



**UNIVERSITI PUTRA MALAYSIA**

***IMMUNOLOGICAL RESPONSES TOWARD CASEOUS LYMPHADENITIS  
RECOMBINANT VACCINE INCORPORATED WITH DIFFERENT  
ADJUVANTS***

**ROSLINDAWANI BINTI MD NOR**

**FPV 2018 29**



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**By**

**ROSLINDAWANI BINTI MD NOR**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra  
Malaysia, in Fulfilment of the Requirements for the Degree of Master of  
Science**

**January 2018**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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**January 2018**

**Chairman : Professor Mohd. Zamri Saad, DVM, PhD**  
**Faculty : Veterinary Medicine**

Caseous lymphadenitis (CLA) is a chronic disease of sheep and goats worldwide. It is caused by a bacterium known as *Corynebacterium pseudotuberculosis* leading to considerable economic losses. CLA was rarely reported in Malaysia until recently, following the importation of goats. Control of CLA via vaccination has not been recommended so far. This was due to the uncertain efficacy of the only commercially available vaccine against CLA, the Glanvac6™ vaccine. In fact, earlier study has revealed that the Glanvac6™ vaccine failed to provide significant protection to goats following challenged by live wild-strain of *C. pseudotuberculosis*. Recombinant vaccines encoding the 31kDa and 40kDa outer membrane proteins were subsequently developed but proved to be ineffective in protecting goats.

This study was conducted to determine the effect of different concentrations of adjuvants; the palm-based oil and the lipopolysaccharide (LPS) of *Pasteurella multocida* B:2 on the ability of a newly developed recombinant CLA vaccine (pET32/LIC-Omp40) to stimulate immune response, hence determined the best concentration of the adjuvant for CLA vaccine preparation. Three different concentrations of each adjuvant were used; the 3%, 5% and 7% of palm-based oil or LPS and were vaccinated into rat model at weeks 0 and 2. The immunoglobulin and cell-mediated immune responses were measured weekly for a period of 10 weeks. The results revealed that rats vaccinated with 3% palm oil adjuvanted recombinant vaccine showed significantly ( $p < 0.05$ ) highest antibody levels. Other groups (the 5% and 7% palm oil and the 3%, 5% and 7% LPS) showed no significant ( $p > 0.05$ ) different in the IgG levels compared with the vaccine without adjuvant. Similarly, the 3% palm oil produced significantly

( $p < 0.05$ ) highest percentage of CD4+ and CD8+ cells compared to other groups. Hence, it is concluded that the best concentration and adjuvant for the CLA recombinant vaccine is the 3% of palm oil.

Then, the efficacy of the newly developed recombinant CLA vaccine containing 3% palm oil adjuvant was compared with the vaccine containing 10% Freund's incomplete adjuvant (FIA) as adjuvant in goats. Nine adult female goats with no history of vaccination against CLA were selected and divided into 3 equal groups consisting of 3 goats per group. Goats of group 1 were vaccinated intramuscularly with the recombinant vaccine containing 10% FIA, Group 2 with the same vaccine containing 3% palm oil as adjuvant and Group 3 with PBS. Booster dose of the respective vaccine was administered two weeks after the first vaccination. All goats were challenged subcutaneously with 1ml of  $10^9$  cfu/ml of live *C. pseudotuberculosis* at week 4. Serum and whole blood sample were collected from all goats before vaccination and at weekly intervals post-vaccination throughout the 12-week study period to determine the immunoglobulin status via enzyme-linked immunosorbent assay (ELISA) and the cell-mediated immunity status via immunofluorescent assay. At the end of the study, all goats were killed by exsanguinations before organ and lymph node samples were collected for bacterial isolation and lesion scoring.

The results revealed that both palm oil adjuvanted and the Freund's adjuvanted recombinant CLA vaccines produced significantly high ( $p < 0.05$ ) antibody levels compared to the control throughout the 12-week study period. Following challenged with live *C. pseudotuberculosis*, the antibody levels of Group 1 increased insignificantly ( $p > 0.05$ ) but Group 2 increased significantly ( $p < 0.05$ ) when compared to the control group. Similarly, Groups 1 and 2 showed significantly ( $p < 0.05$ ) higher percentage of CD4+ compared to the control until week 3 but started to decrease from week 4 until the end of the study period. In contrast, the percentage of CD8+ remained significantly ( $p < 0.05$ ) high throughout the entire study period. Following challenged, *C. pseudotuberculosis* was successfully isolated from all groups of goats. In fact, goats of Groups 1 and 2 showed most severe lesions. This was due to the fact that the adjuvanted vaccines stimulated significant ( $p < 0.05$ ) humoral and cell-mediated immune responses but lasted for a short period of time. It requires consistent booster to maintain the immune response.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia  
sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

