

## MODULATION OF CELL GROWTH AND PPAR- $\gamma$ EXPRESSION IN HT-29 AND COLO 205 CELLS BY CIGLITAZONE

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**Introduction:** Colon cancer is one of the most common malignancies in both men and women in Malaysia. Most therapies for the disease are still unsatisfactory since they cause adverse effects to patients. Current research are now focusing on a better understanding of the tumour response and resistance to treatment, apart from finding drugs that will not cause such adverse effects. The nuclear receptor, peroxisome proliferator-activated receptor (PPAR)- $\gamma$ , is reported to be expressed in various cancer cells, including breast, prostate, and colorectal cancer. PPAR- $\gamma$  is a ligand-dependent transcription factor that regulates expression of genes involved in cellular proliferation, differentiation, and apoptosis. Although this receptor has been shown to be highly expressed in colorectal cancer, its exact role in colorectal carcinogenesis is still unclear.

**Objectives:** This study was carried out to study the possible involvement of PPAR- $\gamma$  in modulating the growth of 2 human colorectal carcinoma cell lines, HT-29 and COLO 205. A synthetic PPAR- $\gamma$  ligand, ciglitazone, which is also a member of the antidiabetic drug thiazolidinediones (TZDs), was used in this study to treat the colorectal cancer cell lines.

**Materials and Methods:** To evaluate whether ciglitazone induced inhibition of cell growth, both cell lines were treated with increasing doses of ciglitazone (2.5–100  $\mu$ M) for 6 to 72 hours. Cytotoxicity was determined by measuring the lactate dehydrogenase (LDH) leakage from the cell membrane. In addition, a fluorescein-conjugated monoclonal antibody against cytokeratin 18 (CK18) that recognizes the caspase-cleaved epitope within the CK18 was used to measure apoptosis by flow cytometry. Additionally, the mRNA expression levels of PPAR- $\gamma$ 1 and PPAR- $\gamma$ 2 from the cell lines were quantified by real-time quantitative PCR technique using specifically developed homologous internal standards for each of the genes. The level of PPAR- $\gamma$  protein was determined by Western blotting.

**Results:** Ciglitazone significantly inhibited the growth of colorectal cancer cell lines in a dose- and time-dependent manner ( $P < 0.01$ ). The EC<sub>50</sub> values of ciglitazone obtained after 48 hours of incubation was about 20  $\mu$ M for HT-29 cells and about 30  $\mu$ M for COLO 205 cells, and these concentrations were then used in subsequent experiments to treat the

corresponding cell lines in a time-dependent manner. Flow cytometry results demonstrated that ciglitazone significantly induced apoptosis in the cell lines ( $P < 0.01$ ). However, real-time PCR results revealed that the levels of PPAR- $\gamma$ 1 and PPAR- $\gamma$ 2 mRNA expression were significantly reduced following treatment with ciglitazone compared to controls ( $P < 0.05$ ). Furthermore, ciglitazone also decreased the level of PPAR- $\gamma$  protein expression as shown by Western blotting.

**Conclusion:** Treatment with ciglitazone suppressed colon cancer cell growth and cell death. It is postulated that the antitumour effects of this synthetic PPAR- $\gamma$  ligand may not depend solely on PPAR- $\gamma$  activation. Since previous reports shown that PPAR- $\gamma$  was upregulated in cancer cells, including colon cancer cells, compared with their normal counterparts, the downregulation of PPAR- $\gamma$  expression following ciglitazone treatment suggested the potential of ciglitazone to be used as an adjuvant for the treatment of colon cancer. However, further research is needed to evaluate the anticancer activity of TZDs, which appear to be both dependent and independent PPAR- $\gamma$  pathway.

Supervisor:  
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## HLA POLYMORPHISM IN MALAY SUB-ETHNIC GROUPS IN PENINSULAR MALAYSIA

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**Materials and Methods:** In this study, the Human Leukocyte Antigen (HLA) class I and II were examined through Sequence Specific Primer (SSP) typing in 176 unrelated individuals from 6 Malay sub-ethnic groups of Peninsular Malaysia: Kelantan ( $n = 25$ ), Minangkabau ( $n = 34$ ), Jawa ( $n = 30$ ), Bugis ( $n = 31$ ), Banjar ( $n = 33$ ), and Rawa ( $n = 23$ ). The common HLA alleles in all the sub-ethnic groups were HLA-A\*24 (26%–48%), HLA-B\*15 (22%–41%), -Cw\*07 (21%–32%), DQB1\*03 (25%–55%), and DRB1\*12 (15%–40%).

