การหาปริมาณเชื้อบีซีจีโตเกียวสายพันธุ์ Type I และ II ในล็อตการผลิตด้วยวิธี Multiplex Real-time PCR

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สถานเสาวภา สภากาชาดไทย กรุงเทพมหานคร

บทคัดย่อ

สถานเสาวภา สภากาชาดไทย ได้นำสายพันธุ์ต้นเชื้อบีซีจีจากประเทศญี่ปุ่นมาผลิตเป็นวัคซีนบีซีจี ตั้งแต่ปี พ.ศ. 2531 โดยทั่วไปเชื้อบีซีจีโตเกียวประกอบด้วย บีซีจีสองสายพันธุ์ คือ สายพันธุ์ type I และ type II โดยที่สายพันธุ์ type I มักพบในปริมาณมากกว่าสายพันธุ์ type II เสมอ โดยสายพันธุ์ type I มีการขาดหายไปของ 22 คู่เบส ในบริเวณยีน RD16 ในการทดลองนี้ได้ตรวจหาปริมาณบีซีจีทั้งสองสายพันธุ์ในตัวอย่างล็อตการผลิต อ้างอิงกับสายพันธุ์ต้นแบบ ด้วยวิธี multiplex real-time PCR ที่พัฒนาขึ้น ผลพบมีบีซีจีทั้งสองสายพันธุ์ในเกือบทุกล็อตการผลิตโดยมีปริมาณค่าเฉลี่ยของสายพันธุ์ type I ร้อยละ 97.26 และ สายพันธุ์ type II ร้อยละ 2.74 การตรวจปริมาณเชื้อด้วยวิธีนี้น่าจะเป็นประโยชน์ในการตรวจหาปริมาณเชื้อบีซีจีในกระบวนการผลิตวัคซีนบีซีจี

คำสำคัญ: วัคซีนบีซีจี Real-time PCR, RD16, *M. bovis*

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Quantification of Bacillus Calmette-Guérin (BCG) Tokyo Type I and II Strains in Commercial Lots using Multiplex Real-time PCR

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Abstract

Japanese bacillus Calmette-Guérin (BCG) strain has been used as a parent strain since 1988 by the Queen Saovabha Memorial Institute, Thai Red Cross Society, to manufacture BCG vaccine in Thailand. In general, the BCG Tokyo strain consists of two BCG subpopulations: type I and type II. The major population is always type I which has a 22-base-pair deletion in the RD16 region. In this study, BCG subpopulations in commercial lots were examined the variation referred to the master seed using modified multiplex real-time PCR in a single tube to quantify two BCG types in commercial lots. Both genotypes were found in almost every lot. The average quantity of type I was 97.26% while that of type II was 2.74%. This method would be useful for quality control of commercial BCG vaccine preparation.

Keywords: BCG vaccine, Real-time PCR, RD16, *M. bovis*

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Introduction

Tuberculosis (TB), caused by the intracellular bacterial pathogen *Mycobacterium tuberculosis*, remains a major cause of mortality and morbidity worldwide. Annually there are an estimated 9.6 million new cases of clinical TB and 1.5 million deaths with the majority of the case being reported from South-East Asia[1]. Bacillus Calmette-Guérin (BCG) is a vaccine produced from an attenuated live strain of *M. bovis*, which has been used since the early 1920s. It is the only approved vaccine for controlling the TB in humans. BCG vaccine provides some level of protection, particularly against TB meningitis and severe forms of disseminated TB in children[2]. However, in the case of severe pediatric TB, such as pulmonary disease in infants, vaccine efficacy is variable[3]. The major daughter strains of BCG used for vaccine production are BCG Danish, Glaxo, Pasteur, Moreau, Tokyo, and Russia. To prevent continuing genetic changes in the strains, the World Health Organization (WHO) recommended that vaccine should not be prepared from any culture with more than 12 passages initially from a defined freeze-dried seed lot[4]. BCG Tokyo-172 strain has been used as a parent strain since 1988 by the Queen Saovabha Memorial Institute, Thai Red Cross Society, for BCG vaccine manufacturing in Thailand. In general, the BCG Tokyo-172 strain consists of two subpopulations, type I and type II[5]. Type I group has the deletion of a 22 base-pair (bp) encoding Rv3405c in the RD16 region[5, 6], whereas type II group has a single base insertion and two single base substitutions in ppsA gene encoding to phenolic glycolipid (PGL) which plays critical roles against the killing activity of host[7]. The BCG Tokyo preparation is mainly composed of the type I subpopulation, with well-known characteristics, such as high viability and good heat stability[8]. In addition, type I subpopulation shows a growth advantage over the type II both in culture media and mice[9].

