

Journal of Advanced Pharmacy Research

Section E: Microbiology & Immunology



Detection of *Mycobacterium tuberculosis* Serum Biomarkers and the Relation with Previous BCG Vaccination

Mohammed Bahey-El-Din^{1*}, Manar Hesham Younes², Taha Ibrahim Zaghoul²

¹ Department of Microbiology and Immunology, Faculty of Pharmacy, Alexandria University, Alexandria, 21521, Egypt.

² Department of Biotechnology, Institute of Graduate Studies and Research (IGSR), Alexandria University, Alexandria, 21526, Egypt.

*Corresponding author: Mohammed Bahey-El-Din, Department of Microbiology and Immunology, Faculty of Pharmacy, Alexandria University, Alexandria, 21521, Egypt. Tel. +201112462729
Email address: m.bahey-el-din@alexu.edu.eg

Submitted on: 24-09-2020; Revised on: 26-10-2020; Accepted on: 06-11-2020

To cite this article: Bahey-Eldin, M.; Younes, M. H.; Zaghoul, T. I. Detection of *Mycobacterium tuberculosis* Serum Biomarkers and the Relation with Previous BCG Vaccination. *J. Adv. Pharm. Res.* **2021**, 5 (1), 241-247. DOI: [10.21608/aprh.2020.44002.1116](https://doi.org/10.21608/aprh.2020.44002.1116)

ABSTRACT

Objectives: Accurate diagnosis of tuberculosis (TB) disease is a cornerstone in the management of disease spread and initiation of proper treatment. Detection of *Mycobacterium tuberculosis* (MTB) antigens in biological fluids might be a reliable way to directly diagnose TB disease. The aim of the current study is to investigate selected TB antigens as potential diagnostic serum biomarkers. **Methods:** We have explored the detection of three MTB antigens, namely Rv2324, Rv2435c and Rv1284, in serum specimens from confirmed pulmonary TB patients. In addition, serum specimens from Bacillus Calmette-Guérin (BCG)-vaccinated healthy adults and BCG-unvaccinated infants were also tested as negative controls. The three antigens were recombinantly expressed in *Escherichia coli* and purified using affinity chromatography. Murine antibodies against the three antigens were raised by mice immunization with corresponding recombinant antigens. TB antigen detection in the serum specimens was carried out by the dot blot test. **Results:** Serum samples from TB patients showed significant reactivity (94.3-100%) with the antigen-specific antibodies. Surprisingly, the three antigens were also detectable in serum specimens (93.1-100%) from healthy adults. On the contrary, the three antigens were barely detectable in sera from BCG-unvaccinated infants (0-14%). **Conclusion:** These findings, supported by recent literature, highlight the significant interference of BCG vaccination with the diagnosis of TB disease by direct antigen detection. Nevertheless, the three tested antigens could represent useful TB serum biomarkers in countries where BCG vaccination is not routinely performed.

Keywords: *Mycobacterium tuberculosis* diagnosis; BCG vaccine; Rv2324; Rv2435c; Rv1284

INTRODUCTION

Tuberculosis (TB) represents one of the top ten causes of death worldwide where about 1.5 million deaths occurred in 2018¹. Furthermore, the growing multi-resistance profile of *Mycobacterium tuberculosis*

(MTB) complicates the antibiotic treatment regimens and renders them longer and more toxic^{1,2}. Early and accurate diagnosis of TB disease is critical for proper treatment and prevention of disease spreading. Several diagnostic approaches are followed to diagnose pulmonary TB disease. Smear staining of *tubercle bacilli*

in sputum specimens and culture methods are the traditional diagnostic approaches in low-resources settings. However, they suffer from low sensitivity and extended incubation time of about 12 weeks, respectively. Tuberculin skin test (TST) detects delayed-type immune response against MTB. However, it suffers from interference by many factors including prior Bacillus Calmette-Guérin (BCG) vaccination besides variability in the interpretation of test results³.

