Determination of Antibody Responses to MPB83 Antigen in BCG-vaccinated Rusa Deer (*Rusa timorensis*)

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Abstract

Bovine tuberculosis (BTB) is a contagious zoonotic disease that causes serious public health concerns. Cervidae are highly susceptible to BTB and it has been diagnosed in various deer species. In order to develop BTB eradication and control program, *Mycobacterium bovis* Bacille Calmette Guerin (BCG) vaccination has been studied in cervids. In this study 15 rusa deer were tested for responsiveness to mycobacterial reaction using a comparative cervical skin test (CCT). The rusa deer were divided into 2 groups; BCG vaccination group, which were inoculated with a single dose of 2×10^6 cfu *M. bovis* BCG Tokyo subcutaneously (n=8) and control group (n=7). The MPB83 protein was constructed and used as a specific antigen for an indirect ELISA to determine antibody levels against BCG vaccination in rusa deer. Sera were collected pre-vaccination and 4, 10, 15, 20 and 25 weeks post-vaccination. The antibody levels at each time point were determined by indirect ELISA using the MPB83 antigen. In the vaccination group, mean difference in optical density (ODs) increased significantly ($p<0.05$) 4 weeks after vaccination. Thereafter, the antibody level continuously declined until the final measurement at 25 weeks. The antibody levels against the MPB83 protein in the vaccination group were higher than in the control group at 4, 10, 15 and 20 weeks post-vaccination and were not different between the positive and negative CCT animals. In the control group, the mean difference in ODs were not significantly different at any measured time point. In conclusion, the immunity response to BCG-vaccination in the rusa deer could be monitored by indirect ELISA using the specific antigen MPB83. Therefore, further studies to determine protective level of antibody against BCG and cell-mediated immune responses should be implemented.

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Introduction

Bovine tuberculosis (BTB) is an important zoonotic disease caused by *Mycobacterium bovis*. BTB is a member of *M. tuberculosis* complex, which is composed of highly pathogenic mycobacteria such as *M. tuberculosis*, *M. africanum*, *M. canettii* and *M. microti* (Broschet al., 2002). In less developed countries, the outbreaks are still common and cause significant economic losses (CFSPH, 2009; Schiller et al., 2010). *M. bovis* has a wide range of host whilst cattle are the primary host species for BTB; other domestic and wild animals are also susceptible. Wildlife can act as maintenance hosts for BTB and it can then spill over to domestic animals and also humans (OIE, 2009; Palmer, 2008). Zoonotic cases of BTB have been previously reported from domestic animals and wildlife (Kaneene and Thoen, 2004; Cosivi et al., 1998; Dalovisio et al., 1992; Cawthorn, 1994; Fanning and Edwards, 1991).

BTB has been found both in free-living and captive cervidae (Mackintosh et al., 2004; OIE, 2009). Clinical signs can be sub-acute or chronic with variable rates of disease progression. Infected deer may become severely affected within a few months or may take several years to develop the clinical signs, which are related to lesions in the animal (OIE, 2009). In order to eradicate the occurrence of BTB, effective monitoring and diagnosis are required in management and control programs. However, vaccination has been described as an additional alternative tool for preventing infection, disease and transmission. The attenuated *M. bovis* Bacille Calmette Guerin (BCG) has been studied for the prototype vaccine to control BTB in cervids such as white-tailed deer (*Odocoileus virginianus*) and red deer (*Cervus elaphus*) (Palmer et al., 2007; Waters et al., 2003; Griffin et al., 1999).

MPB83 is a lipoprotein antigen associated with cell surface of *M. bovis* (Wiker, 2009; Harboe et al., 1998; Vosloo et al., 1997). During infection this protein is highly expressed by *M. bovis* and strongly recognized by immune system (McNair et al., 2001; Waters et al., 2006). Conserved MPB83 protein expression is only found in species of *M. tuberculosis* complex (MTC) and also in BCG. By this specific expression, MPB83 has been posed as a specific antigen to tuberculosis diagnosis (Wiker, 2009).