## TINDAKBALAS IMUNOLOGI KE ATAS VAKSIN REKOMBINAN BISUL NODUS LIMFA DENGAN ADJUVAN YANG BERLAINAN

Oleh

**ROSLINDAWANI BINTI MD NOR**

**Januari 2018**

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Bisul nodus limfa (CLA) merupakan sejenis penyakit kronik yang sering menyerang biri-biri dan kambing di seluruh dunia. Penyakit ini berpunca daripada sejenis bakteria yang dikenali sebagai *Corynebacterium pseudotuberculosis* sehingga mengakibatkan kerugian besar kepada ekonomi. Kes CLA di Malaysia jarang dilaporkan, tetapi semakin meningkat kebelakangan ini berikutan aktiviti pengimportan kambing. Pengawalan CLA menggunakan vaksin belum pernah diusulkan setakat ini. Ini adalah kerana vaksin *Glanvac6<sup>TM</sup>*, satu-satunya vaksin sedia ada dipasaran untuk melawan CLA masih belum diyakini keberkesanannya. Tambahan pula, kajian sebelum ini telah merumuskan bahawa vaksin *Glanvac6<sup>TM</sup>* gagal untuk memberikan perlindungan menyeluruh terhadap kambing. Walaupun vaksin rekombinan protein selaput luar 31kDA dan 40kDA telah dihasilkan, namun terbukti masih belum berkesan untuk melindungi kambing kerana ketidakupayaannya menjana antibodi yang mencukup dan tindak balas sel perantara.

Kajian ini dijalankan untuk mengenal pasti kesan perbezaan kepekatan adjuvan, iaitu minyak sawit dan lipopolisakarida (LPS) dari *P. multocida* B:2 ke atas keupayaan vaksin rekombinan CLA (pET32/LIC-Omp40) untuk merangsang imuniti, sekaligus mengenal-pasti kepekatan terbaik bagi adjuvan dalam proses penyediaan vaksin CLA. Tiga kepekatan adjuvan yang berbeza telah digunakan, iaitu 3%, 5% dan 7% minyak sawit atau LPS dan diuji ke atas tikus ujikaji. Tahap gerakbalas imunoglobulin dan sel perantara direkodkan dalam tempoh 10 minggu. Keputusan mendapati bahawa vaksin dengan 3% minyak sawit menunjukkan tahap antibodi yang tertinggi ( $p < 0.05$ ). Sebaliknya, kumpulan lain (5% dan 7% minyak sawit dan 3%, 5% dan 7% LPS) tidak menunjukkan perbezaan yang nyata ( $p > 0.05$ ) berbanding vaksin tanpa

adjuvan. Begitu juga, 3% minyak sawit telah merangsang peratusan sel CD4+ dan CD8+ tertinggi ( $p < 0.05$ ) berbanding kumpulan lain. Oleh itu, adalah disimpulkan bahawa kepekatan adjuvan terbaik untuk vaksin rekombinan CLA ialah 3% minyak sawit.

Seterusnya, vaksin rekombinan CLA baharu dengan adjuvan 3% minyak sawit telah dibandingkan dengan 10% Freund's incomplete adjuvant (FIA) ke atas kambing. Sembilan ekor kambing betina yang tidak pernah diberi vaksin CLA dipilih dan dibahagikan kepada 3 kumpulan yang sama rata. Kumpulan pertama disuntik secara intraotot dengan vaksin rekombinan yang mengandungi 10% adjuvan daripada FIA. Kumpulan kedua menerima suntikan yang sama dengan adjuvan 3% minyak sawit dan kumpulan tiga menerima suntikan PBS sebagai kumpulan kawalan. Dos tambahan bagi setiap vaksin diberikan dua minggu selepas suntikan pertama. Pada minggu keempat, kesemua kambing dicabar dengan 1ml inokulum mengandungi  $10^9$  cfu/ml *C. pseudotuberculosis* hidup. Serum dan sampel darah kambing diambil sebelum vaksinasi dan setiap minggu sepanjang tempoh 12 minggu kajian. Hal ini bertujuan untuk mengenal pasti status imunoglobulin melalui ujian imunoterapi terangkai enzim (ELISA) dan juga status ketahanan sel perantara melalui *immunofluorescent assay*. Di akhir kajian ini, kesemua kambing disembelih dan sampel organ dan nodus limpa diambil untuk pengasingan bakteria dan penilaian lesi.