**Results:** The Malay sub-ethnic groups studied showed close relationship to each other and to Asian populations despite specific differences between them. Banjar, Jawa, and Bugis Malays showed no significant differences to each other, which could be a result of their related origin from the islands around the Java Sea. Besides sharing in the most common haplotype found, phylogenetic and principal coordinate (PCO) analysis showed a genetic similarity between Minangkabau

and Rawa Malays. This could be a consequence of their common origin from Sumatera. The Kelantan Malays, show statistical significant difference with the other groups and revealed differences for the most frequent haplotypes, which could be related to their different origin, and the different populations influence along time. Statistical analysis on the Malay sub-ethnic groups HLA data also revealed credible forensic parameters for forensic applications.

**Conclusion:** The HLA data obtained from this study can be applied for vaccine development, searching for suitable donor for transplantation, disease association studies, and as a guideline for infectious disease prevention programs

*Supervisor :*  
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### THE DEVELOPMENT OF A CANDIDATE TUBERCULOSIS DNA VACCINE EXPRESSING Mtb8.4 AND Ag85B OF MYCOBACTERIUM TUBERCULOSIS

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**Introduction:** Tuberculosis (TB) is still one of the major health problems worldwide. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis*, Bacille Calmette–Guerin (BCG). However, the efficacy of BCG vaccine continues to be debated. Therefore, a more effective vaccine against TB is urgently needed. DNA vaccination is a new approach to the control of infectious agents.

**Materials and Methods:** In this study, a DNA vaccine encoding the candidate TB antigens Mtb8.4 and Ag85B was developed using assembly PCR. Balb/c mice were immunized intramuscularly with 50 µg of the DNA vaccine, pNMNO23, containing the 2 antigens in each hindleg.

**Results:** Reactivity against the Ag85B peptides, P1, and P3 as well as Mtb8.4 showed a consistent Th1 type of immune response by virtue of the increased expression of IL-2, IFN-γ and IgG2a. Splenocytes from immunized mice were also found to proliferate more aggressively when stimulated with the antigens compared to the vector alone. In order to improve the vaccine efficacy, a preliminary prime-boost approach was used. Priming with pNMNO23 and boosting with recombinant BCG (rBCG) in Balb/c mice was carried out. Flow cytometric intracellular cytokine analyses of splenocytes from mice immunized with the DNA-rBCG prime-boost regime showed that both CD4<sup>+</sup> and CD8<sup>+</sup>T cells showed an increase in IL-2 and IFN-γ production following stimulation with either antigens at significantly higher levels than those immunized with rBCG-DNA prime-boost.

**Conclusion:** The data obtained from this study suggested that DNA vaccination in combination with the prime-boost approach provide a potential strategy for

developing a candidate vaccine against TB.

*Supervisor:*  
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### MAGIC POLYMER GEL DOSIMETRY USING X-RAY COMPUTED TOMOGRAPHY: A FEASIBILITY STUDY

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**Introduction:** The aim of this project was to carry out feasibility study of developing Methacrylic and Ascorbic Acid in Gelatin Initiated by Copper (MAGIC) polymer gel dosimetry system by utilizing helical multislice X-ray computed tomography (CT) available in Hospital Universiti Sains Malaysia to determine dose.

**Materials and Methods:** The MAGIC gel was prepared based on the formulation proposed in the literature by Fong et al. (2001), with some modifications. The characteristics of the gel were studied for its water-equivalent properties. The mass density ( $\rho$ ) was determined based on Archimedes' principle. The weight fraction of elemental composition and the effective atomic number ( $Z_{eff}$ ) were calculated. The electron density was also measured with 90-degree scattering angle at room temperature. The linear attenuation coefficient ( $\mu$ ) of unirradiated gel, irradiated gel, and water were determined using Am-241 based on narrow beam geometry.

**Results:** The measured linear attenuation coefficient of unirradiated MAGIC gel and water was found to be 0.84 (SD 0.04)  $\text{cm}^{-1}$  and 0.85 (SD 0.02)  $\text{cm}^{-1}$  respectively. The results showed that the MAGIC gel is water-equivalent. The data obtained using irradiated gel showed a linear relationship between linear attenuation coefficient and absorbed dose in the range 2–40 Gy. The protocol for CT imaging to obtain the best quality image of irradiated MAGIC gel was determined for evaluating dose information. The irradiated gel placed inside the cylindrical water phantom was scanned using various available scan parameters (kV, mA, and reconstruction algorithm) with the field of view 25 x 25 cm and 5 mm slice thickness. The signal to noise ratio (SNR) and standard deviation (SD) were the parameters chosen to determine the image quality after image averaging process was carried out. The image that has the highest SNR and lowest SD was the best quality image, and the corresponding scan parameters were used as the protocol for scanning the irradiated gel. The scan parameters of 140 kV and 400 mA with 5 mm slice thickness, 1000 ms exposure time, standard reconstruction algorithm and 25 x 25 cm field of view were chosen as scanning protocol. Using this scanning protocol, the irradiated MAGIC gels of different doses were imaged to establish relation between average CT numbers and doses. A linear relation was found