The World Health Organization (WHO) endorses the use of Xpert MTB/RIF® (Cepheid, USA) to detect the presence of MTB and rifampicin resistance¹. Other molecular techniques such as line probe assays and TB-loop-mediated isothermal amplification (TB-LAMP) besides interferon-gamma release assay (IGRA) are also recognized by WHO¹. However, the latter methods have their own limitations in addition to their relative high cost especially in low-income countries where TB is endemic. Serological tests involving detection of antibodies against TB antigens are not recommended for TB diagnosis due to poor specificity and sensitivity^{4,5}.

Another important approach to diagnose TB is the direct detection of TB antigens in biological fluids⁶. Detection of lipoarabinomannan (LAM) of MTB in urine samples is used for the diagnosis of TB in patients co-infected with Human Immunodeficiency Virus (HIV) but not recommended in HIV-negative patients¹. Detection of TB-specific biomarkers in biological fluids is a promising area which can speed up TB diagnosis as it offers a definitive confirmation of active disease. Thus, the search for potential TB biomarkers is important to ultimately establish a useful TB diagnostic test. In the current study, we have investigated the detection of three TB biomarkers, namely Rv2324, Rv2435c and Rv1284, in serum specimens as a direct method for TB diagnosis. The selection of the latter biomarkers was based on the study made by Commandeur and colleagues who made a genome wide approach for recognition of *in-vivo* expressed MTB antigens in four mouse infection models⁷. It was revealed that 16 MTB antigens, including the three antigens of the present study, were expressed *in-vivo* and were able to elicit immune response, rendering them potential vaccine candidates⁷. Hence, we reasoned that these antigens are potential TB serum biomarkers which can be looked for in serum specimens for detection of active TB disease. We also chose these three antigens due to their relatively small molecular weights which would facilitate their successful recombinant expression in *Escherichia coli*. Up to our knowledge, this is the first report exploring the direct detection of these three biomarkers in serum as a means for TB diagnosis.

MATERIAL AND METHODS

Serum samples

Seventy serum samples were collected from confirmed, newly diagnosed patients with MTB pulmonary disease. All patients were hospitalized at Elmaamoura Chest Hospital, Alexandria, Egypt. Diagnosis was confirmed by microscopical examination of sputum samples for acid fast bacilli and/or by chest X-ray examination of infected subjects. The age of patients ranged from 20 to 68 years with a median age of 44 years, where 44 patients (62.8%) were males. In addition, 58 serum samples were collected from tuberculosis (TB)-negative healthy volunteers who were previously Bacillus Calmette-Guérin (BCG)-vaccinated and none of them had a history of exposure to TB. The age of the healthy volunteers ranged from 15 to 52 years with a median age of 33 years, where 35 patients (60.3%) were males. Moreover, 43 serum samples were collected from newborn infants from El-Shatby University Hospital for Obstetrics and Gynecology. Newborn infants were not vaccinated with BCG and thus were used as negative controls where 19 samples (44.2%) were obtained from male newborns. The serum collection process was performed following national ethical guidelines and in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and upon informed consent from individual patients/guardians and approval by the research ethics committee of Alexandria University.

Bacterial strains, plasmids and culture conditions

A clinical isolate of MTB was obtained from a positive culture on Lowenstein-Jensen (L J) medium from the microbiology laboratory at Elmaamoura Chest Hospital in Alexandria. *Escherichia coli* DH5- α was used as a cloning host while *E. coli* M15 (pREP4) (Qiagen) was used as an expression host for the tested antigens in the present study. Luria Bertani (LB) broth (Lab M, UK) was used to culture *E. coli* with incubation at 37°C under shaking conditions.

Polymerase chain reaction (PCR) for amplification of MTB genes

The mycobacterial antigenic determinant genes *Rv2324*, *Rv2435c* and *Rv1284* were amplified using PCR. Briefly, few colonies of MTB, grown on L J slant and suspended in 200 μ l sterile distilled water, were boiled for 20-25 min, in a boiling water bath⁸. The suspension was then centrifuged and the supernatant, which contained MTB genomic DNA, was used as a template. PCR amplification of target genes was carried out using MyTaq® Hot start master mix (Bioline, UK) following manufacturer's instructions and using the corresponding primers described in **Table 1**.