In Thailand, rusa deer (*Rusa timorensis*) are introduced for meat and velvet production in the deer farm business. Because Thailand is a clinical disease area of BTB, effective management and control program of BTB in rusa deer farms is required. In this study, the BCG vaccination in rusa deer was studied and humoral immune responses after BCG vaccination were determined by using the MPB83 protein as a specific antigen.

Materials and Methods

MPB83 protein cloning and purification: A primer pair was designed to amplify the mature MPB83 protein gene based on the coding sequences of *M. bovis* MPB83 gene in NCBI GenBank (D64165); MPB83F (5′-GGC<u>ATATG</u>GCGTTCTTAGCGGG-3′) and MPB83R (5′-TTCT<u>CGAG</u>TCACTGTGCCGGGGGC-3′). *Nde*I and *Xho*I restriction sites are underlined. The MPB83 gene excluding the signal sequences was amplified using *M. bovis* BCG strain Tokyo genomic DNA template (supported by Department of Microbiology, Faculty of Science, Mahidol University). PCR conditions were initially denatured at 94°C for 1 min, followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, and final extension at 72°C for 5 min. The PCR product was ligated into pGEM-T easy vector (Promega®, USA) and transformed to competent cells *E.coli* DH5α by heat shock. Positive colonies were selected by blue/white screening and PCR analysis. Recombinant plasmids were extracted by QIAGEN Plasmid Mini kit (Qiagen, Germany) and confirmed by restriction enzyme digestion. The recombinant pGEM-MPB83 and pET28a(+) expression vectors (Novagen®) were digested by *Nde*I and *Xho*I. Then, the MPB83 gene was sub-cloned into the pET28a(+). After selection and confirmation, the recombinant pET28a-MPB83 plasmid was transformed to *E.coli* BL21 expression cells. To confirm that the recombinant plasmid was cloned in proper translation, a sequence analysis was performed using a 3730XL DNA sequencer (Applied Biosystems Inc., Macrogen laboratory, Korea). Prokaryotic expression of recombinant protein was performed by inoculating the recombinant bacteria to LB Broth containing 50ug/ml Kanamycin sulfate and allowing them to grow overnight at 37°C in a shaker incubator. The bacteria were sub-cultured at 37°C shaking until OD_{600} reached to 0.5. Then, 0.5 mM of isopropyl β-D thiogalactoside (IPTG) was added and the bacteria were allowed to continue growing at 37°C shaking for 4 h. Exogenous protein expression was analyzed by 15% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

After expression, the recombinant MPB83 protein was partially purified. The cultured cell pellets were washed 2 times with 8 M urea, then once with 1X PBS. The pellets were resuspended in 1X STE buffer and 10% sarcosine was added to the 1.25% final concentration. The solution was sonicated 3 times for 5 min each. Supernatant was collected and Triton X-100 was added to the 0.05% final concentration. Thereafter, the partially purified protein lysate was analyzed on a 15% SDS-PAGE.

Determination of MPB83 recombinant protein in tuberculosis detection: Tuberculosis-positive bovine sera were used to determine the ability of recombinant MPB83 protein in tuberculosis detection. In western blot analysis, recombinant proteins on SDS-PAGE gel were blotted on nitrocellulose membrane. The membrane was incubated at 37°C for 1 h in a blocking solution (5% skim milk in PBS, 0.05% Tween 20), then incubated with TB-positive bovine sera (dilute 1:500) for 1 h, followed by HRP-conjugated goat anti-bovine IgG (dilute 1:500; KPL) for 1 h. The binding antibody was visualized by 3, 3′-diaminobenzidine tetrahydrochloride (DAB) staining. Thereafter, the reaction was stopped by soaking the membrane in distilled water.

For the indirect ELISA method, a 96-well ELISA plate was coated with the lysed recombinant MPB83 (positive antigen) and the pET28a(+) protein (control antigen) at 4°C overnight. The plate was then incubated with blocking solution (0.5% casein) for 1 h.