between average CT numbers and doses in the range 2–40 Gy with CT number (HU)-dose sensitivity of 0.30 (SD 0.02) HU Gy<sup>-1</sup>. In order to verify the usefulness of the CT based gel dosimetry to measure dose, the percentage depth dose (PPD) and isodose curve (beam profile) of 8 x 8 cm field size photon beam from 6 MV linear accelerator were measured. The measured PDD and isodose curves were compared with that calculated in water using radiotherapy treatment planning computer system (TPS). The disagreement of irradiated gel PDD compare to TPS at 5 cm and 10 cm depth were found to be + 1.8% and + 2.1%, respectively. The maximum disagreement of gel PDD compare to TPS calculation in the water was + 3%. The maximum disagreement of gel isodose curves compare to TPS calculation in the water at 5 cm and 10 cm measurements were + 10% and + 11.6%, respectively.

**Conclusion:** The results show that the CT based MAGIC gel dosimetry system using HUSM CT scanner could determine the dose of high energy photon in the range 2–40 Gy.

*Supervisor:*

*Professor Dr Ahmad Bin Zakaria*

## THE ALLELE AND GENOTYPE VARIATIONS OF FIFTEEN SHORT TANDEM REPEAT (STR) LOCI IN MALAY, CHINESE, INDIAN, AND JAVANESE GROUPS OF THE MALAYSIAN POPULATION

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**Materials and Methods:** Allele and genotype frequencies for 15 short tandem repeats (STR) loci (D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX, and FGA) among 185 Malays, 216 Chinese, 195 Indians, and 109 Javanese were determined.

**Results:** For these 4 distinct groups investigated, the observed heterozygosity (HO) at these STR loci ranged from 56.0% to 82.1%. The values of the combined power of discrimination of these loci for the Malays, Chinese, Indians, and Javanese were 1 in 4.362 x 10<sup>18</sup>, 1 in 6.268 x 10<sup>18</sup>, 1 in 2.370 x 10<sup>18</sup>, and 1 in 2.543 x 10<sup>17</sup>, respectively. The values of the polymorphism information content (PIC) ranged 0.52–0.91. The probability of paternity for the Malays, Chinese, Indians, and Javanese were 0.99999932, 0.99999937, 0.99999971, and 0.99999888 respectively. The distribution patterns of the STR alleles were different in each group, especially the Indian group. The genetic data indicated the Penta E locus to be the most highly polymorphic and the TPOX locus the least polymorphic. Several loci demonstrated deviations from the Hardy–Weinberg Equilibrium (HWE) when tested with chi-square goodness-of-fit and exact test. Comparison of the population databases showed close genetic relationships

between the Malay and Javanese groups and with greater variations when compared with the Chinese and Indian groups. The overall co-ancestry coefficients,  $\theta$ , were estimated and the value ranged 0.005–0.015, which is consistent with the National Research Council II recommended value of 0.03. The pair-wise comparisons between the various groups of population based on co-ancestry identity were performed and were demonstrated by using phylogenetic tree.

**Conclusion:** The results showed that the genetic relationship between the Malays and Javanese are very close, and supported the possibility that there were genetic ties between the Malays and Chinese. The result also clearly showed that genetically the Indians were different from the Malays, Chinese, and Javanese.

*Supervisor:*

*Dr S Panneerchelvam*

## IMMUNOGENICITY STUDY OF DNA VACCINE AND DNA VACCINE CARRIER EXPRESSING VP1 OF ENTEROVIRUS 71 IN THE PRIME BOOST VACCINATION STRATEGY

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**Introduction:** Enterovirus 71 (EV71) is a highly infectious causative agent of hand, foot, and mouth disease (HFMD) in children and could lead to severe neurological complications. In Malaysia, the first epidemic occurred in 1997 in Sarawak and caused 34 deaths due to severe neurological syndrome. There is currently no vaccine available against EV71. Vaccination is considered the most effective means to control EV71 infection.

**Materials and Methods:** A candidate vaccine was developed, involving the construction a synthetic VP1 gene of EV71 fused to a ubiquitin (UbGR) gene and cloning into a DNA vaccine vector with a strong eukaryotic promoter known as pVAX1, to create the candidate DNA vaccine pVaxUbVP1. The immunogenicity of the constructed DNA vaccine was evaluated in BALC/c mice involving 2 methods of delivery, as a naked DNA vaccine delivered intramuscularly or delivered orally via the live attenuated bacteria *Salmonella typhi* Ty21a, of which the recombinant strain carrying pVaxUbVP1 was designated as StUbVP1. Both candidate vaccines were used in homologous and heterologous prime boost approaches: Formats A (pVaxUbVP1 alone), B (StUbVP1 alone), C (StUbVP1 as primer vaccine and pVaxUbVP1 as booster), and D (pVaxUbVP1 as primer vaccine and StUbVP1 as booster).