In 2007, Shibayama *et al*. established the method to quantify the percentage of two BCG Tokyo types by singleplex real-time PCR. They examined the master seed lots and commercial lots for quantification of two subpopulations. They showed that three master seed lots and four commercial lots contained both type I and type II subpopulations. Type II subpopulation found in commercial lots ranged from 1.5% to 7.4%, while type II found in master seed lots were 3.6% to 55.1%. The results indicated that the two variant BCG strains contained in both master seed lots and commercial lots with the less type II population than type I and also the decrease of type II[11]. However, this established singleplex real-time PCR had the limitation of only one target DNA sequence in one reaction.

Real-time detection of PCR products is based on the detection of a fluorescent signal produced by reporter molecule which increased as the target gene amplification. TaqMan assays employ a sequence-specific, fluorescently

labeled oligonucleotide probe called the TaqMan probe. The probe contains a fluorescent reporter at the 5' end and a quencher at the 3' end. The probe hybridizes to the target gene and the dsDNA-specific 5'——>3' exonuclease activity of Taq cleaves off the reporter. When the reporter is separated from the quencher, the free reporter fluoresces. The resulting fluorescence signal is proportional to the amount of amplified product in the sample. The good amplify efficiency of the assay should be 90-105%, the coefficient of determination (R^2) of the standard curve should be > 0.980, and the quantification cycle (Cq) values of the replicates should be similar[10].

BCG vaccine manufactured by the Queen Saovabha Memorial Institute, Thai Red Cross Society has been produced from the BCG Japan strain for 30 years. The WHO declared that BCG vaccine subpopulations in commercial lots were examined the variation referred to the master seed. In addition, to improve the BCG types quantification method, multiplex real-time PCR targeting multiple sequences at once was developed.

Materials and methods

BCG vaccine lots (freeze-dried vaccines)

Commercial lots of BCG vaccine (0.05 mg/sample) used in this study were FB01114, FB01915, FB02115, FB03012 and a bulk vaccine sample from the Queen Saovabha Memorial Institute. *M. bovis* DNA from each lot was extracted by Genomic Extraction kit (Qiagen, Germany). Fifty nanograms of extracted DNA were further used as the DNA template in this study.

PCR and DNA sequencing of RD16 region (Rv3405c)

To identify the nucleotide sequence of RD16 gene of two BCG subpopulations specific PCR was performed by using a primer set, RD16-F (5'-GGC TGG TGTT TCG TCA CTT C-3'), and RD16-R (5'-ACA TTG GGA AAT CGC TGT TG-3')[6]. PCR amplification was proceeded on a thermocycler (MWG Biotech, U.S.A.) by using 35 cycles of denaturation at 94°C for 2 minutes, annealing at 50°C for 30 minutes, extension at 72°C for 30 minutes and the final extension at 72°C for 7 minutes. The 50 uL PCR reaction mixture contained 23 uL of nuclease-free H_2O, 25 uL of 2X Master Mix (Vivantis, Malaysia), 1 uL of primers and 1 uL of genomic *M. bovis* BCG. After amplifying the RD16 gene (data not shown), the PCR products were sequenced (1st Base DNA sequencing service, Seri Kembangan, Malaysia). The nucleotide sequences of the products were published on GenBank, CP014566.1 (BCG type I) and KX424965.1 (BCG type II)[13] and were aligned by CLC bio software (Qiagen, Germany), Fig. 1.