Table 1. Oligonucleotide primers used in the present study

Primer code	Description	^a Sequence (5'-3')
MTB7	<i>Rv2324</i> forward primer	TATAGGATCCGATGGACCGCCTGGATGACAC (<i>Bam</i> HI)
MTB8	<i>Rv2324</i> reverse primer	ATTA <u>AAAGCTT</u> CTAGCGGTCGATGAGGTTTGACA (<i>Hind</i> III)
MTB1	<i>Rv2435c</i> forward primer	TGAAGGATCCGATGGTGC GGCCAATCCG (<i>Bam</i> HI)
MTB2	<i>Rv2435c</i> reverse primer	ATTA <u>AAAGCTT</u> TTATGATCGCTCCGACAATCG (<i>Hind</i> III)
MTB14	<i>Rv1284</i> forward primer	TAATGGATCCGATGGTTACCGACGACTACCTGGC (<i>Bam</i> HI)
MTB15	<i>Rv1284</i> reverse primer	TAATA <u>AGCTT</u> CTAGGGCGTGACCTCGTTGA (<i>Hind</i> III)

^a Restriction sites are underlined, while enzyme names are given in parentheses.

Design of primers was based on published sequences of MTB H37Rv strain found in the *Mycobacterium* database (<https://mycobrowser.epfl.ch/>). Primers were designed with flanking *Bam*HI and *Hind*III restriction sites (Table 1) to enable directional cloning in the multiple cloning site (MCS) of pQE31 plasmid vector. In case of *Rv2435c*, only the C-terminal part, which includes the cyclase domain, was cloned in order to avoid the two hydrophobic transmembrane domains found at the N-terminal⁹.

Cloning of *M. tuberculosis* genes in *E. coli*

The resulting purified PCR products were double digested with *Bam*HI and *Hind*III (ThermoScientific, USA), then ligated to a similarly digested pQE31 plasmid using T4 DNA ligase (ThermoScientific, USA). Chemically competent *E. coli* DH5- α was subsequently transformed with the ligation reaction mixture. Transformants were plated onto LB agar containing ampicillin (100 μ g/ml) as a selection antibiotic. Positive clones were grown, and the cultures were subjected to plasmid extraction using GeneJET Plasmid Miniprep Kit (ThermoScientific, USA). The extracted recombinant plasmids (pQE31-*Rv2324*), (pQE31-*Rv2435c*) and (pQE31-*Rv1284*) were then used to transform chemically competent *E. coli* M15 (pREP4) as an expression host. Transformants were plated onto LB agar containing ampicillin (100 μ g/ml) and kanamycin (25 μ g/ml) and positive clones were confirmed by colony PCR. Finally, one positive clone of each construct was stored at -80°C. Correct sequences of cloned genes were confirmed using commercial DNA sequencing service offered by Eurofins Genomics, Germany.

Protein expression and purification using metal affinity chromatography

For the purpose of overexpression of mycobacterial antigens, cultures of *E. coli* M15 (pREP4) containing recombinant pQE31 derivative plasmids

carrying the mycobacterial genes were induced with isopropyl- β -D-thiogalactopyranoside (IPTG) for 3h at 37°C. Under native conditions, cells were lysed by bead beating as previously described¹⁰. Since pQE31 adds an N-terminal His tag to expressed proteins, metal affinity chromatography (using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads) was used for protein purification from cell lysate following the protocol recommended by Qiagen (The QIAexpressionist™). The eluted proteins were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Raising antigen-specific polyclonal antibodies in mice

Each of the purified antigens (*Rv2324*, *Rv2435c* and *Rv1284*) was thoroughly mixed with equal volume of incomplete Freund's adjuvant (IFA) (BD Bioscience). For each protein separately, two female Swiss albino mice (six-weeks old) were immunized intraperitoneally with 0.2ml of the protein/IFA mixture containing 10 μ g protein, on days 1, 15 and 30. Three weeks after the last booster dose, serum samples were collected from the immunized mice using the submandibular bleeding technique^{11,12}. Animal immunization procedures were in accordance with the EU Directive 2010/63/EU for animal experiments and were ethically approved by the Animal Care and Use Committee (ACUC) of the Faculty of Pharmacy, Alexandria University.

