Original article

Assessment of BCG vaccine immune response in a sample of Egyptian infants

**Background:** In Egypt, tuberculosis (TB) is considered the third most important public health problem after schistosomiasis and hepatitis C. We sought to investigate the immune response induced by the currently used Bacillus Calmette-Guerin (BCG) vaccine in a sample of Egyptian infants. **Methods:** A cross sectional study comprising 25 healthy BCG vaccinated infants, 14-24 months old was carried out. They were 15 boys and 10 girls. These infants were subjected to clinical and laboratory evaluation including blood counts, tuberculin intradermal test and in-vitro assessment of T cell response to purified protein derivative (PPD) and phytohemagglutinin (PHA) stimulation with measurement of IFN-γ in the supernatant of cultured mononuclear cells using the AssayMax Human interferon-gamma (IFN-γ) ELISA Kit (Assaypro), USA. **Results:** Among enrolled infants, 76% had BCG scar and 28% had positive tuberculin test. IFN-γ levels after PPD stimulation (median (IQR): 0.13 (0.09-0.44) ng/ml) and after PHA stimulation (median (IQR): 1 (0.99-1) ng/ml) were significantly higher than basal levels (median (IQR): 0.08 (0.05-0.22) ng/ml), p= 0.001. Only five infants (20%) had failed IFN-γ response after PPD stimulation and these infants showed also lower PHA stimulated IFN-γ response (z=-2.18, p=0.03), in comparison to those with PPD stimulated IFN-γ response. BCG scar positive and negative groups were comparable in their immunological parameters. Infants with positive tuberculin test results (n=7) showed significantly higher IFN-γ levels after PPD stimulation in comparison to the negative tuberculin group (n=18) (z=-2.09, p= 0.036). **Conclusion:** In this small cohort, it appears that the current BCG vaccination in Egypt results in an acceptable level of immune response. Absent BCG scar does not indicate failed immunization. Further longitudinal studies on a large number of infants are recommended to estimate the real clinical protection conferred by the currently used vaccine.

Keywords: BCG vaccine, Tuberculosis, Tuberculin skin test, Purified protein derivative, Phytohemagglutinin, interferon gamma.

**INTRODUCTION**

Tuberculosis (TB) is considered to be responsible for 2 million deaths every year despite being a treatable airborne infectious disease. Due to its infectious nature, chronic progression and long treatment, TB is a great burden for society. Moreover, the emergence of multi-drug resistant TB and the currently associated human immunodeficiency virus (HIV) worldwide epidemic has led to even greater concern. Treating and preventing TB have become a permanent challenge since the ancient times. Bacillus Calmette-Guérin (BCG) is the only vaccine available today in practice and has been used for more than 90 years with wide safety range records. However, its efficacy remains controversial.

In Egypt, TB is addressed and handled as a health problem affecting large sectors in the society, especially the poor and the vulnerable. In 2013, prevalence rate of TB was 27 per 100,000 populations. BCG vaccination became compulsory in all governorates of Egypt since 1974. The main strains of BCG vaccine currently used in Egypt are the Indian strains (Serum Institute of India) and Denmark strains (Staten Serum Institute).

According to the CDC reports for BCG vaccination, the presence of post vaccination scar or size of a tuberculin skin-test reaction does not predict whether BCG will provide any protection against TB disease. Furthermore, the size of a tuberculin skin-test reaction in a BCG-vaccinated person is not a factor in determining whether the reaction is caused by previous BCG vaccination or
current Mycobacterium tuberculosis (Mtb) infection.  

Studies in both animals and humans have demonstrated that interferon gamma (IFN-γ) is critical for immunity to Mtb. For this reason, IFN-γ responses to relevant antigens are widely used as the best available correlates of protective immunity in the evaluation of new vaccines for TB. There is an increasing interest in the use of in vitro assays based on IFN-γ production as an indicator of a protective response, to provide a more accurate correlate of natural and vaccine-induced protection as well as a diagnostic tool for infection with M. tuberculosis and other species of mycobacteria.

Methods to measure IFN-γ production by cultured peripheral blood cells in response to mycobacterial antigens include reverse transcription–polymerase chain reaction (RT–PCR) of mRNA, fluorescence-activated cell sorter (FACS) analysis of stained intracellular cytokines, and enzyme-linked immunospot assay (ELISPOT) or enzyme-linked immunosorbent assay (ELISA) of supernatants from undiluted whole blood culture.