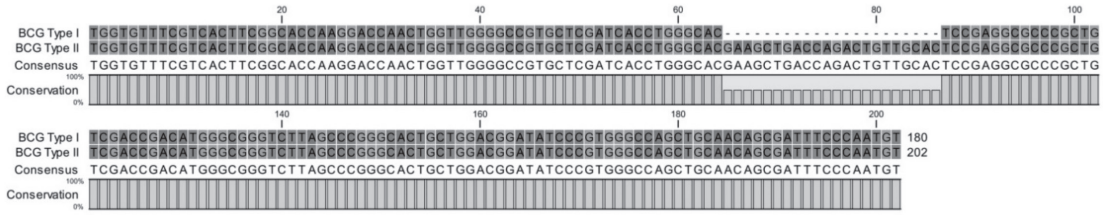

Fig. 1 Sequences and alignment differences of each BCG subpopulation in RD16 region[5] by CLC bio software (Qiagen, Germany). This information was used to design probes and primers for multiplex real-time PCR.

Design of primers and probes for multiplex real-time PCR and quantitative PCR (qPCR)

The nucleotide sequences of RD16 region from each BCG subpopulation in Fig. 1 were used in a mutation assay (Bio-Rad, U.S.A.) to design specific TaqMan probe and primers used for multiplex real-time PCR (from NCBI Reference Sequence: CP014566.1 (BCG type I) and KX424965.1 (BCG type II)[13]. Hexachlorofluorescein (HEX) probe fluorophore was used to hybridize RD16 sequence of type I subpopulation, whereas carboxyfluorescein (FAM) probe was used for that of type II. Probes and primers were designed by web design software, shown in dMDS803069376 code (Bio-Rad, U.S.A.)[14]. The reaction was carried out in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, U.S.A) in 20 uL volume with TaqMan assay using 10 uL of 2X SsoAdvanced universal probes supermix (FAM and HEX), 1 uL of 20 pmol of primers, 8 uL of nuclease-free H_2O and 1 uL of 50 ng DNA sample. The multiplex real-time PCR condition was performed as follows: enzyme activation step at 95°C for 10 minutes and 40 cycles in these steps: denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minute and extension at 72°C for 10 seconds.

The standard curve of real-time PCR

The RD16 PCR product of each type was cloned into the pTG19-T vector (Vivantis, Malaysia). Then, type I and type II-recombinant plasmids were used as the DNA template for generating the standard curve of BCG type I (HEX), type II (FAM) and also as the positive control. Measurement of plasmid concentration was performed by determining the optical density (O.D.) at 260 nm by using DU® 650 spectrophotometer (Beckman Coulter, U.S.A.). The plasmid DNA of two BCG subpopulations initiated at the same concentration of 2.15×10^8 copies/uL. The serial dilutions of two BCG types were 2.15×10^8, 2.15×10^6, 2.15×10^4, 2.15×10^2 and 2.15×10^1 copies/uL. The number of copies was calculated using the following formula; the number of copies = (ng × 6.022 × 10^{23}) / (length × 1 × 109 × 650); ng is the amount of DNA in nanogram; 6.022 × 10^{23} = Avogadro's number; length is the length of

DNA fragment in base pairs[12]. The standard curve values were done in triplicate.

Results

Verification of real-time PCR system

The primers and probes designed for BCG types detection were HEX (type I) and FAM (type II). HEX was specified to type I with the deletion of 22 bp in RD16 region while FAM was specified to type II with the complete sequences of RD16 region (Fig. 1). The recombinant plasmid templates of both BCG types were used as the positive control. The qPCR with BCG type I plasmid template gave a single DNA peak of HEX with no significant amplification peak of DNA type II (FAM). Likewise, the qPCR with BCG type II plasmid template gave a single peak of FAM with no peak of HEX (data not shown). For negative control, the reaction mixture without DNA, no significant amplification peak was detected. Performance of real-time PCR was initially tested with diluted recombinant plasmid DNA of BCG type I and II. The standard curves for each type shown in Fig. 2 and Fig. 3 were generated from triplicated values with low variation. The coefficient of variation of HEX (type I) was 0.03 and FAM (type II) was 0.10 (Fig.2).

