

## Effectiveness of the Bacillus Calmette-Guerin vaccine in an Indonesian population with D543N *NRAMP1* polymorphism

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### Abstract

**Introduction:** Bacille Calmette-Guerin (BCG) vaccination remains a routine immunization in primary care in tuberculosis (TB)-endemic areas, though several studies found that its efficacy was inconclusive. Natural resistance-associated macrophage protein 1 (*NRAMP1*) polymorphism has been shown to result in higher susceptibility to TB. Information on genetic susceptibility in populations will be useful in planning the application of the BCG vaccine. The present study explored BCG efficacy in a rural Timor population with specific *NRAMP1* polymorphism in a TB-endemic region of eastern Indonesia.

**Methodology:** A case-control study with 64 newly diagnosed pulmonary TB patients and 65 healthy controls was performed. BCG scars were examined by a physician. *NRAMP1* polymorphism was evaluated using molecular methods.

**Results:** Half of the subjects (65; 50.4%) had a clear presenting BCG scar on the upper arm, suggesting a successful BCG vaccination. Among the subjects, D543N *NRAMP1* polymorphism, history of contact with TB patients, and not having a clear BCG scar on the upper arm tended to be significantly association with active TB. The significant differences were more profound when subjects were divided based on presenting BCG scar. Subjects without clear BCG scars had significant association with developing TB disease ( $p = 0.014$ ). In multivariate analysis, history of previous contact with TB patients and unclear presenting BCG scar were associated with active TB (OR 9.2; 2.0–43.8 95% CI, OR 4.8; 2.1–11.0 95% CI, respectively).

**Conclusions:** BCG vaccination in our population was effective for TB protection, especially in highly endemic areas of TB, regardless genetic susceptibility.

**Key words:** BCG vaccination; tuberculosis; *NRAMP1* polymorphism.

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### Introduction

Bacillus Calmette-Guerin (BCG) vaccine is still the only vaccine routinely used in primary care as a specific protection against TB, as recommended by the World Health Organization [1]. Meta-analysis reported that BCG vaccine is still beneficial for TB prevention, although such efficacy varies across populations [2]. Recently, a need for new vaccine was declared. A new vaccine will potentially provide a better immunity effect; however, the current public health issue of tuberculosis is related to HIV, which significantly reduces the host's immune capacity [3-5]. Although a new vaccine is being developed, new vaccines will not be able to immediately substitute the current BCG, as

the safety and cost in a large population must be taken into consideration [6].

Indonesia is one of the high burden countries with TB; the country ranks fifth worldwide for disease prevalence. Based on a national survey, the coverage of BCG vaccination in 2011 was estimated to be 98%. However, some discrepancy was seen, as two areas with the highest TB prevalence, Papua and East Nusa Tenggara, had low BCG vaccination coverage: 63.8% and 77.7%, respectively [7,8]. A national control program was implemented to combat TB; however, a strong recommendation for vaccination was not included in the control program statement [9].

Interestingly, genetic studies demonstrated that some genes in a specific population might be involved in susceptibility to TB (*e.g.*, *NRAMP1*) [10,11]. This might explain, using the host-agent-environment (HAE) model, why 90% of people infected by *Mycobacterium tuberculosis* (Mtb) do not develop TB disease [12]. In the western and central parts of Indonesia, *NRAMP1* gene polymorphism seems not to be related to TB susceptibility [13,14], though our previous study in the eastern region showed otherwise [15].

The aim of this study was to evaluate whether routine BCG vaccination was still beneficial in preventing adult pulmonary TB in an endemic area in subjects with genetic susceptibility to TB.

## Methodology

### *Study design*

A case-control study, as part of a TB study in Kupang, was conducted [15]. Sixty-four patients newly diagnosed with pulmonary TB confirmed by positive smear for acid-fast bacilli were included, following the national guideline for TB diagnosis [9]. Another 65 healthy individuals without symptoms and history of TB were also recruited in this study as controls.