Enzyme-linked immunosorbent assay (ELISA) and western blot

ELISA and western blot were carried out to confirm reactivity of the raised polyclonal antibodies. ELISA test was carried out as previously described¹³. Western blot was carried out as previously described with slight modifications¹⁴. Briefly, after SDS-PAGE, the gel containing purified recombinant proteins was blotted against a nitrocellulose membrane. The raised mouse antigen-specific polyclonal IgG antibodies (1/1000 dilution) was used as primary antibody.

Peroxidase-labeled anti-mouse IgG antibody (KPL, USA) was used as secondary antibody. Signals were detected using chemiluminescent ECL substrate (GE Healthcare, USA).

Dot blot test for qualitative detection of mycobacterial antigens

The dot blot test was used for the detection of the studied antigens in serum specimens as previously described with slight modification¹³. Briefly, a nitrocellulose membrane was divided into small square sections using a marker, according to the number of tested samples. An aliquot (15µl) of each serum sample was spotted onto the membrane, so that serum samples of TB patients, BCG-immunized healthy adults and BCG-unimmunized newborns were all spotted on different positions on the same membrane. The membrane was left to dry for few minutes then it was blocked by immersion in 5% skimmed milk for 3h at room temperature. The membrane was subsequently washed followed by addition of the raised antigen-specific polyclonal antibodies for 1h at 1/1000 dilution in skimmed milk. After membrane wash, the peroxidase-labeled anti-mouse IgG antibody (KPL, USA) was added for 1h at 1/15000 dilution. Afterwards, the membrane was thoroughly washed and the peroxidase substrate 0.05% DAB (3, 3'-diaminobenzidine tetrahydrochloride), prepared in phosphate buffered saline pH 7.4, was mixed with 1µl of hydrogen peroxide 30% per ml DAB reagent immediately before addition to the membrane.

RESULTS

Successful expression of recombinant MTB antigens and raising of antigen-specific antibodies

The mycobacterial genes *Rv2324*, *Rv2435c* and *Rv1284* were successfully PCR-amplified. All genes produced single bands at expected sizes upon detection by agarose gel electrophoresis. Sizes of amplicons of *Rv2324*, *Rv2435c* and *Rv1284* were 438 bp, 795 bp and 492 bp, respectively. The genes were subsequently cloned in pQE31 plasmid vector and ultimately transformed into *E. coli* M15 (pREP4) expression host. Sequencing data showed full identity with sequences of the corresponding mycobacterial genes as recorded in the *Mycobacterium* database. The cloned genes were expressed upon induction with IPTG and the corresponding proteins were purified. Results of SDS-PAGE showed successful protein expression in *E. coli* strains where the proteins appeared pure at expected sizes following Ni-NTA metal affinity chromatography (Figure 1).

The western blotting results confirmed the reactivity of polyclonal antibodies that were raised through animal immunization with their corresponding

recombinant antigens (Figure 2). In addition, ELISA assay showed that these raised polyclonal antibodies had appreciably good titers of 32,000 for anti-Rv2324 serum and 4000 for both anti-Rv2435c and anti-Rv1284 sera. These raised murine antibodies were employed in the subsequent dot blot test to detect mycobacterial antigens in the tested sera of TB patients, healthy adults and newborns.