We sought to investigate the immune response and hence the protection induced by the currently used BCG vaccine in a small group of healthy Egyptian infants.

METHODS
This cross-sectional study comprised 25 healthy BCG-vaccinated Egyptian infants aged between one and two years who were enrolled from the Outpatient Clinic of Children’s Hospital, Faculty of Medicine, Ain Shams University. The exclusion criteria included the following: 1) Infants not previously BCG vaccinated or those received the vaccine outside Egypt, 2) Infants with active infection at enrolment, or having features suggestive of active tuberculosis or in close contact with a known tuberculous patient, 3) Infants with any clinical or laboratory data suggestive of primary or secondary immunodeficiency, 4) Infants recently vaccinated with live attenuated vaccines especially MMR within the last 4-6 weeks before enrolment.

All enrolled infants were subjected to full history taking with special emphasis on history of infections or chronic illness, vaccination history, or complication from vaccines. Infants were also evaluated for their growth parameters, exclusion of infection or any chronic illness and examined for the presence of BCG scar.

Ethical considerations: The study was conducted in accordance with the ethical standards of the Helsinki Declaration of 1964 as revised in 2008 and the study protocol gained approval from the local Ethical committee of Pediatric department, Faculty of Medicine, Ain Shams University. Informed written consents were obtained from parents or caregivers of enrolled infants.

Laboratory investigations: Five milliliters of venous blood were collected from each infant under complete aseptic conditions and used for:

1- Complete blood count (CBC): EDTA (K3EDTA) vacutainer with concentration of 1.2 mg of the anhydrous salt per ml of blood was used. The CBC was done using the automated cell counter coulter® LH 750 cell counter (Coulter Corporation, Florida, USA), with manual white cells differential count.

2- Lymphocytes’ culture and incubation for 5 days in three conditions: basal (non-stimulated; negative control), Mycobacterium tuberculosis purified protein derivative (PPD) stimulation, and phytohemagglutinin (PHA) stimulation (as positive control), followed by measurement of IFN-γ in the supernatant of cultured mononuclear cells in these three conditions. Heparinized, preservative-free vacutainers were used. Samples were processed within 2 hours of collection as described below.

- Reagents for in vitro stimulation of peripheral blood mononuclear cells (PBMCs):
Sterile cell culture RPMI 1640w/ L-Glutamine and Fungizone antifungal were provided by Lonza (Walkersville, USA). Penicillin / streptomycin and Gentamycin were obtained from Sigma (St. Louis, Mo.). Fetal bovine serum (FBS) and Phytohemagglutinin (PHA) were provided by (Invitrogen, Ave Carlsbad, USA). Mycobacterium tuberculosis purified protein derivative (PPD) (strength: 5 tuberculin units (TU) per 0.1 mL) was provided by Span Diagnostics (SACHIN 394230 (Surat) INDIA). Ficoll-Hypaque was supplied by Amersham Biosciences (Buckinghamshire, UK).

- Isolation of PBMCs and lymphoproliferation assay:
Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Ficoll-Hypaque density gradient centrifugation method. Cells were treated with red blood cell lysing buffer (155 mM NH4Cl, 10 mM NaHCO3, and 0.1 mM EDT; pH 7.4) to lyse red blood cells. PBMCs freshly harvested from the studied infants were incubated in 96-well tissue culture plates (2×10² cells in 0.2 ml/well) in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units of penicillin G per ml, 100 µg/ml of streptomycin and 0.25 µg/ml of fungizone. Cells were cultured with medium alone
(basal condition) and with stimulation by PHA (1 ug/ml) and Mycobacterium tuberculosis PPD (100,000 unit/ml) at 37°C with 5% CO2. The cultures were incubated for 5 days.

**IFN-γ detection by ELISA:**
AssayMax Human IFN-gamma ELISA Kit obtained from ASSAYPRO (St. Charles, USA) was used for detection of IFN-γ in the culture supernatant. At the end of the incubation period, cell-free supernatants were collected and frozen at -70°C until cytokine levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA), to assess the T lymphocyte function in response to the in-vitro stimulation. Polyclonal antibody specific for human IFN-γ has been pre-coated into a 96 -well microplate. IFN-γ in standards and samples is sandwiched by immobilized antibodies and biotinylated polyclonal antibody specific for IFN-γ, which is recognized by a streptavidin – peroxidase conjugate. All unbound material is then washed away, and a peroxidase enzyme substrate is added. The color development is stopped, and the intensity of color is measured at optical density (450nm) with reference filter 570 nm by using an ELISA reader then the concentrations of samples were determined from the standard curve. The kit lowest detection limit is 0.01 ng/ml while the kit linearity is up to 1.0 ng/ml. All cases show positive lymphocyte proliferative function with variable concentrations of IFN-γ.