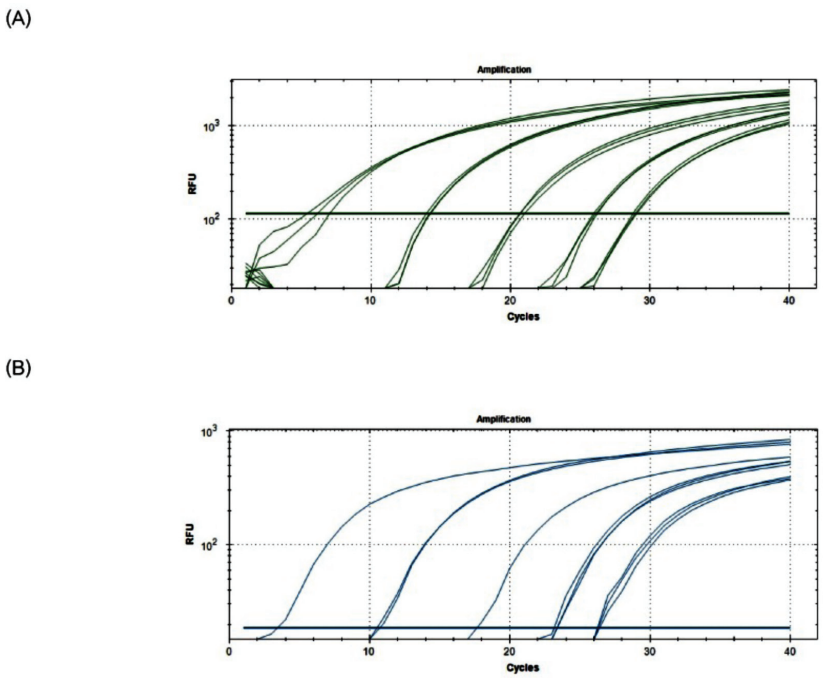

Fig. 2 DNA amplification standard curve, (A) BCG type I (HEX) and (B) BCG type II (FAM) in log scale between relative fluorescence units (RFU) and cycles numbers.

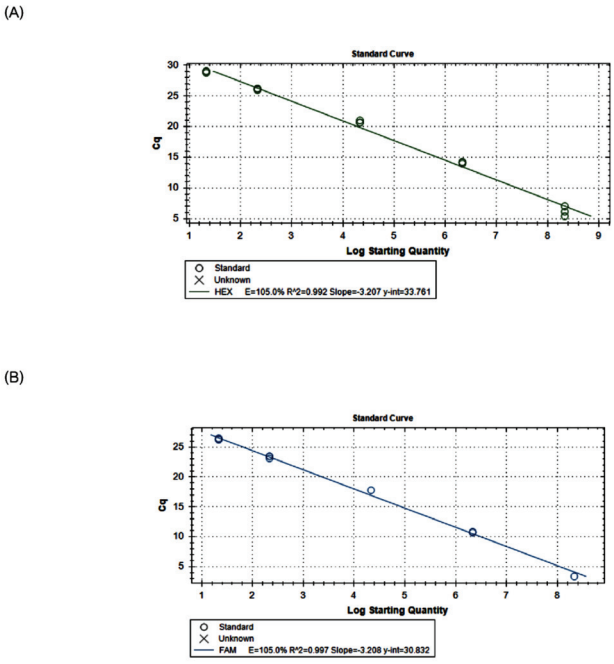

Fig. 3 Standard curves between log starting quantity and Cq (quantification cycle). (A) amplification of BCG type I (HEX) and (B) amplification of BCG type II (FAM).