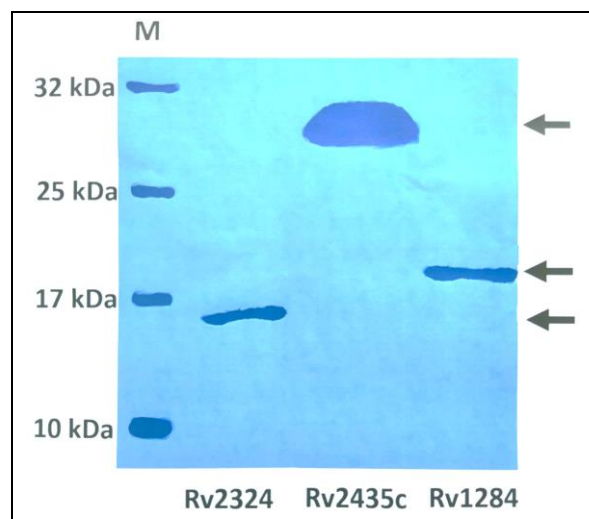


Figure 1. SDS-PAGE of purified MTB antigens. Eluates of affinity-purified His-tagged *Rv2324* (16.35 kDa) (A), *Rv2435c* (30kDa) (B) and *Rv1284* (18kDa) (C) were examined by SDS-PAGE. M: lane of protein molecular weight marker.

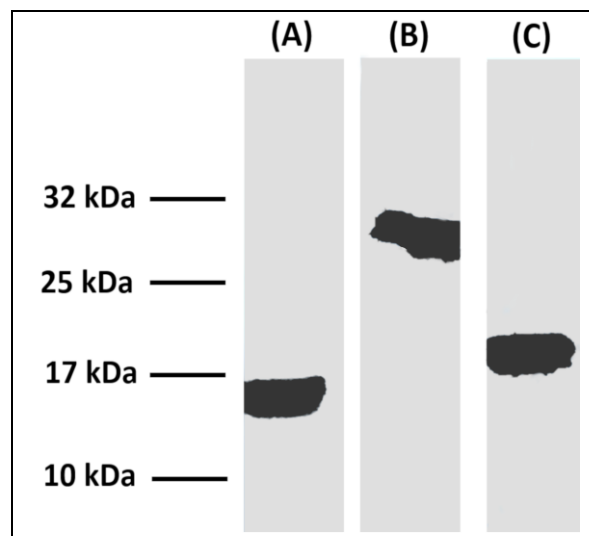


Figure 2. Western blot of MTB antigens. Reactivity of the raised murine antibodies was confirmed against the corresponding antigens *Rv2324* (A), *Rv2435c* (B) and *Rv1284* (C), respectively.

Mycobacterial antigen detection in serum samples

The presence of the mycobacterial antigens Rv2324, Rv2435c and Rv1284 was checked in serum specimens using the dot blot test. The serum of TB patients and healthy subjects (BCG-vaccinated) showed 100% positive results in dot blot test in case of detection Rv2435c and Rv1284 antigens. Furthermore, Rv2324 was also detectable in 94.3% and 93.1% of tested serum samples from TB patients and BCG-vaccinated healthy adults, respectively. In contrast, serum samples from newborns (BCG-unimmunized) did not react significantly with the polyclonal antibodies raised against the tested mycobacterial antigens. The percentage of positive results in newborn serum samples were 14%, 7% and 0% concerning the detection of Rv2324, Rv2435c and Rv1284, respectively. The difference between antigen detection in serum samples from newborns was statistically significant when compared with serum samples from TB patients and healthy BCG-immunized subjects (p value < 0.001 by Fisher's exact test). Statistical analysis was carried out using GraphPad InStat version 3.10, GraphPad Software, San Diego, California, USA. Number of positive samples among total number of serum samples tested in the three categories of the study are shown in **Table 2**. Representative dot blot results of the detection of Rv2324 antigen are shown in **Figure 3**.