To detect changes in IFN-γ after PHA or PPD stimulation, folds of increase in IFN-γ levels were calculated as follows:

**Fold of increase:** IFN-γ level after stimulation divided by the initial value (basal)

3- Tuberculin intradermal test:
Tuberculin skin test was performed using purified protein derivative (5 TU/0.1ml, VACSERA, Egypt). Skin intradermal testing was carried out on the volar surface of the forearm following standard procedures. The skin was cleansed with 70% alcohol before applying the skin test. The test was performed by intradermal injection of 0.1 ml of the antigen. When placed correctly, the injection should produce a pale elevation of the skin (a wheal) 6 to 10 mm in diameter. After 48 to 72 hours, a positive reaction for tuberculin skin test consists of induration ≥ 5 mm.

**Statistical Analysis**
Data were analysed using SPSS (version 22) statistical software package. Frequencies were described as number and percentage. Arithmetic mean and standard deviation were calculated for parametric data while median and interquartile range were used for non-parametric ones. Comparison of non-parametric groups were done using Mann- Whitney U test. Spearman’s correlation test was used for correlating non-parametric variables. A probability value \( p \leq 0.05 \) was considered significant.

**RESULTS**
At time of enrollment, the age of infants ranged between 14 to 24 months (mean ± SD: 19.6 ± 3.2 months). They were 15 boys (60%) and 10 girls (40%). Their weight percentiles ranged between 2.9 to 97.3 centiles (mean ± SD: 46.86± 3.44), while the length percentiles ranged between 11.3 to 74.5 centiles (mean ± SD: 43± 20.3).

**BCG scar and tuberculin skin test results:**
None of the enrolled infants gave history of BCG vaccine related complications. BCG scar was observed in 19 out of the 25 enrolled infants (76%). Concerning tuberculin intradermal skin test results, induration diameters ranged between 0 and 13 mm, mean±SD: 3.8±4.6 mm with median (IQR): 2 (0-10) mm. Induration diameters 5 mm or less were considered negative. Seven out of the 25 infants (28%) had positive tuberculin skin test (with induration diameter range 10-13 mm and median diameter of 10 mm (table 1).

| Tuberculin test result | Present | Absent |
|------------------------|---------|--------|
| BCG scar               | 19 (76%)| 6 (24%)|
| Positive BCG scar      | 6 (31.6%)| 1 (16.7%)|
| Negative BCG test       | 13 (68.4%)| 5 (83.3%)|

**In-vitro response to PPD and PHA stimulation**
Studied infants showed significant elevation in INF-γ levels after in-vitro PPD and PHA stimulation (table 2). PPD stimulation resulted in increased INF-γ production with a range of increase 0.13-16.7 folds compared to basal levels, median (IQR): 2 (1.22-3), while PHA stimulation resulted in increased INF-γ production with range of increase 1.3-38 folds, median (IQR): 8.9 (3.2-18.4) folds compared to basal levels.

Among the PPD stimulated samples, 5 did not show any elevation of INF γ production. Their main immunological characteristics are shown in table 3.

Patients with failed response to PPD stimulation (n=5) had comparable age (z= -0.07, p= 0.97),
weight centiles (z= -1.33, p=0.19), absolute lymphocyte counts (z=0, p=1) and tuberculin test diameters (z= -0.77, p=0.45) but lower folds of increase in INF γ production after PHA stimulation (z= -2.18, p=0.03) in comparison to those who were responsive to PPD-stimulation

Variation of infants’ parameters in relation to BCG scar and Tuberculin test results

Clinical and laboratory parameters of BCG scar positive (n=19) and negative (n=6) groups were compared, and no significant differences were found between the 2 groups in terms of age, weight and height centiles, absolute lymphocyte counts, tuberculin test induration diameters, basal and PPD- and PHA-stimulated IFN γ levels. On the other hand, infants with positive tuberculin test results (n=7) showed significantly higher IFN γ levels after PPD stimulation in comparison to the negative tuberculin group (n=18) (z= -2.09, p= 0.036) (figure 1), while infants in the two groups were comparable in the rest of clinical and laboratory parameters.