The TB-positive bovine sera were diluted by 2-fold dilution (starting from 1:200 to 1:409600) and followed by the HRP-conjugated goat anti-bovine IgG as a secondary antibody. The TMB (3, 3', 5, 5' tetramethyl-benzidine) substrate was added and then the reaction was stopped using 0.01% sodium dodecyl sulfate. Optical densities (ODs) were analyzed at 650 nm by ELISA reader (Sunrise™, Tecan group Ltd., Switzerland) to evaluate antibody levels.

Animal vaccination: Fifteen healthy rusa deer, aged between 2 to 4 years old, were obtained from a herd with no history of tuberculosis. Prior to the experiment, all deer were tested for responsiveness to mycobacterial antigens by the comparative cervical skin test (CCT). Briefly, three areas of 3x3 cm were clipped at the right cervical region. The 2000 IU *M. bovis* purified derivative protein (PPDb) (Bovituber®, synbiotics), 2500 IU *M. avium* purified derivative protein (PPDa) (Avituber®, synbiotics) and 0.1 ml normal saline solution were injected intradermally in each separated locations. Skin thickness at each injection sites was measured at 72 h after administration. All rusa deer were non-reactive to PPDb and classified as BTB negative. However, a positive reaction to PPDa was found in 9 deer, assuming that these animals were perhaps previously exposed to other environmental mycobacteria.

The vaccination started in a week after the CCT. The rusa deer with PPDa-positive and negative results were divided into 2 groups; vaccinated group (n=8) and control (non-vaccinated) group (n=7). In the vaccinated group, a single dose of 2×10^6 cfu *M. bovis* BCG strain Tokyo was administered by subcutaneous injection. Serum samples were collected at pre- and 4, 10, 15, 20 and 25 weeks post-vaccination in both vaccination and control groups.

MPB83 antibody detection in BCG vaccinated rusa deer: Antibody levels against the BCG vaccination in rusa deer were determined by the indirect ELISA using recombinant MPB83 antigen as previously described. In this step, horseradish peroxidase (HRP)-conjugated protein A was used to detect the antibody of rusa deer. The antibody levels were reported as different ODs between rMPB83 protein and control bacterial protein.

Statistical analysis: Means of different ODs between the vaccination and control groups were statistically analyzed by Student's *t*-test. The mean difference in ODs between pre-and post-vaccination were analyzed by a paired *t*-test. The freely available software StatPlus: mac LE, a statistical analysis program for Mac OS. Version 2009 (Analyst Soft Inc., www.analystsoft.com) was used for all analyses. Differences between groups were considered significant if probability values of *p*<0.05 were obtained.

Results

The recombinant MPB83 protein was successfully cloned into the pET28(a+) expression vector. A positive clone was expressed in the *E. coli*s

system with an estimated molecular weight of 25-kDa. The western blot analysis and indirect ELISA using recombinant MPB83 antigen revealed the specificity and sensitivity of this protein to detect the TB-positive bovine sera and indicated the ability to use this for tuberculosis detection (Figs 1 and 2). In CCT analysis, the responses to *M. bovis* PPD were negative in all deer. However, 9 deer showed positive reaction to *M. avium* PPD. The deer were classified into 2 groups based on their CCT results: the vaccinated group, which included 3 negative and 5 positive PPDa responses; and the control group, which included 3 negative and 4 positive PPDa responses.
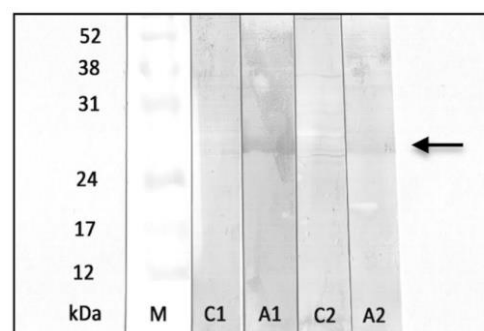
Figure 1 Western blot analysis of TB-positive sera against recombinant MPB83 protein (Lanes A1 and A2) indicating 25 kDa protein band, compared with the control *E.coli* BL21 protein (Lanes C1 and C2); Lane M: protein marker.