Fig. 3 showed the standard curve in log scale between BCG type I (HEX) and type II (FAM) in the same time of reaction.

The efficiency of the standard curve of type I and type II was 105%. R^2 of type I and type II were 0.992 (HEX) and 0.997 (FAM), respectively, indicating that this method is optimized.

Determination of the amount of two BCG subpopulations in vaccine commercial lots

Fig. 4 showed that the amount of type II (FAM) was less than that of type I (HEX) during the PCR reaction at the same time. The other lot results are shown in Table 1. The results in Table 1 showed the proportion of each BCG type in each commercial lot which was derived from the average starting quantity (SQ) value indicating the quantity of HEX and FAM in qPCR. The percentage of each type was calculated from the summation of both types as 100%. The average quantity of type I obtained from 5 lot samples was 97.26% while that of type II was 2.74%. Notably, BCG type II in FB01915 lot was obviously not found.

Discussion

There are some research studies that use a singleplex real-time PCR technique for quantitation of two BCG types[11, 15]. In the study of Shibayama *et al.*, the total amount of both types was determined using TaqMan probe specified to the conserved region of two BCG types (I, II). The type II was parallelly deter-

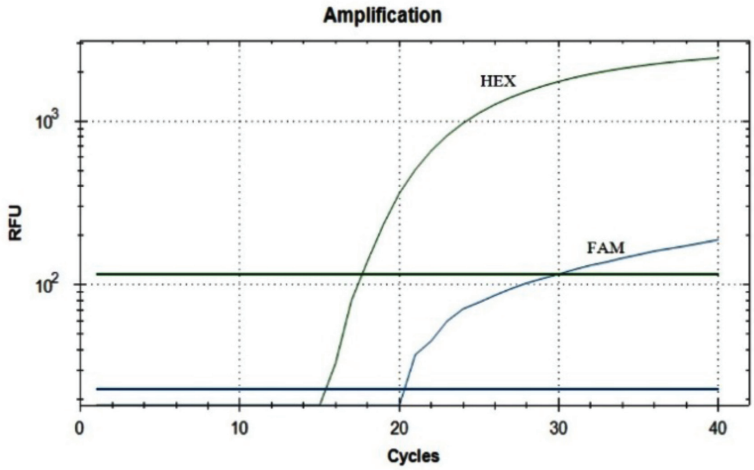

Fig. 4 qPCR of HEX and FAM probes in FB01114 lot as the example result of BCG commercial lots.

mined in another tube using type II TaqMan specific probe. The percentage of variant strain type II in the mixture of two variant strains was calculated by using the total amount of DNA as the references[11]. However, a singleplex real-time PCR using two tubes required more DNA templates and many preparation steps which increased the contamination opportunities and reagent costs. Therefore, the multiplex real-time PCR was established in this study. This method can detect the amount of BCG type I and II in a single tube. Two specific TaqMan probes and two primer sets were used in the same tube in real-time PCR reaction in which both BCG subpopulations could be quantified in a single test. The benefit of this experiment is the ability to detect two variant strains in the same amount of time.

The quantification of BCG type I and II strains in commercial lots produced by the Queen Saovabha Memorial, Thai Red Cross Society, have not been studied. The variation of two BCG types existing in commercial lots which have been prepared for 30 years should be confirmed. This is the first study using multiplex real-time PCR in a single tube to quantify amount of two BCG types. The results of multiplex real-time PCR showed that the amount of BCG type II in commercial vaccine lots was less than that of BCG type I (Table 1), except for FB01915 lot which did not have BCG type II. The average of BCG type I was 97.26% and BCG type II was 2.74%, which were consistent with the study of Shibayama *et al.*[11] showing that the main BCG subpopulation in commercial lots was type I. In the commercial lot, the positive control of both standard curves (type I, II) did not show cross contaminated DNA peak of the other type in the reaction (data not shown). However, some lots did not contain BCG type II which may be due to this BCG Tokyo172 preparation