Correlates with in-vitro IFN γ levels after PPD stimulation

PPD stimulated IFN γ levels correlated positively with Tuberculin test induration diameters (r=0.52, p=0.008) (figure 2). Also, fold of increase in IFN γ after PPD stimulation correlated positively with folds of IFN γ rise after PHA stimulation (r=0.542, p= 0.005) (figure 3).

Table 2. INF-γ levels, basal, after PPD and PHA stimulation among the studied infants

| INF-γ levels (ng/ml) | Basal level | PPD stimulation | PHA stimulation | P1 | P2 | P3 |
|----------------------|-------------|-----------------|-----------------|----|----|----|
| Range                | 0.01-0.65   | 0.03-1          | 0.08-1          |    |    |    |
| Median (IQR)         | 0.08 (0.05-0.22) | 0.13 (0.09-0.44) | 1 (0.99-1)     | P=0.015 | P<0.001 | P<0.001 |

Kruskal Wallis test; P1: comparison between baseline and PPD stimulated INF-γ levels; P2: comparison between baseline and PHA stimulated INF-γ levels; P3: comparison between PHA and PPD stimulated INF-γ levels

Table 3. Immunological parameters of the 5 infants with failed INF-γ response to PPD stimulation

| Patients | BCG scar | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 |
|----------|----------|-----------|-----------|-----------|-----------|-----------|
| Tuberculin induration diameter | present | 0 mm | 2 mm | 0 mm | 2 mm | 10 mm |
| Folds of INF-γ change after PPD stimulation | 0.71 | 0.13 | 0.19 | 0.75 | 0.27 |
| Folds of INF-γ increase after PHA stimulation | 5.9 | 1.5 | 2.8 | 3.6 | 4.5 |

IFN-γ: Interferon gamma

![Figure 1. Interferon gamma after PPD stimulation according to tuberculin test results](image)
BCG vaccine immune response among infants

Figure 2. Correlation between PPD stimulated IFN γ levels and Tuberculin test induration diameters

Figure 3. Correlation between folds of rise in IFN γ after PPD stimulation and after PHA stimulation

DISCUSSION

Tuberculosis vaccinology represent one of the most evolving fields in medical research. After its advent in 1921, the live attenuated BCG vaccine remains the most widely used vaccine until today despite its limitations and highly variable efficacy in providing protection against childhood pulmonary TB. 

Our results showed that only 76% of enrolled infants had BCG scar, while the remaining 24% had scar failure versus 17% (in 100 studied Sudanese infants), and 18% (in 150 studied Egyptian children) as reported by Kheir et al11 and Beshir et al12 respectively, who used the Danish strain of BCG vaccine. It has been reported that scar failure may occur in 10% to 20% of BCG vaccinated infants and is more common with immunization within 48 hours of life.13 Different sample size and variation of age at time of vaccination might partially explain the difference noted between our and their results. Pang et al, 2015 in their study of 27,517 Chinese infants who were BCG vaccinated in the first 3 months of life, demonstrated that the presence of a BCG scar is a good indicator of successful immunization among vaccinated infants, but lack of a scar is not predictive of a poor immune response.14 Worth to note that, in Egypt, BCG vaccine is given soon after birth which might explain the increased incidence of scar failure.
Development of BCG scar depends on the strain used, injected dose and technique of administration. Other factors like quality of vaccine, proper transport, storage or injection technique, racial factors and undiagnosed underlying immune disorder in infants may be also responsible for the absence of scar formation. In our study, both groups with and without BCG scar were comparable in terms of their clinical and immunological parameters.

Tuberculin test is thought to have low sensitivity and specificity for previous BCG vaccination or TB infection. In our study, 72 % of the tested infants had negative results for tuberculin test, further emphasizing that tuberculin test is not a good indicator of BCG immune response. Individuals who receive BCG vaccine, may show a positive tuberculin reaction, 2-3 months following vaccination, which then wanes with time. There is no correlation between post vaccination tuberculin reaction and the degree of protection against TB. Previous work by Gaisford and Griffiths suggested that a larger dose of PPD is required to elicit a tuberculin response in BCG vaccinated infants than in adolescent children, but carries the risk of severe reaction, while a study performed by Fallah et al, 2012 showed that even the number of live organisms in the vaccine used is another factor in determining the tuberculin response.