### *BCG scar and NRAMP1 polymorphism*

A list of questions to determine the subjects' socio-demographics profiles was used. Blood samples were taken after all subjects completed an informed consent form. The BCG vaccination area was examined by a physician through the presenting BCG scar on the upper arm [16]. The presence of typical BCG scar findings (*i.e.*, round scar with minimum 2 mm in diameter, paler than surrounding skin) was observed in the deltoid part of the upper arms, and classified as a clear or unclear scar.

Peripheral blood was drawn using an ethylenediaminetetraacetic acid (EDTA) tube. Peripheral blood mononuclear cells (PBMCs) then separated and genomic DNA was obtained using a Blood Mini Kit (Qiagen, Hilden, Germany). A single nucleotide polymorphism was found in position 1703G→A in exon 15 (D543N), causing a substitution from aspartate to asparagines at codon 543. The single polymorphism was determined using polymerase chain reaction (PCR) or restriction fragment length polymorphism (RFLP) as previously described.

### *Statistical analysis*

Statistical analysis was performed using SPSS for Windows version 11.5. The data were tested for normality using the Kolmogorov-Smirnov normality test, with  $p > 0.05$  assumed to be in normal distribution. Independent *t*-test and Chi-square tests were performed to assess the statistical significance in numeric and nominal data, respectively. Multivariate analysis using backward logistic regression was performed to assess the further association with active TB based on an initial  $p$  value  $> 0.2$  and current theory that might support our expectancy. This study was approved by the ethical committee of the Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

## Results

### *Demographic characteristic of participants*

A total of 64 active pulmonary TB patients were included in this study, along with 65 healthy individual as controls. There were no significant differences in age and sex among both groups.

There was a significant difference in hemoglobin levels in both groups, where patients with active TB, as expected, had lower hemoglobin levels compared to the controls ( $11.2 \pm 2.1$ ,  $14.7 \pm 1.6$ , respectively). Patients in the active TB group also tended to have a history of previous contact with TB patients ( $p < 0.001$ ), as shown in Table 1. A clear presenting BCG scar was profound in 65 subjects (50.4%), both patients and controls. History of previous contact with TB patients was seen in 22 subjects (17.1%). Both variables had significant association with active TB (Chi-square test,  $p < 0.05$ ).

### *Single nucleotide polymorphism D543N NRAMP1 gene*

In the present study, there was no D543N *NRAMP1* data from 14 control subjects. The missing data had no significant differences for age and sex ( $p > 0.05$ ). The distribution of D543N *NRAMP1* polymorphism was reported in a previous study [15].

### *BCG scar and NRAMP1 single polymorphism*

All subjects (patients and controls) were then separated into two groups: with and without presenting BCG scar. The differences in D543N *NRAMP1* distribution and its association with active TB cases were analyzed (Table 2). In vaccinated individuals, there was no difference in total number of active TB. However, subjects without a clear presenting BCG scar had significant association with active TB ( $p = 0.014$ ).

**Table 1.** Characteristics of the respondents

Variables	Active TB n = 64	Non-TB n = 65	Crude OR (95% CI)	P value
<i>Age (year ± SD)</i>	31.6 ± 11.4	32.5 ± 11.7	1.2 (0.50–2.8)	0.693
15-44 years; n (%)	52 (81.2)	51 (78.5)		
45-65 years; n (%)	12 (18.8)	14 (21.5)		
<i>Gender</i>				
Male; n (%)	40 (62.5)	28 (43.1)	2.2 (1.1–4.4)	0.027
Female; n (%)	24 (37.5)	37 (56.9)		
<i>BCG scar</i>				
Yes (%)	19 (29.7)	46 (70.8)	0.2 (0.1–0.4)	0.000
No (%)	45 (70.3)	19 (29.2)		
<i>Hb g/dL</i>				
	11.2 ± 2.1	14.7 ± 1.6		
<i>Anemia<sup>a</sup></i>				
Yes (%)	46 (71.9)	3 (4.6)	NA	0.000
No (%)	18 (28.1)	62 (95.4)		
<i>Contact<sup>b</sup></i>				
Yes (%)	19 (29.7)	3 (4.6)	8.7 (2.4–31.3)	0.000
No (%)	45 (70.3)	62 (95.4)		