**Results:** The results indicated that total IgG levels in serum was significant in Formats A and D whereas IgG subclasses assay showed that IgG2a levels were higher than

IgG1 levels in both immunization formats. Production of in vitro IFN- $\gamma$  was significant in mice vaccinated using Formats A, B, and D, whereas IL-4 production was relatively low in all groups of immunization but shows a significant increase in Format D. The percentage of intracellular cytokine (IFN $\gamma$ , IL-2, and IL-4) production by CD4<sup>+</sup> and CD8<sup>+</sup> population of T cells showed a moderate to high response in Formats A and D. The analyses also showed that the use of pVaxUbVP1 in a homologous prime boost format (Format A) resulted in a Th1 type of immune response whereas using Format D (pVaxUbVP1 as primer vaccine and StUbVP1 as booster) gave a mixed Th1–Th2 types of immune response.

**Conclusion:** The pVaxUbVP1, used alone in a homologous prime boost approach or as the primer vaccine in a heterologous prime boost immunization format together with StUbVP1, showed potential for further development as a vaccine against EV71.

*Supervisor:*

*Professor Dr Zainul Faziruddin Zainuddin*

### CONSTRUCTION OF RECOMBINANT BCG EXPRESSING THE VP1 ANTIGEN OF ENTEROVIRUS 71 FOR THE DEVELOPMENT OF A CANDIDATE VACCINE

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**Introduction:** Enterovirus 71 (EV71), the causative agent of outbreaks of hand, foot, and mouth disease (HFMD) in children require urgent control due to the high number of cases. Vaccination is one of the most effective methods to control disease outbreaks.

**Materials and Methods:** In this study, a recombinant BCG vaccine candidate was constructed against EV71. The recombinant BCG (rBCGV1) expresses a synthetic gene encoding the VP1 protein of EV71 fused to ubiquitin complex (UbGR), which was constructed using the technique of assembly PCR. The synthetic gene was codon optimized for expression mycobacterium. The AgB5A promoter and signal peptide sequence from *M. tuberculosis* was used to drive the expression and secretion of the synthetic gene.

**Results:** The expression of the UbGR-VP1 fusion protein was confirmed by Western blotting using rabbit polyclonal antibody specific to the VPI protein and was found in the cell pellet of the recombinant BCG. rBCGV1 showed the ability to induce moderate antibody production BALB/c (H-2d) mice when sera from immunized mice were tested against purified UbGR-VP1 fusion protein. IgG2a subclass antibody was shown to be induced at a significantly higher level than IgG1. Splenocytes obtained from rBCGV1 immunized mice showed significant higher level of lymphocyte proliferation

when stimulated with UbGR-VP1 compared to control. Analyses of intracellular cytokines show that CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells from rBCGV1-immunized mice were stimulated by UbGR-VP1 protein to express significant levels of IL-2, IFN- $\gamma$ , and IL-4 when compared to the control. Extracellular cytokine analyses also showed significantly higher levels of IFN- $\gamma$  compared to control.

**Conclusion:** Overall, the immunogenicity studies results suggested that the rBCGV1 enhanced the stimulation of immune system towards the Th1 pathway. Data from this study also suggested the potential of rBCGV1 to be developed as a vaccine, and further studies must be carried out to evaluate the efficacy of this candidate vaccine.

*Supervisor:*

*Professor Dr Zainul Faziruddin Zainuddin*

### THE EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR- (PPAR- $\gamma$ 1 AND PPAR- $\gamma$ 2) IN NAIVE AND MEMORY CD4<sup>+</sup> T LYMPHOCYTES

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**Introduction:** Peripheral CD4<sup>+</sup> T cells can be divided into 2 functional groups based on the expression of distinct isoforms of the surface molecule that contains an intracellular 2-domain phosphatase portion, known as CD45. Memory T cells express the lowest molecular weight CD45RB isoform, whereas naive T cells express CD45RA (human) or CD45RB (mouse) isoforms. CD45 is a protein tyrosine phosphatase which plays an important role in TCR-mediated signaling through its activation of Lck by dephosphorylating the regulatory Tyr505. Human naive and memory CD4<sup>+</sup> T cells differ in the requirements for activation and magnitude of the cellular responses. The nuclear receptor, peroxisome proliferator-activated receptor (PPAR)- $\gamma$  has been reported to be involved in regulating the activities of immune cells such as macrophages or monocytes and T lymphocytes.

**Materials and Methods:** Given their roles in immune regulation, the current study was carried out to determine the expression of PPAR- $\gamma$  in human naive and memory CD4<sup>+</sup> T cells, since it is possible that PPAR- $\gamma$  may be differentially expressed in the different isoforms of CD45. In addition, the differential signaling patterns and cytokine secretion of these subsets of T cells may require engagement with PPAR- $\gamma$  isoforms—a possibility that has not been explored thus far. To further dissect the role of PPAR- $\gamma$  in the regulation of naive and memory CD4<sup>+</sup> T cell activation, the PPAR- $\gamma$  agonist ciglitazone, was used to modulate the activation status of naive and memory CD4<sup>+</sup> T cells, as well as the expression of PPAR- $\gamma$  and selected cytokines.