The indirect ELISA was used to determine the antibody responses to BCG vaccination in rusa deer. After vaccination, the antibody specific to MPB83 antigen was detected in the serum within 4 weeks. In comparison with pre-vaccination, the antibody levels increased significantly (*p*<0.05) in 4 weeks post-vaccination and then continuously declined during the following 21 weeks post-vaccination (Fig 3). For the comparison between the vaccination and control groups, the antibody levels against MPB83 protein in the BCG-vaccinated group were significantly higher than the control group in 4, 10, 15, 20 weeks post-vaccination (p<0.05). In the unvaccinated group, the mean ODs were not different across time periods (Fig 3).

Based on the CCT results within groups, in the vaccination group the antibody levels of the positive PPDa animals were higher than the negative PPDa animals. However, there was no significant difference between the groups (Fig 4). In the control group, the results of both positive and negative CCT were also similar (Fig 4).

Discussion

Bovine tuberculosis is a contagious disease that can affect a wide range of hosts including domestic livestock, humans and wildlife. Wildlife species acts as an important reservoir of infection that can spill over into domestic livestock and humans leading to economic and zoonotic problems. Thus, control of the disease in the maintenance host should prevent the disease with long-term persistence (Buddle et al., 2006). The principle aim for control of bovine

tuberculosis is to eliminate the risk and prevent the establishment of infection. Therefore, sensitive and specific tests for BTB diagnosis are important for BTB surveillance and monitoring programs.

In this study, the recombinant MPB83 protein was cloned and expressed to be applied for BTB detection as a specific antigen. Tuberculosis detection with the TB-positive bovine sera indicated that the recombinant MPB83 protein exhibited the sensitivity and specificity for TB detection. MPB83 is the glycosylated lipoprotein expressed by *M. bovis*. It has been recognized as the sensitive and specific antigen for BTB diagnosis (Wiker, 2009). The study of MPB83 revealed that it could differentiate the infection between BTB and Paratuberculosis, caused by *M.*