\*All data were in normal distribution; <sup>a</sup>Anemia defined by HB threshold (male subjects 13.0 g/dL (>15 years of age), female subjects were 12.0 g/dL (>15 years of age, not pregnant); <sup>b</sup>Subjects who reported having contact with tuberculosis patients; NA: not analyzed

**Table 2.** Distribution of D543N *NRAMP1* polymorphism among participants with and without BCG scars

D543N NRAMP1	Presenting clear BCG scar			Presenting unclear BCG scar		
	Active TB n = 19	Non-TB n = 35	P value	Active TB n = 45	Non-TB n = 16	P value
G/G	8 (42.1)	19 (54.3)	0.39	15 (33.3)	11 (68.8)	0.014
G/A and A/A	11 (57.9)	16 (45.7)		30 (66.7)	5 (31.2)	

\*Total subjects for polymorphism analysis were different from other variables above – 64 in case group and 51 in control group, as 14 controls were missing a DNA sample. G/A and A/A were combined since only one subject (control) had A/A allele; Chi-square test: significant if p < 0.05.

**Table 3.** Multivariate analysis of TB becoming active

Variables	Adjusted OR (95% CI)	P value
Contact with TB	9.2 (2.0–43.8)	0.005
Not having BCG scar	4.8 (2.1–11.0)	<0.001
D543N <i>NRAMP1</i> polymorphism	1.9 (0.8–4.6)	0.134

In multivariate analysis, history of previous contact with TB patients and presenting BCG scar was associated with active TB (OR 9.2 2.0–43.8 95% CI vs. OR 4.8 2.1–11.0 95% CI, respectively) (Table 3).

## Discussion

In our study, only half of the subjects (50.4%), both patients and controls, had a clear presenting BCG scar. It seemed that rural people had a lower exposure to the routine immunization. Based on a national survey, areas with higher numbers of uneducated and rural people tend to have lower coverage of BCG vaccination [8]. Our study also confirms that the role of genetic susceptibility to TB was prominent only in adult subjects presenting an unclear BCG scar. After adjusting for other cofounders, those with history of contact with TB patients and those who did not have a clear BCG scar were more likely to develop TB disease.

Our study assessed pulmonary TB in a population over 15 years of age and provided evidence that genetic susceptibility in our population could explain the high prevalence of TB in this area. The genetics of the population in remote East Nusa Tenggara are less likely to change because of low migration numbers, though our previous finding did not meet the Hardy-Weinberg equilibrium [15]. Our study used presenting BCG scar in the deltoid region of the upper arm as an indicator of successful vaccination. This method was used and evaluated by previous studies [17,18]. Approximately 10% of people previously immunized with the BCG vaccine do not have a presenting BCG scar [19]. The sensitivity and repeatability of scar readings were good. However, the success of the vaccination to protect from TB was not dependent on the formation of the scar only. There were other factors externally and internally that protect from TB, such as the quality of the management of the vaccine, the time the vaccination is administered, and the time of assessing the BCG scar (the longer ago the patient had been vaccinated, the less effective the protection). When the scar formation is expected to be stabilized, approximately four years since vaccination, a scar reading might give approximately 80% sensitivity and further could describe the induction of immune responses [17,20]. In Brazil, when cross-checked with a patients' vaccination card, scar reading offered good sensitivity and specificity [21]. The decline of the sensitivity over time could underestimate the exact number of people in this study who were vaccinated in childhood [17]. Another limitation of this study was that we did not assess molecular immunity using

tuberculin skin testing (TST) [22] and we did not quantify immune response by measuring IFN- $\gamma$  production [23]. However, since our study was conducted in a highly endemic area, the result of skin testing could have been affected by infection post-vaccination, as we recruited adult subject in this population-based study. In addition, we could not obtain exact vaccination records from our subjects. Policy in Indonesia states that every baby born must be given BCG vaccination soon after birth. The rule that every delivery must be accompanied by trained health personnel was implemented in 2009, so most of our subjects did not have record of post-partum immunization.