**Results:** From real-time PCR, it was observed that unstimulated naive and memory CD4<sup>+</sup> T cells did not express PPAR- $\gamma$ 1 and PPAR- $\gamma$ 2, whereas stimulated naive and memory CD4<sup>+</sup> T cells express high levels of these receptors, with PPAR- $\gamma$ 2 expression being higher than PPAR- $\gamma$ 1 in both cell types ( $P < 0.01$ ). In addition, the PPAR- $\gamma$ 1 expression was higher in stimulated memory compared with stimulated naive CD4<sup>+</sup> T cells ( $P < 0.05$ ), whereas there was no significant difference between PPAR- $\gamma$ 2 expression in both types of stimulated cells. The addition of the PPAR- $\gamma$  agonist, ciglitazone significantly increased the expression of PPAR- $\gamma$ 1 by about 61-fold and 175-fold in stimulated naive and memory CD4<sup>+</sup> T cells, respectively ( $P < 0.01$  in each). In contrast to PPAR- $\gamma$ 1, the addition of ciglitazone significantly decreased the expression of PPAR- $\gamma$ 2 by about 650-fold and 140-fold in stimulated naive and memory CD4<sup>+</sup> T cells, respectively ( $P < 0.01$  in each). In addition, the expression levels of TGF- $\beta$  and IL-1 $\beta$  gene were higher in unstimulated naive and memory CD4<sup>+</sup> T cells, but were decreased in their stimulated state ( $P < 0.01$ ). IL-8 gene was expressed at low levels in unstimulated but elevated in stimulated naive and memory CD4<sup>+</sup> T cells ( $P < 0.01$ ). However, there were no significant differences in the levels of these cytokines between naive and memory CD4<sup>+</sup> T cells of both states. IL-2, IFN- $\gamma$ , IL-5, IL-13, TNF- $\alpha$ , GM-CSF, and IL-6 were only expressed in stimulated naive and memory CD4<sup>+</sup> T cells, but not in their unstimulated state. The expression levels of IL-2 and IL-13 were significantly higher in stimulated naive compared with stimulated memory CD4<sup>+</sup> T cells ( $P < 0.01$ ). In contrast, the expression levels of IFN- $\gamma$  were significantly higher in stimulated memory as compared to stimulated naive CD4<sup>+</sup> T cells ( $P < 0.05$ ). However, there were no significant differences in the expression of IL-5, IL-6, TNF- $\alpha$ , and GM-CSF between both stimulated cell types. The addition of ciglitazone decreased the expression levels of TGF- $\beta$ , IL-1 $\beta$ , IL-8, IL-2, IFN- $\gamma$ , IL-5, TNF- $\alpha$ , and GM-CSF in stimulated memory and naive CD4<sup>+</sup> T cells. The induction of PPAR- $\gamma$ 1 and suppression of PPAR- $\gamma$ 2 expression in naive and memory CD4<sup>+</sup> T cells in the presence of ciglitazone suggested that the PPAR- $\gamma$  isoforms may have different functions in T cell regulation.

**Conclusion:** The expression of selected cytokine genes in activated naive and memory CD4<sup>+</sup> T cells is consistent with previous studies. The exact mechanism of how PPAR- $\gamma$  inhibit cytokine expression in stimulated naive and memory CD4<sup>+</sup> T cells and which PPAR- $\gamma$  isoforms is responsible for this effect remain uncertain. It is possible that PPAR- $\gamma$  inhibit the expression of cytokine genes in these stimulated cell subsets via interacting with NF- $\kappa$ B, AP-1, and STATs, which are important transcription factors for these cytokines, as shown by previous studies in other cells.

*Supervisor:*  
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## CLONING, EXPRESSION AND IMMUNOGENICITY OF RECOMBINANT BACILLE CALMETTE-GUERIN (BCG) CONTAINING T AND B CELL EPITOPES OF MYCOBACTERIUM TUBERCULOSIS

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**Introduction:** Tuberculosis (TB) remains as one of the leading causes of morbidity and mortality in humans. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis*, Bacille Calmette–Guerin (BCG). However, the efficacy of BCG continues to be debated. The BCG protection against adult pulmonary TB ranged 0%–80 % in randomized control trials. In addition, the rising rates of multi-drug resistant *M. tuberculosis* have worsened the situation. Thus, an improved TB vaccine is urgently needed. Recombinant BCG (rBCG) is one of the most potential approaches in evoking the immune response against TB.