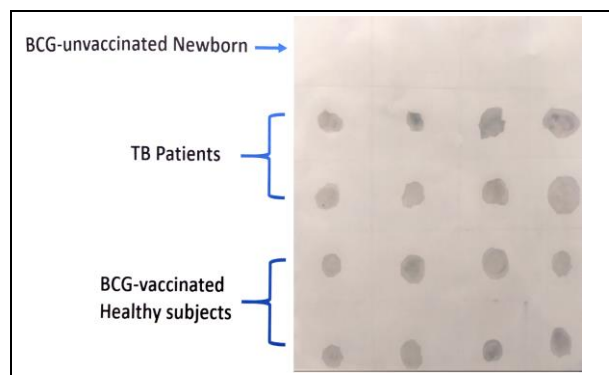


Figure 3. Dot blot detection of Rv2324 in serum specimens. Representative results of dot blot test for Rv2324 detection in serum samples from TB patients, BCG-vaccinated healthy subjects and BCG-unvaccinated newborns. Murine Rv2324-specific antibodies were used to detect Rv2324 in the serum specimens by dot blot test.

DISCUSSION

The issue of TB diagnosis is an important area that is increasingly requiring much research and improvement. Direct TB antigen detection in specimens such as urine, sputum and blood would represent a definitive diagnosis of active TB disease¹⁵. However,

search for useful TB antigen biomarkers is required to attain better sensitivity and specificity for TB diagnosis¹⁵.

In the present study, the dot blot test was used to detect three mycobacterial antigens, namely Rv2324, Rv2435c and Rv1284, in serum specimens. Serum specimens from TB patients showed high reactivity in the dot blot test where 94.3-100% of tested sera showed positive results. This indicated the presence of the three tested antigens in the serum of TB patients. Surprisingly, serum specimens from healthy subjects showed similar reactivity with the raised antigen-specific antibodies where 93.1-100% reacted positively. On the contrary, specimens from BCG-unvaccinated newborn infants did not react significantly with the raised antigen-specific antibodies where only 0-14% of the samples reacted positively. These results can be interpreted based on the status of BCG vaccination of the tested individuals. BCG vaccination is compulsory in Egypt where all children must be vaccinated within the first few weeks after birth. Consequently, all adults in Egypt are BCG-vaccinated. This is the reason we used sera from BCG-unvaccinated newborn as a negative control since there are no BCG-unvaccinated adults in Egypt.

The function of the tested mycobacterial proteins was checked based on the data available for *M. tuberculosis* H37Rv strain on the *Mycobacterium* database (<https://mycobrowser.epfl.ch/>). Rv1284 is a beta-carbonic anhydrase¹⁶ while Rv2324 is a putative transcriptional regulatory protein, probably belongs to AsnC-family. Rv2435c is a putative adenylyl- or guanylyl-cyclase⁹. Our bioinformatic analysis revealed conservation of these proteins among *M. tuberculosis* and *M. bovis* BCG with identity of 99-100%. This means the three tested TB antigens are present in *Mycobacterium bovis* BCG strain as well. These results raised the point that the positive reactivity observed with the sera of adult healthy subjects was actually due to BCG vaccination rather than active TB disease. This interesting finding might indicate that the attenuated BCG vaccine strain is still viable in the bodies of BCG-vaccinated adults and is expressing its proteins at detectable levels in blood. Interestingly, adult healthy subjects were BCG-vaccinated very long ago, during their early infancy, and it seems weird that the attenuated BCG strain is still living in their bodies in adulthood. More intriguingly, a relatively recent study by Markova and colleagues in 2015 suggested that BCG could be found as cell wall-deficient forms (L-forms) in the blood of BCG-vaccinated healthy adults with no history of exposure to TB¹⁷. They could cultivate the proposed L-form bacteria *in vitro* through a special multi-step approach. When they examined the cultivated L-forms genetically, it was confirmed that the L-forms were of mycobacterial origin in about 46.4% (45 out of 97 samples) of tested L-forms. They eventually proposed

Table 2. Summary of dot blot test results for the detection of TB serum biomarkers