Furthermore, we did not establish whether the patients had any concomitant diseases (including HIV, diabetes, and other comorbidities). The recruitment subjects as controls were based on the absence of history of TB and typical TB symptoms (productive cough, fever, night sweats, etc.), as we did not perform chest X-rays.

In our previous study, we found that the East Nusa Tenggara population might have genetic susceptibility to TB, especially in D543N *NRAMP1* polymorphism [15]. This finding might explain the high prevalence of TB disease. Similar to another study, our finding suggests that BCG vaccination and history of contact TB might play a significant role in TB disease development [24]. In the Chinese Han population, *VDR* and *NRAMP1* gene polymorphisms were found to be significantly associated with TB. The findings in that study also had about the same percentage of people who had received a BCG vaccination. Therefore, in settings where TB is endemic and there are high exposure rates and genetic susceptibility to TB, BCG vaccination still might provide protection against TB.

A study of animals demonstrated interesting findings; as shown in a rat model, BCG induced immune protection [25]. Furthermore, this protection was profound in early response in mice with genetic susceptibility (the *NRAMP1* gene) [26]. In a mouse model, delayed-type hypersensitivity (DTH) was stronger in susceptible mice than resistant ones, although the production of nitride oxide and TNF- $\alpha$  was lower in susceptible mice. In that experiment, using *M. vaccae* to control allergic asthma, *NRAMP1* expression might have had a role in T cell-mediated responses [27]. This finding suggests that a susceptible individual would also have a stronger immune response if exposed previously with vaccination.

Regardless of genetic polymorphism, our study demonstrated the effectiveness of BCG vaccination. A previous study conducted in Java showed that BCG vaccination only had a significant association with severity of TB. In BCG scars, the proportion of mild-moderate TB was higher than severe TB. In addition, there was no significant association between mild-moderate TB and the control group [28]. Although some studies reported that BCG efficacy might decline over the years, and moreover that BCG might not give protection to people over 15 years of age [29,30], our study produced contrary results. In our subjects who were over 15 years of age, the efficacy of BCG vaccination was significant. The same results were found in another study that focused on American Indians and Alaska natives [31].

As described previously, our study gave another point of view about the efficacy of the current BCG vaccine. The host-agent-environment concept implies the importance of the host's genetics to be taken into consideration in the explanation of the development of tuberculosis. Based on our findings, prevention through specific protection by BCG vaccination to prevent adult pulmonary TB was still effective in rural areas with a high prevalence of tuberculosis. Current strategy to eliminate TB emphasizes DOTS (directly observed treatment short course), as stated in Indonesia's national strategy to combat TB [32]. However, studies in different parts of the world reveal that there are many barriers to the success of DOTS treatment (*i.e.*, geographical conditions, patients' knowledge, homeless status, alcohol intake, etc.) [33,34]. Thus, in primary-care settings, a prevention-through-immunization program in Indonesia could be highly cost-effective in comparison to treatment [35].

## Conclusions

BCG vaccination is still an effective method of protection against TB, even in genetically susceptible people, as demonstrated with D543N *NRAMP1* polymorphism in this population-based study. We strongly recommended broadening the coverage of BCG vaccination, especially in endemic areas, including the eastern part of Indonesia. A TB control program could give more support to the importance of BCG vaccination.

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## Author's contributions

IP did the data analysis, drafted the first manuscript, and wrote the final manuscript. TAP designed the study, lead the data collection and data analysis, and wrote the final manuscript. IS and AAB supported the data analysis. EK designed the study. AM designed the study and did the data collection in the laboratory. ES did the data collection in the laboratory, analyzed the data, and wrote the final manuscript. All authors read and approved for the final version of the manuscript.

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