**Materials and Methods:** In this study, 2 different types of rBCG were constructed: rBCG expressing T cell epitopes from *M. tuberculosis* Ag85B antigens and Mtb8.4 protein (rBCG018) or a combination of the antigens fused to B cell epitopes from ESAT-6, CFP10, and MTP40 proteins (rBCG032). Polyclonal anti-Mtb8.4 was successfully raised in rabbit and subsequently used for rBCG expression. Immunogenicity study of the vaccine constructs were used for immunization of Balb/c mice. Specific IgG response was obtained against the ESAT-6 and CFP10 in the sera of rBCG032-immunized mice.

**Results:** Splenocytes from these mice showed a high response against the Ag85B antigens and the Mtb8.4 protein, whereas splenocytes from rBCG018-immunized mice elicited a lower response against Ag85B epitopes and a high response against Mtb8.4 protein. Mice immunized with the rBCG strains produced a Th1 pattern of response against the T cell epitopes. Six weeks after the final immunization, the rBCG constructs were recovered from spleen, lung, liver, and peritoneal washout. The presences of both constructs in the colonies grown from the organ were detected by PCR.

**Conclusion:** The data obtained from this study demonstrated that T and B epitopes expressed in a single rBCG construct induced appropriate humoral and cellular immune responses against immunogenic epitopes from *M. tuberculosis*.

*Supervisor:*  
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## DEVELOPMENT OF RECOMBINANT MYCOBACTERIUM BOVIS BACILLE CALMETTE–GUERIN (rBCG) EXPRESSING THE 22 kDa SERINE REPEAT ANTIGEN (SE22) OF PLASMODIUM FALCIPARUM

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**Introduction:** The *Plasmodium falciparum* serine repeat antigen (SERA) is one of the promising blood stage malarial vaccine candidates.

**Materials and Methods:** In this study, we have developed a recombinant *Mycobacterium bovis* Bacille Calmette Guerin (rBCG) expressing a synthetic 22 kDa protein (SE22) from the 47 kDa N-terminal domain of SERA using assembly PCR. This synthetic gene was driven by the 65 kDa heat shock protein (hsp65) of *M. tuberculosis* and the signal peptide from MPT63 *M. tuberculosis*. Immunoblotting analysis using a monoclonal antibody against SE47 revealed that the SE22 protein was detected in the cytoplasm. The rBCG carrying SE22 protein (rBCG/SE22) was administered intraperitoneally into Balb/c mice to determine the humoral and cellular immune responses against the SE22 antigen.

**Results:** Significant SE22-specific IgG and IgG subclasses were observed after immunization with the rBCG/SE22. In addition, the lymphocytes proliferative response to SE22 antigen was significantly higher in the rBCG/SE22 vaccinated group compared to the control groups. The expression of cytokines (IL-2, IL-4, and IFN- $\gamma$ ) in CD4<sup>+</sup> and CD8<sup>+</sup> splenocytes were also detectable following stimulation with SE22. The rBCG expressing SE22 antigen induced a mixed Th1/Th2 response. The IFA results showed that rBCG could induce SE22 specific antibodies in mice and react against SE22 protein expressed on the merozoites.

**Conclusion:** These results indicate that the rBCG/SE22 could enhance and regulate both humoral and cellular immune responses, therefore it is proposed as a potential vaccine.

Supervisor:  
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## THE ANTICANCER MECHANISM OF IBUPROFEN AND INDOMETHACIN IN COLORECTAL CANCER CELLS

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**Introduction:** Ibuprofen and indomethacin are among the frequently studied non-steroidal anti-inflammatory drugs (NSAIDs) for their anticancer activities. Besides being non-selective cyclooxygenase (COX)-2 inhibitors, both NSAIDs are also direct ligands for peroxisome proliferators-activated receptor (PPAR)- $\gamma$ . However, the precise mechanism(s) of action whereby both NSAIDs exert their anticancer effect remain unclear.

**Objectives:** In this study, the effects of both NSAIDs in constitutively COX-2-expressing (HCA-7 and HT29) and non-constitutively COX-2-expressing (HCT116) cell lines were investigated with the initial aim of determining the NSAID growth inhibitory effect, as well as the effective concentration to inhibit 50% of cell growth (EC<sub>50</sub>) of each NSAID in each cell line, assessed by using lactate dehydrogenase (LDH) release assay.

**Materials and Methods:** The apoptosis mechanism was then investigated using M30 CytoDEATH assay prior to flow cytometry analysis. The apoptotic-related proteins, such as caspase-8, -9, -3, and -7, were also investigated using Western blot analysis, whereas the modulation of mRNA expression of relevant molecular targets such as COX-2, c-myc,  $\beta$ -catenin, TCF-4, and PPAR subtypes ( $\alpha$ ,  $\delta$ ,  $\gamma$ 1, and  $\gamma$ 2) mRNA was quantified using real-time PCR analysis.