Antigen biomarker	No. of positive results/total number of tested sera (%)		
	TB patients	Healthy BCG-vaccinated subjects	Newborns (BCG-unvaccinated)
Rv2324	66/70 (94.3%)	54/58 (93.1%)	6/43 * (14%)
Rv2435c	70/70 (100%)	58/58 (100%)	3/43 * (7%)
Rv1284	70/70 (100%)	58/58 (100%)	0/43 * (0%)

* : Statistically significant (p value <0.0001) by Fisher's exact test when compared with samples from TB patients and BCG-vaccinated healthy subjects.

that these L-forms are most likely due to persisting BCG vaccine¹⁷. Our results corroborate the proposal of Markova and co-workers and draw attention to the potential persistence of attenuated vaccine strains in human body. This persistence can interfere with diagnosis of active infection of virulent pathogens when common conserved antigens are detected as in our case. We recently reported the potential use of mycothiol acetyltransferase (Rv0819) as a TB serum biomarker to diagnose active TB disease using the dot blot test¹³. Although Rv0819 is a conserved common antigen in BCG and MTB, it was detected only in sera of TB patients but not in BCG-vaccinated healthy adults. The specificity of Rv0819 detection by the dot blot test reached 100% with no false-positive results¹³. This apparent discrepancy could be explained based on difference of the level of expression of Rv0819 between the BCG L-forms and the virulent *M. tuberculosis* where it seems that only the latter expressed Rv0819 at a level high enough to be detectable in patients' blood. It is noteworthy that BCG L-forms with its unusual morphology and phenotype is expected to have mutations and DNA rearrangements, with subsequent changed proteomic profile, under the long-term stressful survival conditions in human blood¹⁷.

BCG vaccination is reported to interfere with tuberculin skin test (TST) by eliciting a false positive induration reaction at the intradermal injection site³. This reaction is due to delayed-type hypersensitivity reaction resulting from the immune response of BCG-vaccinated individuals. On the other hand, we were detecting MTB antigens in the present study rather than measuring the immune response against them. Up to our knowledge, such interference by BCG in tuberculosis antigen detection has not been previously reported.

CONCLUSION

Overall, the results of the present study highlight the potential interference caused by BCG vaccine which may happen during detection of TB antigens in blood of BCG-vaccinated individuals. The previously reported BCG L-forms¹⁷ seems to be a

significant reality which should be taken into consideration during future design of TB diagnostics where discriminating antigens should be selected. Furthermore, the three tested antigens Rv2324, Rv2435c and Rv1284 might be useful TB serum biomarkers in populations where BCG vaccination is not routinely performed as in the case of North America.

Acknowledgement

We thank staff members of Elmaamoura Chest Hospital and El-Shatby University Hospital for their help in collection of serum samples. We also thank Hend Zeitoun for help and support.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

1. WHO | Global tuberculosis report 2019. World Health Organization (WHO) **2019**, Available from: https://www.who.int/tb/publications/global_report/en/ [Accessed 24th Sep. 2020]
2. Koch ,A.; Cox, H.; Mizrahi, V. Drug-resistant tuberculosis: challenges and opportunities for diagnosis and treatment. *Curr. Opin. Pharmacol.* **2018**, *42*, 7–15.
3. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. *Am J Respir Crit Care Med.* **2000**, *161*, 376–95. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10764337>
4. Rasolof, V.; Chanteau, S. Field evaluation of rapid tests for tuberculosis diagnosis. *J. Clin. Microbiol.* **1999**, *37*, 4201.
5. Ratanasuwat, W.; Kreiss, J.K.; Nolan, C.M.; Schaeffler, B. A.; Suwanagool, S.; Tunsupasawasdikul, S.; Chuchottaworn, C.; Dejsomritrutai ,W.; Foy, H.M. Evaluation of the MycoDot test for the diagnosis of tuberculosis in HIV seropositive and seronegative patients. *Int. J. Tuberc. Lung Dis.* **1997**, *1*, 259–64.
6. Bekmurzayeva, A.; Sypabekova, M.; Kanayeva, D. Tuberculosis diagnosis using immunodominant,