The higher response to PPD stimulation in the tuberculin positive group in our study, might reflect a more efficient BCG response, more activation and/or better functioning of T cells in the responsive group, but does not necessarily imply a lack of response in the tuberculin negative one. Several factors might have affected the response to PPD stimulation whether related to the functional status of the immune system or the environmental exposure to mycobacteria.

In our series, 20 infants (80 %) responded to in-vitro stimulation by PPD with increased INF-γ production, denoting effective immune response to BCG vaccine with median increase of 2 folds compared to their baseline INF-γ levels. These results agree with the reported BCG vaccine efficacy in protection against pulmonary TB, ranging 50-80 %. A previous study by Weir et al., 2008 on 148 scar negative-BCG vaccinated-UK adolescents, showed increased in-vitro INF-γ production at 3 months (87 % responders) and 12 months (74 % responders) after BCG vaccination with the Danish strain. In their study, INF-γ levels increased by 12.4 folds and 7.5 folds at 3 and 12 months respectively in comparison to non-vaccinated controls, with gradual reduction of response till 14 years post-vaccination. These results all together denote that BCG vaccine strains used in Egypt do provide comparable efficacy to the other strains in use. Worth to not that our infants were aged between 14-24 months and INF-γ was measured after in-vivo stimulation of T cells, unlike the INF-γ in-vivo production by both T cells and macrophages. Furthermore, the kits used in our study for INF-γ measurement, has an upper limit of detection of 1 ng/ml, denoting the possibility of the presence of higher INF-γ levels that were not detected. These factors might explain the lower values in our study in comparison to others.

In the current study, 5 infants showed failed INF-γ response to PPD stimulation, and four them had negative tuberculin test. However, 4 of these infants had evident BCG scar. Those infants had also lower INF-γ production in response to PHA stimulation as well. Failed INF-γ response to PPD in these 5 infants might be a consequence of reduced function of T cells, whether transiently or as a congenital defect or might reflect a reduced ability to generate new longer term central memory cells due to high environmental mycobacterial exposure, leading to overstimulation of an activated effector cell population “blocking hypothesis”, resulting in the absence of in-vitro response to PPD stimulation despite positive tuberculin test. Earlier assessment of immune response to the vaccine within 3 months after vaccination may give more accurate results about their actual response to the vaccine. Meticulous clinical follow-up of these infants is essential for detection of early signs that can be suggestive of T cell immune defects. Meanwhile, after exclusion of underlying phagocytic or T cell defects, these 5 infants might be candidate for booster BCG or new TB vaccination especially in highly endemic countries, an issue that warrants further studies.

**STUDY LIMITATIONS**

Small sample size and the lack of control group of non-vaccinated infants are the main points of limitation in our study. The cross-sectional nature of the study and the limitation of the kits results to a maximum of IFN γ levels of 1 ng/ml were additional limiting factors. We could not use BCG for T cell stimulation in our study due to lab regulation issues.
CONCLUSION
In conclusion, our study showed that BCG vaccination applied in the extended program of immunization in Egypt, results in an acceptable level of immune response in 80 % of vaccinated infants as evidenced by the in-vitro results. Absent BCG scar doesn’t necessarily imply failed BCG vaccination or immunodeficient state. Further longitudinal studies with larger sample size are recommended with follow up of the enrolled infants to estimate the real clinical protection conferred by BCG vaccine against development of TB in correlation with the in-vitro test results. In highly endemic regions, vaccinated infants with absent scar and failed in-vitro response to PPD should be evaluated for further decisions concerning booster or new BCG vaccination.