**Results:** The results demonstrated that both NSAIDs produced remarkable inhibition on the growth of all 3 cell lines tested. The inhibitory effect occurred in a concentration- and time-dependent manner, with indomethacin (EC<sub>50</sub> value > 100  $\mu$ M) being more potent compared to ibuprofen (EC<sub>50</sub> value > 1000  $\mu$ M). Furthermore, the ability of both NSAIDs in inhibiting the growth of cells is likely not to be associated with COX-2 expression. The evidence from M30 CytoDEATH assay suggested that the major mode of cell death caused by both NSAIDs was caspase-dependent apoptosis. This evidence was further supported by Western blot analysis, which indicated that the induction occurred via caspase-9-dependent pathway, whereas the real-time PCR analysis showed that both NSAIDs appear to modulate gene expression via a variety of different molecular targets in COX-2-dependent and/or independent pathway(s) depending on the colorectal cancer (CRC) cell type. However, alteration of TCF-4 and PPAR- $\gamma$ 1 mRNA expression are likely essential for both NSAIDs to induce apoptosis. Thus, Wnt and PPAR- $\gamma$  signaling pathways may be involved in mediating the apoptosis induced by both NSAIDs in CRC cells. In addition, PPAR- $\delta$  was found to be another essential molecular target for indomethacin-induced CRC cell apoptosis.

**Conclusion:** This study may provide additional information and evidence of the various mechanisms and actions of NSAIDs in human CRC cells, which may be useful in selecting effective apoptotic drugs against specific CRC types. As shown in this current study, as well as others, both NSAIDs have anti-CRC activities and are potential anti-CRC agents. Further studies on the effect of both NSAIDs on CRC cells are important, as drugs may be developed as chemotherapeutic agents for human CRC.

**Supervisor:**

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## THE DEVELOPMENT AND EVALUATION OF A NASBA SYSTEM FOR THE DIAGNOSIS OF CHOLERA USING ELISA AND BIOSENSOR METHODS

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**Introduction:** Cholera is a diarrheal disease caused by *Vibrio cholerae*. Cholera is potentially lethal if not diagnosed on time. Hence, early detection is crucial for patient treatment and containment of outbreak. Conventional culture and biochemical tests are laborious, time-consuming, and lacking of sensitivity. Although molecular-based methods are rapid, more sensitive and specific, they require expensive equipments and cold storage of reagents. Furthermore, DNA-based tests, such as PCR, do not distinguish between viable and nonviable cells. Nucleic acid sequence-based amplification (NASBA) is an isothermal RNA amplification technique that specifically detects viable cells. The objective of this study was to develop and evaluate a thermostabilized cholera-NASBA-ELISA assay with biosensor detection for *V. cholerae* based on the *lolB* gene.

**Materials and Methods:** RNA transcripts as positive control were first constructed and specific primers and probes were designed. NASBA and ELISA conditions were optimized and the analytical specificity was tested with 41 reference strains comprising of *V. cholerae*, *Vibrio* spp., and enteric pathogens. The analytical sensitivity was tested with serial dilutions of RNA transcripts and *V. cholerae* cells. Clinical evaluation of the assay was performed using spiked stool samples ( $n = 200$ ). Subsequently, biosensor detection for the NASBA-ELISA assay was optimized and the results compared to spectrophotometry. The NASBA mix was thermostabilized by freeze-drying, and its stability at different temperatures was determined periodically. In addition, suitability of *lolB* mRNA as a viability indicator was investigated by subjecting cultures to lethal treatments and detecting the NASBA signal. The optimized cholera-NASBA-ELISA assay detected amplicons using fluorescein-labeled probes and TMB/HRP signal.

**Results:** The analytical specificity of the assay was 100%, while the analytical sensitivity was 102 molecules/ $\mu$ l RNA transcripts and 10 CFU/ml cells. Clinical evaluation gave 100% sensitivity, 84.52% specificity, 89.92% PPV, and 100% NPV. Biosensor detection was comparable to spectrophotometry, yielding similar analytical sensitivity level, excellent correlation ( $R_2 < 0.964$ ) and near perfect kappa agreement (95.1%,  $\kappa = 0.828$ ). Thermostabilization of the NASBA mix was able to preserve its stability at 8 °C and -20 °C

for 2 months. In the viability assay, *lolB* mRNA was detected even after 48 hours post-treatment, therefore precluding its use as a viability indicator.

**Conclusion:** We have, for the first time, developed a sensitive cholera-NASBA-ELISA assay with biosensor detection that can be performed using simple equipments within 4 hours. The dry NASBA mix reduces multiple pipetting steps and facilitates transportation and storage. The test is suitable for use as a rapid diagnostic test or screening test in the field.