- secreted antigens of *Mycobacterium tuberculosis*. *Tuberculosis* **2013**, *93*, 381–8.
7. Commandeur, S.; van Meijgaarden, K.E.; Prins, C.; Pichugin, A.V.; Dijkman, K.; van den Eeden, S.J.F.; Friggen, A.H.; Franken, K.L.M.C.; Dolganov, G.; Kramnik, I.; et al. An Unbiased Genome-Wide *Mycobacterium tuberculosis* Gene Expression Approach To Discover Antigens Targeted by Human T Cells Expressed during Pulmonary Infection. *J. Immunol.* **2013**, *190*, 1659–71.
 8. Tortoli, E.; Nanetti, A.; Piersimoni, C.; Cichero, P.; Farina, C.; Mucignat, G.; Scarparo, C.; Bartolini, L.; Valentini, R.; Nista, D.; et al. Performance Assessment of New Multiplex Probe Assay for Identification of *Mycobacteria*. *J. Clin. Microbiol.* **2001**, *39*, 1079–84.
 9. Shenoy, A.R.; Visweswariah, S.S. *Mycobacterial* adenylyl cyclases: Biochemical diversity and structural plasticity. *FEBS Lett.* **2006**, *580*, 3344–52.
 10. Bahey-El-Din, M.; Griffin, B.T.; Gahan, C.G.M. Nisin inducible production of listeriolysin O in *Lactococcus lactis* NZ9000. *Microb. Cell Fact.* **2008**, *7*, 24.
 11. Golde, W.T.; Gollobin, P.; Rodriguez, L.L. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. *Lab. Anim. (NY)*. **2005**, *34*, 39–43.
 12. Shehat, M.G.; Bahey-El-Din, M.; Kassem, M.A.; Farghaly, F.A.; Abdul-Rahman, M.H.; Fanaki, N.H. Recombinant expression of the alternate reading frame protein (ARFP) of hepatitis C virus genotype 4a (HCV-4a) and detection of ARFP and anti-ARFP antibodies in HCV-infected patients. *Arch. Virol.* **2015**, *160*, 1939–52.
 13. Zeitoun, H.; Bahey-El-Din, M.; Kassem, M.A.; Aboushleib, H.M. Mycothiol acetyltransferase (Rv0819) of *Mycobacterium tuberculosis* is a potential biomarker for direct diagnosis of tuberculosis using patient serum specimens. *Let. Appl. Microbiol.* **2017**, *65*, 504–11.
 14. Bahey-El-Din, M.; Casey, P.G.; Griffin, B.T.; Gahan, C.G.M. *Lactococcus lactis*-expressing listeriolysin O (LLO) provides protection and specific CD8+T cells against *Listeria monocytogenes* in the murine infection model. *Vaccine* **2008**, *26*, 5304–5314.
 15. Flores, L.L.; Steingart, K.R.; Dendukuri, N.; Schiller, I.; Minion, J.; Pai, M.; Ramsay, A.,; Henry, M.; Laal, S. Systematic review and meta-analysis of antigen detection tests for the diagnosis of tuberculosis. *Clin. Vaccine Immunol.* **2011**, *18*, 1616–1627.
 16. Nienaber, L.; Cave-Freeman, E.; Cross, M.; Mason, L.; Bailey, U.M.; Amani, P.; Davis R.A.; Taylor, P.; Hofmann, A. Chemical probing suggests redox-regulation of the carbonic anhydrase activity of mycobacterial Rv1284. *FEBS J.* **2015**, *282*, 2708–2721.
 17. Markova, N.; Slavchev, G.; Michailova, L. Presence of mycobacterial L-forms in human blood: Challenge of BCG vaccination. *Hum. Vaccines Immunother.* **2015**, *11*, 1192–200.