Table 1 Quantification data with the results of BCG type proportion

Lot samples	HEX		FAM		Proportion of BCG type	
	Cq	SQ	Cq	SQ	HEX, type I (%)	FAM, type II (%)
FB01114	17.61	108767.93	20.02	2339.19	97.89	2.11
FB01915	16.91	179894.60	not found	not found	100.00	0.00
FB02115	17.13	153537.32	22.90	296.10	99.81	0.19
FB03012	16.56	158806.77	27.05	13558.80	92.13	7.87
a bulk sample	17.30	135886.15	18.96	5001.21	96.45	3.55
					97.26 (average)	2.74 (average)

Cq = quantification cycle, SQ =starting quantity

mainly consisted of type I subpopulation[6]. Although type II was still present in every preparation including the seed lot, the proportion of type II subpopulation could decrease during passaging until not found[9]. It is possible that some conditions of lot preparation, such as nutrient components and temperature for culture, might affect the proportions of certain subpopulations. In addition, propagation of seed lots, subculturing for mass production and freeze-drying techniques might be involved in the generation of mutations and the accumulation of genetic variants within the substrain[15]. In case of the production of immune response, type I seems to be greater than type II, as determined from IFN-gamma secretion by sensitized spleen cells and BCG therapy for bladder cancer which found only the type I in every case[17]. Moreover, PGL, produced by type I but not type II, demonstrated the inhibition of the host innate immune response. The loss of PGL was responsible for the increase in the release of TNF-alpha and interleukins 6 and 12 *in vitro*, accordingly, PGL deficiency showed a phenotype with low virulence/pathology[18].

Rv3405c in the RD16 region is the common marker for differentiating the subpopulations of BCG-172 strain. In the present study, the results showed the difference of the PCR product which 180 bp could be amplified by using primers specific for type I subpopulation, whereas, 202 bp by primers specific for type II (data not shown). Furthermore, the nucleotide sequence of each PCR product was identified. Consistent with the previous study by Thaveekarn *et al.*[5], there were 22 bp differences between the two types (GAA GCT GAC CAG ACT GTT GCA C) as seen in Fig. 1. BCG Tokyo-172 strain normally exhibited two subpopulations. The RD16 region found in BCG Tokyo-172 type I strain showed a characteristic 22-bp deletion but type II was full length complete in this region. BCG Connaught and BCG Pasteur strain had the complete sequence of this region[6]

those were missing in BCG Moreau strain[6, 16]. Therefore, the RD16 region can be used to distinguish BCG Tokyo strain from the others.

In this study, multiplex real-time PCR showed 105% efficiency of the standard curve and R^2 was 0.992 for FAM probe. In the case of HEX probe, the efficiency of the standard curve was 105% and R^2 was 0.997. These results with $R^2 > 0.980$ indicated that the quantitative PCR (qPCR) assay of this study was optimized, accurate, with high amplification efficiency and consistency across replicate reaction. The R^2 could be used to evaluate whether qPCR assay was optimized, and also R^2 value of a standard curve presents how well the experimental data fit the regression linearity. For the amplify efficiency, closing to 100% (90-105%) is the best indicator of a reproducible assay. The efficiency of the standard curve in this experiment was 105% (HEX and FAM) indicating that qPCR assay of this study was reproducible.

In conclusion, the multiplex real-time PCR in a single tube can determine the amount of two BCG types in each lot, in which most of the vaccine commercial lots still had two BCG subpopulations corresponding to the master seed. This technical development can be used for the detection of the actual component in BCG vaccine in commercial lots, which is useful for quality control aspects of BCG vaccine products of the Queen Saovabha Memorial Institute, Thai Red Cross Society. However, to examine the immunogenicity and the appropriate proportional amount of type I and II subpopulation in BCG vaccine in Thailand, a further study should be required.

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