon SV. Microarray analysis of Mycobacterium microti reveals deletion of genes encoding PE-PPE proteins and ESAT-6 family antigens. Tuberculosis (Edinb). 2004;84(3–4):159–66 [CrossRef].

[33] Marmiesse M, Brodin P, Buchrieser C, Gutierrez C, Simoes N, Vincent V, Glaser P, Cole ST, Brosch R. Macro-array and bioinformatic analyses reveal mycobacterial `core` genes, variation in the ESAT-6 gene family and new phylogenetic markers for the Mycobacterium tuberculosis complex. Microbiol (Reading). 2004;150(Pt 2): 483–96 [CrossRef].

[34] Yip MJ, Porter JL, Fyfe JA, Lavender CJ, Portaels F, Rhodes M, et al. Evolution of *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria from a common *Mycobacterium marinum* progenitor. J Bacteriol 2007;189(5):2021–9 [CrossRef].

[35] Mve-Obiang A, Lee RE, Umstot ES, Trott KA, Grammer TC, Parker JM, Ranger BS, Grainger R, Mahrous EA, Small PLC. A newly discovered mycobacterial pathogen isolated from laboratory colonies of Xenopus species with lethal infections produces a novel form of mycolactone, the Mycobacterium ulcerans macrolide toxin. Infect Immun. 2005;73(6):3307–12 [CrossRef].

[36] van Ingen J, de Zwaan R, Dekhuijzen R, Boeree M, van Soolingen D. Region of difference 1 in nontuberculous mycobacterium species adds a phylogenetic and taxonomical character. J Bacteriol. 2009;191(18):5865–7 [CrossRef].

[37] Tortoli E. Microbiological features and clinical relevance of new species of the genus Mycobacterium. Clin Microbiol Rev. 2014;27(4):727–52 [CrossRef].

[38] Huber CA, Ruf MT, Pluschke G, Käser M. Independent loss of immunogenic proteins in Mycobacterium ulcerans suggests immune evasion. Clin Vaccine Immunol. 2008;15(4):598–606 [CrossRef].

[39] Electronic calalogue „Vidal“. Instruction for use for Diaskintest®. Available at: https://www.vidal.ru/drugs/diaskintest_28611. Accessed August 20, 2021.

[40] Kobashi Y, Mouri K, Yagi S, Obase Y, Miyashita N, Okimoto N, et al. Clinical evaluation of the QuantiFERON-TB gold test in patients with non-tuberculous mycobacterial disease. Int J Tuberc Lung Dis 2009;13(11):1422–6.

[41] Ra SW, Lyu J, Choi CM, Oh Y-M, Lee S-D, Kim WS, Kim DS, Shim TS. Distinguishing tuberculosis from Mycobacterium avium complex disease using an interferon-gamma release assay. Int J Tuberc Lung Dis. 2011;15(5):635–40 [CrossRef].

[42] Mazurek, G.H.; Jereb, J.; Vernon, A.; LoBue, P.; Goldberg, S.; Castro, K.; IGRA Expert Committee; Centers for Disease Control and Prevention (CDC). Updated guidelines for using Interferon Gamma Release Assays to detect *Mycobacterium tuberculosis* infection - United States, 2010. *MMWR Recomm Rep*. **2010**; *59(RR-5)*, 1-25.

[43] Krasilnikov IV. Modern methods for mass early diagnosis of tuberculosis infection. Medical Alphabet 2013;1(6):33–5.

[44] Flint JL, Kowalski JC, Karnati PK, Derbyshire KM. The RD1 virulence locus of Mycobacterium tuberculosis regulates DNA transfer in Mycobacterium smegmatis. Proc Natl Acad Sci USA 2004;101(34):12598–603 [CrossRef].

[45] Coros A, Callahan B, Battaglioli E, Derbyshire KM. The specialized secretory apparatus ESX-1 is essential for DNA transfer in Mycobacterium smegmatis. Mol Microbiol. 2008;69(4):794–808 [CrossRef].