avium paratuberculosis (Map) (Marassi et al., 2010). Previous studies of antibody responses in experimental *M. bovis* infection in cervids revealed that MPB83 was a sero dominant antigen which could be detected early as the cell-mediated immune response (Harrington et al., 2008; Waters et al., 2004; Waters et al., 2005). In white-tailed deer and reindeer, the antibodies against MPB83 antigen were recognized in serum by multi antigen print immunoassay (MAPIA) as early as 4 weeks after experimental infection (Waters et al., 2004; Waters et al., 2005). In red deer-elk hybrid challenged with *M. bovis*, the antibodies to MPB83 were also detected by immunoblot assay and MAPIA at 30 and 44 d post-infection, respectively (Harrington et al., 2008).
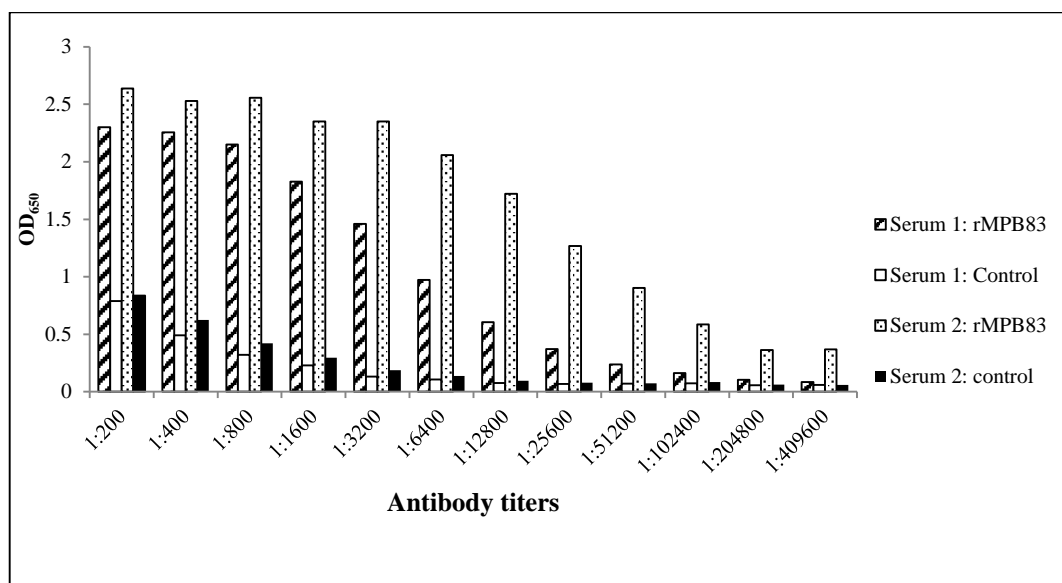
Figure 2 Indirect ELISA showing antibody levels of two TB-positive sera against recombinant MPB83 protein, compared with control background of *E.coli* BL21 protein.
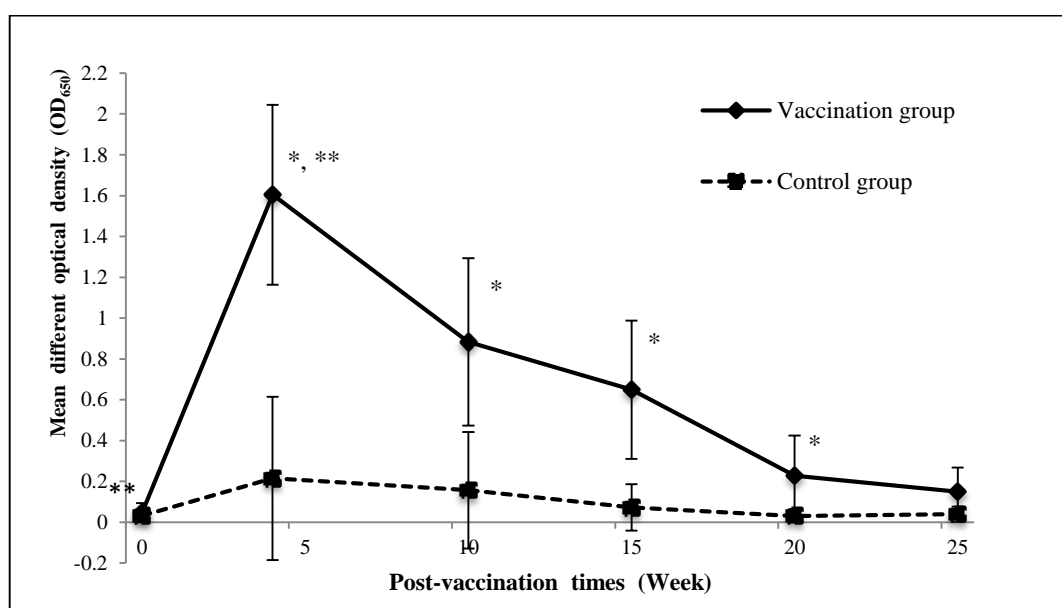
Figure 3 Mean different optical density (OD_{650}) of vaccinated and control rusa deer during pre-vaccination (week 0) and post-vaccination (weeks 4-25). (*) Mean ODs of BCG vaccinated group were different from control group ($p<0.05$). (**) In BCG vaccinated group, mean OD of 4 weeks post-vaccination was significantly increased from pre-vaccination ($p<0.05$).

In this present study, the humeral immune responses to MPB83 antigen were evaluated in BCG-vaccinated rusa deer. The subcutaneous-vaccination with BCG could induce the antibody responses. In the vaccinated rusa deer, the antibody levels against recombinant MPB83 protein increased significantly after 4 weeks post-vaccination and were greater than the control group. The studies of antibody responses in BCG-vaccinated deer evaluated by ELISA had been previously determined in elk and white-tailed deer (Waters et al., 2003; Palmer et al., 2007). The antibody responses to crude mycobacterial antigens (such as *M. bovis* PPD and Proteinase K-digested whole cell sonicate of *M. bovis* BCG) of BCG-vaccinated elk were detected at 2 weeks post-vaccination and then declined after 4 weeks before the second booster of BCG (Waters et al., 2003). In comparison with BCG vaccination in white-tailed deer, the antibody specific for Lipoarabinomannnan (LAM)-enriched mycobacterial antigen was greater in 9 weeks post-vaccination (Palmer et al., 2007). Both previous studies indicated that the second booster of BCG at 6 weeks could provide the greater level of antibodies.