REFERENCES
1. Luca S, Mihaescu T. History of BCG Vaccine. Maedica (Buchar) 2013; 8(1): 53-8.
2. Ottenhoff TH, Kaufmann SH. Vaccines against tuberculosis: where are we and where do we need to go? PLoS Pathog 2012; 8(5):e1002607.
3. WHO/ Tuberculosis country profile, 2015. Accessed at: http:// www. WHO.int/tb/country/profiles/en/. Last updated 2015. Last visited 11/2015.
4. Saad-Hussein A, Mohammed AM. Trend of application of World Health Organization control strategy of tuberculosis in Egypt. J Epidemiol Glob Health 2014; 4(3):195-202.
5. Weir RE, Fine PE, Nazareth B, Floyd S, Black GF, King E et al. Interferon-gamma and skin test responses of school children in south east England to purified protein derivatives from mycobacterium tuberculosis and other species of mycobacteria. Clin Exp Immunol 2003; 134(2):285-94.
6. Wang L, Turner MQ, Elwood RK, Schulzer M, FitzGerald JM. A meta-analysis of the effect of Bacille Calmette Guerin vaccination on tuberculosis skin test measurements. Thorax 2002; 57(9):804-9.
7. Finan G, Ota MO, Mardhant A, Newport MJ. Natural variation in immune responses to neonatal BCG vaccination in Cohort of Gambian infants. PLoS One 2008; 3(10):e3485.
8. Fordham C. Vaccines for prevention of tuberculosis. Accessed at https://www.uptodate.com/contents/vaccines-for-prevention-of-tuberculosis. Last updated 2/2021. Last visited 08/2021.
9. CDC/fact sheet for tuberculin skin testing. Accessed at: https://www.cdc.gov/tb/publications/factsheets/testing/skintesting.pdf. Last updated 9/ 2020. Last visited 7/2021.
10. Fatima S, Kumar A, Das G, Dwivedi VP. Tuberculosis vaccine: A journey from BCG to present. Life Sci. 2020 Jul 1;252:117594.
11. Kheir AE, Alhaj AA, Ibrahim BA. The sensitivity of BCG scar as an indicator of previous vaccination among Sudanese infants. Vaccine 2011; 29(46): 8189-91.
12. Bebhir MR, Zidan AE, El-Saadny HF, Ramadan RA, Karam NA, Amin EK, et al. Evaluation of the immune response to Interferon gamma release assay and tuberculin skin test among BCG vaccinated children in east of Egypt: A Cross-Sectional Study. Medicine (Baltimore). 2016 Apr;95(17): e3470.
13. Surekha RH, Vijayalakshmi V, Sunil K, Laksbmi K, Suman L, Murthy K. Cell mediated immunity in children with scar-failure following BCG vaccination. Indian pediatri 1998; 35: 123-7.
14. Pang Y, Kang W, Zhao A, Liu G, Du W, Xu M et al. The effect of bacille Calmette-Guérin vaccination at birth on immune response in China. Vaccine 2015; 33(1):209-13.
15. Roth A, Gustafson P, Nhasa A, Djana Q, Poulsen A, Barly ML et al. BCG vaccination scar associated with better childhood. Int J Epidemiol 2005; 34(3): 540-7.
16. Grabenstein J. Delayed-hypersensitivity testing: guide to product selection. Hosp Pharm 1990; 25: 1102-7.
17. Floyd S, Ponnighaus JM, Bluiss L, Warnodrff DK, Kasumba A, Mogha P, et al. BCG scars in northern Malawi: sensitivity and repeatability of scar reading, and factors affecting scar size. Int J Tuberc Lung Dis 2000; 4(12):1133-42.
18. Black GF, Weir RE, Floyd S, Bluiss L, Warnodrff DK, Crampin AC, et al. BCG induced increase in interferon-gamma response to mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: two randomised controlled studies. Lancet 2002;359(9315):1393-1401.
19. Weir RE, Gorak-Stolinska P, Floyd S, Lalor MK, Stenson B, Brandon K, et al. Persistence of the immune response induced by BCG vaccination. BMC Infect Dis. 2008; 25:8:9.
20. Gaisford W, Griffiths M. A freeze-dried vaccine from isoniazid-resistant BCG: A clinical investigation. Br Med J 1961; 1(5238):1500-1
21. **Fallah F, Eslami G, Pourbaba R, Goudarzi H, Taheri S, Marhamati N.** The role of CFU of BCG vaccine in Tuberculin skin reaction. Arch Dis Child 2012; 97:520.

22. **Parthasarathy A.** Controversies in BCG immunization. Indian J Pediatr 2003; 70(7):585-6.

23. **Weir RE, Gorak-Stolinska P, Floyd S, Lalor MK, Stenson S, Branson K, et al.** Persistence of the immune response induced by BCG vaccination. BMC Infect Dis 2008; 25;8:9.

24. **Pitt JM, Blankley S, McShane H, O’Garra A.** Vaccination against tuberculosis: How can we better BCG? Microb Pathog 2013 May; 58:2-16.

25. **Haile M, Källenius G.** Recent developments in tuberculosis vaccines. Curr Opin Infect Dis 2005; 18(3):211-5.