**Supervisor:**

Dr P. Lalitha

## DEVELOPMENT OF RECOMBINANT MYCOBACTERIUM BOVIS BACILLE CALMETTE-GUERIN (rBCG) EXPRESSING THE 19 kDa C-TERMINUS OF MEROZOITE SURFACE PROTEIN-1 (MSP-1C) AND THE 22 kDa OF SERINE REPEAT ANTIGEN (SE22) OF PLASMODIUM FALCIPARUM AS A POTENTIAL BLOOD-STAGE MALARIAL VACCINE

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**Introduction:** Recombinant *Mycobacterium bovis* Bacille Calmette-Guerin (rBCG) expressing the 19 kDa C-terminus of merozoite surface protein-1 (MSP-1C) and a 22 kDa protein (SE22) from the 47 kDa N-terminal domain of serine repeat antigen (SERA) of *Plasmodium falciparum* is a potential blood-stage malarial vaccine candidate. In the present study, the MSP-1C and SE22 were synthetically generated in favour of mycobacterial codon usage by assembly PCR. More importantly, the synthetic MSP-1C was mutated at various sites to induce the production of inhibitory but not blocking antibodies as previously reported.

**Materials and Methods:** The MSP-1C and SE22 fragments were cloned into a shuttle plasmid to facilitate expression by BCG. The expression of the blood-stage epitopes were driven by the heat shock protein 65 (*hsp65*) promoter from *M. tuberculosis* and the signal peptide from the MPT63 *M. tuberculosis* antigen. Expression of the recombinant clones were detected by specific monoclonal antibodies using Western blotting: SE47 mAb against the SE22 and 12.10 and 1E1 mAbs against the MSP-1C.

**Results:** The SE22 successfully reacted with SE47 mAb while the MSP-1C protein reacted with the inhibitory mAb 12.10, but not the blocking of mAb 1E1. The immunization of BALB/c mice with the rBCG elicited specific humoral responses against both blood-stage epitopes with a mixed Th1/Th2 profile. Immunized sera containing high levels of specific IgG2a against both epitopes (as determined by ELISA) were reactive with fixed *P. falciparum* merozoites as demonstrated

by the indirect immunofluorescence assay (IFA). In addition, the antibody titres against the MSP-1C and SE22 epitopes appeared to be correlated with the levels of inhibition of merozoite invasion of erythrocytes in vitro. Furthermore, the lymphocyte proliferative response to MSP-1C and SE22 from rBCG-immunized mice was significantly higher than the control groups. The expression of intracellular cytokines (IL-2, IL-4, and IFN- $\gamma$ ) in CD4<sup>+</sup> and CD8<sup>+</sup> cells were also detectable following in vitro stimulation with both epitopes. Preliminary and long-term in vivo stability analyses showed that the rBCG were stable in spite of being a non-integrative plasmid.

**Conclusion:** This study demonstrated that a single construct expressing a combination of 2 blood-stage epitopes of *P. falciparum* induced appropriate humoral and cellular responses against the parasites, paving the way for the construction of a potential blood-stage malarial vaccine.

*Supervisor:*  
Professor Dr Norazmi Mohd. Nor

**Conclusion:** These results suggested that VCUSM1 and VCUSM2 are the least toxic, highly immunogenic, promising vaccine candidates against *V. cholerae* O139 Bengal.

*Supervisor:*  
Professor Dr Zainul Faziruddin Zainuddin

## DEVELOPMENT AND EVALUATION OF hema MUTANTS OF *VIBRIO CHOLERA* O139 BENGAL AS A POTENTIAL VACCINE

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**Objectives:** The study aimed to describe the development of live attenuated oral vaccine strains VCUSM1 and VCUSM2 against *Vibrio cholerae* O139 Bengal. These strains are metabolic auxotrophs and require exogenous aminolevulinic acid (ALA) for their survival.

**Materials and Methods:** The auxotrophy was achieved by mutating a house-keeping gene hema that encodes for glutamyl tRNA reductase, an important enzyme of the C5 pathway of ALA biosynthesis. Experiments carried out in infant mice and adult rabbits have shown that these vaccine strains are good colonizers of the small intestine. Subsequent experiments have revealed that these strains shed for a maximum of 4 days in the stool and elicit greater than a 4-fold rise in vibriocidal antibodies in the vaccinated rabbits. Rabbits vaccinated with VCUSM1 and VCUSM2 were fully protected against subsequent challenges with virulent wild type.

**Results:** Dose optimization studies have shown that as little as  $1 \times 10^6$  CFU of VCUSM1 and VCUSM2, given orally 2 weeks apart, yielded 100% protection against subsequent challenge. Experiments carried out in ligated ileal loops of rabbits have shown that these strains are not absolutely non-reactogenic; however, they are 2.5-fold less toxic at a dose of  $1 \times 10^6$  CFU. VCUSM1 and VCUSM2 survived no longer than 6 days in environmental waters as compared with the wild type, which was still detectable on day 20 post-inoculation.