The positive CCT reaction with PPDa indicated that the animals might have prior exposure to the environmental mycobacteria. After BCG vaccination in rusa deer, the antibody responses to MPB83 were similar in both positive and negative PPDa responses. However, the antibody levels of the positive PPDa group seemed to be higher than the negative PPDa group. Previous studies suggest that prior exposure to environmental mycobacteria such as *M. avium* primed the host immune responses in cattle (Thom et al., 2008; Howard et al., 2002). Exposure to *M. avium* prior to BCG vaccination resulted in higher interferon gamma (IFN-γ) producing cells responding to PPD antigen at the time of *M. bovis* infection (Thom et al., 2008; Howard et al., 2002) and the protective efficacy of BCG in cattle not being modulated (Thom et al., 2008).

In conclusion, the indirect ELISA using MPB83 protein obtained by this study can be used for monitoring antibodies in BCG-vaccinated rusa deer. Therefore, studies of the protective level of antibody against BCG and the responses of cell-mediated immunity in rusa deer should be undertaken. Moreover, the indirect ELISA using MPB83 protein may have high potential to be used as an alternative TB screening tool for non-BCG vaccinated animals especially in wildlife.
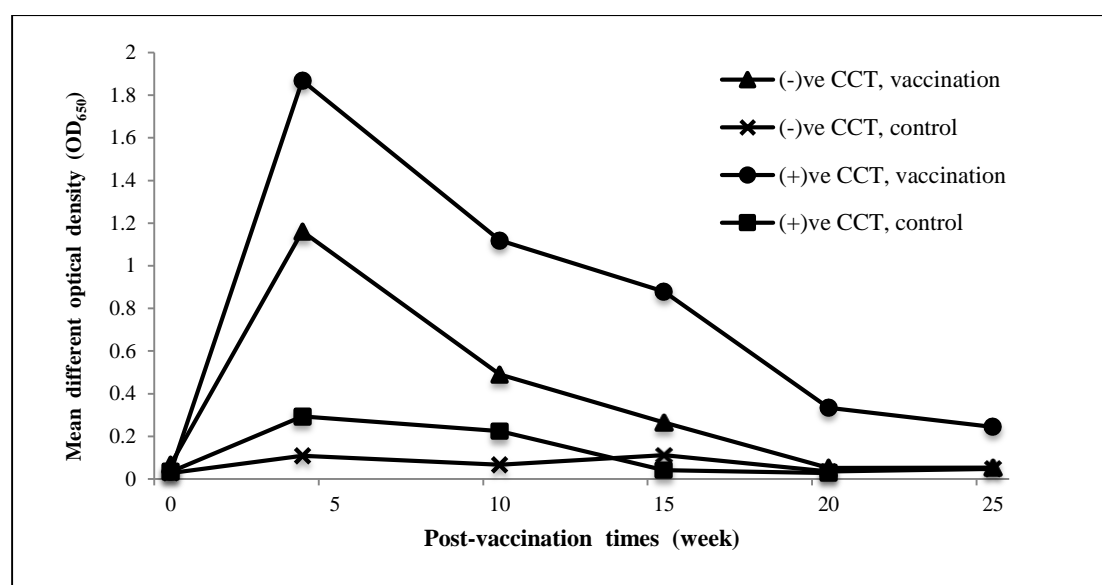

Figure 4 Comparison of antibody level in BCG-vaccinated group and control group based on the CCT results.

Acknowledgements

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บทคัดย่อ

การศึกษาระดับแอนติบอดีต่อแอนติเจน MPB83 ในกวางรูซ่า (*Rusa timorensis*) ที่ได้รับวัคซีนบีซีจี

สิรินารถ ชัยชนะทอง[1, 2] วรวิทย์ วัชชวัลคุ[3] ชญานิศ ประสานวงศ์[4] เมลานี สุขสำราญทวีรัชต์[4]
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วัณโรค (Bovine tuberculosis) เป็นโรคติดต่อระหว่างสัตว์และคนที่ร้ายแรงและก่อให้เกิดปัญหาที่รุนแรงต่อการจัดการระบบสาธารณสุข สัตว์ตระกูลกวางมีความไวต่อการเกิดวัณโรคค่อนข้างสูง และพบการรายงานการเกิดวัณโรคในกวางหลายชนิด ในการควบคุมและป้องกันโรค พบว่าได้มีการศึกษาการใช้วัคซีนบีซีจีในการกำจัดและควบคุมป้องกันวัณโรคในสัตว์ตระกูลกวาง การศึกษาครั้งนี้ได้ศึกษาผลการทำวัคซีนบีซีจีในกลุ่มตัวอย่างกวางรูซ่าจำนวน 15 ตัว โดยก่อนการทำวัคซีน กวางทั้งหมดได้รับการทดสอบปฏิกิริยาความไวต่อเชื้อมัยโคแบคทีเรียโดยวิธี comparative cervical skin test (CCT) จากนั้นทำการแบ่งกลุ่มกวางรูซ่าออกเป็น 2 กลุ่ม ได้แก่ กลุ่มที่ทำวัคซีนบีซีจี (n=8) และกลุ่มควบคุม (n=7) กวางในกลุ่มที่ทำวัคซีนได้รับการฉีดวัคซีนบีซีจี สเตรนโตเกียว ในขนาด 2×10^6 cfu เข้าทางใต้ผิวหนัง หลังจากนั้นทำการเจาะเก็บซีรั่มในกวางทั้งสองกลุ่มที่ 4, 10, 15, 20 และ 25 สัปดาห์ แล้วนำไปวิเคราะห์หาระดับแอนติบอดีต่อแอนติเจน MPB83 ที่ผลิตขึ้นในการทดลองด้วยวิธี indirect ELISA จากการทดลองฉีดวัคซีนบีซีจี ในกวางรูซ่า พบว่าค่าเฉลี่ยของระดับแอนติบอดีต่อ MPB83 มีค่าเพิ่มขึ้นในสัปดาห์ที่ 4 หลังทำวัคซีนอย่างมีนัยสำคัญ (*p*<0.05) เมื่อเปรียบเทียบกับก่อนทำวัคซีน จากนั้นระดับแอนติบอดีลดลงเรื่อยๆ ภายในระยะเวลา 25 สัปดาห์หลังทำวัคซีน เมื่อเปรียบเทียบระหว่างกลุ่มที่ทำวัคซีนบีซีจีและกลุ่มควบคุม พบว่ากลุ่มที่ทำวัคซีนบีซีจีมีค่าเฉลี่ยของระดับแอนติบอดีต่อ MPB83 มากกว่ากลุ่มควบคุมในสัปดาห์ที่ 4, 10, 15 และ 20 หลังการทำวัคซีน และระดับแอนติบอดีไม่แตกต่างกันระหว่างกลุ่มกวางที่ให้ผลบวกและกลุ่มกวางที่ให้ผลลบต่อ CCT สำหรับในกลุ่มควบคุม พบว่าระดับของแอนติบอดีไม่มีความแตกต่างกันตลอดระยะเวลาที่ทำการทดลอง การศึกษานี้ชี้ให้เห็นว่า วิธีการ Indirect ELISA โดยใช้แอนติเจน MPB83 สามารถใช้ติดตามระดับภูมิคุ้มกันภายหลังจากการทำวัคซีนบีซีจีในกวางรูซ่าได้ อย่างไรก็ตามควรมีการศึกษาเกี่ยวกับระดับภูมิคุ้มกันจากการทำวัคซีนบีซีจีที่ให้ผลในการควบคุมป้องกันโรค และภูมิคุ้มกันทางด้านเซลล์ที่เกิดขึ้นจากการทำวัคซีนในกวางรูซ่า

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