Hasil kajian menunjukkan bahawa vaksin CLA rekombinan dengan adjuvan minyak sawit dan adjuvan Freund menghasilkan tahap antibodi lebih tinggi ( $p < 0.05$ ) berbanding kumpulan kawalan sepanjang tempoh 12 minggu kajian dijalankan. Selepas dicabar dengan *C. pseudotuberculosis*, tahap antibodi kumpulan 1 meningkat tetapi tidak signifikan ( $p > 0.05$ ) sementara kumpulan 2 meningkat signifikan ( $p < 0.05$ ) berbanding kumpulan kawalan. Begitu juga kumpulan 1 dan 2 menunjukkan nilai CD4+ yang lebih tinggi ( $p < 0.05$ ) berbanding kumpulan kawalan sehingga minggu ketiga sebelum mula menurun selepas minggu keempat sehingga kajian selesai dijalankan. Sebaliknya, peratusan CD8+ kumpulan 1 dan 2 kekal tinggi ( $p < 0.05$ ) sepanjang tempoh kajian. Meskipun begitu, *C. pseudotuberculosis* telah berjaya diasingkan daripada semua kumpulan kambing. Malah, kumpulan 1 dan 2 menunjukkan kesan serangan paling serius. Kedua-dua vaksin beradjuvan telah merangsang gerakbalas humoral dan sel perantara yang signifikan ( $p < 0.05$ ) tetapi hanya dalam jangka masa yang singkat. Tindak balas imun ini perlu dikekalkan dengan vaksin tambahan yang konsisten.

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I certify that a Thesis Examination Committee has met on 4 January 2018 to conduct the final examination of Roslindawani Binti Md Nor on her thesis entitled "Immunological Responses to A Recombinant Vaccine Incorporated with Different Adjuvants" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Degree of Master of Science.

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## LIST OF ABBREVIATIONS

%	Percentage
α	Alpha
β	Beta
γ	Gamma
δ	Delta
°C	Degree celcius
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
AGID	Agar gel immunodiffusion test
APC	Antigen Presenting Cells
API	Analytical profile index
BHI	Brain-heart infusion
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
Cfu	Colony forming unit
CMI	Cell mediated immunity
DNA	Deoxyribonucleic acid
Dntp	Deoxynucleotide triphosphate
DVS	Department of Veterinary Service
EDTA	Ethylene-diamine-tetraacetic acid (disodium salt)
ELISA	Enzyme linked immunosorbent assay
Fc	Fragment crystallizable
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocyanate
g	Gram
h	Hour
IFN	Interferon
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
kb	Kilobase pair
kDa	KiloDalton
kg	Kilogram
LB	Luria-Bertani
L	Liter
M	Molar
MDP	Muramyl dipeptide

MgCl <sub>2</sub>	Magnesium chloride
MHC	Major histocompatibility complex
min	Minutes
ml	Milliliter
mm	Milimeter
MW	Molecular weight
NaOH	Sodium hydrogen peroxide
OIE	World Animal Health Organization
PBS	Phosphate buffer saline
PBS-T	Tween 20 in phosphate buffer saline
PCR	Polymerase chain reaction
pH	Puissance hydrogen (hydrogen ion concentration)
PLD	Phospholipase D
rDNA	Ribosomal deoxyribonucleic acid
Rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
s	Seconds
<i>Taq</i>	<i>Thermus aquaticus</i> YT-1
TBE	Tris-boric EDTA
Tc	T cytotoxic cell
Th	T helper cell
Th0	naive T helper cell
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
U	Unit
UV	Ultra-violet
V	Voltan/volt
v/v	Volume per volume
w/v	Weight per volume

## CHAPTER 1

### INTRODUCTION

Caseous lymphadenitis (CLA) is a chronic contagious disease of sheep and goats worldwide (Abdullah *et al.*, 2013) leading to losses due to disruption in trade of animals and animal products (Williamson, 2001). Furthermore, there are condemnations of skin and carcasses due to abscesses besides losses in reproductive efficiency and in wool, meat and milk production (Guimaraes *et al.*, 2011). In fact, CLA became a significant political issue in 1920s when mutton carcasses that were imported into Great Britain from a number of countries were found to be affected by the disease (Baird and Fontaine, 2007). Dissemination of this disease throughout the world occurred through importation of infected animals (Baird and Fontaine, 2007).

Caseous lymphadenitis was rarely reported in Malaysia until recently, following the importation of goats. Despite the sero-positivity of CLA in ruminant farms in Malaysia, cases with clinical signs of CLA were rarely encountered (Abdullah *et al.*, 2013). Nevertheless, the true prevalence of this disease in Malaysia has not been estimated, partly due to the lack of adequate and available diagnostic tests for live sheep and goats. Nevertheless, control of CLA in Malaysia is by culling of the infected animals (Komala *et al.*, 2008). This control policy has been suggested by Renshaw *et al.* (1979) since affected animals serve as reservoir of the infection. However, the culling policy is not popular among small holder farmers due to the high value of the animals. With the current perceived increasing incidence of CLA in Malaysia, the Department of Veterinary Services Malaysia (DVS) had decided to reduce the incidence by restricting the movements of goats. This is because CLA is usually introduced into a herd through the purchase of new animals (Kuria *et al.*, 2001; Adza Rina *et al.*, 2013) and spread within a herd is mainly by direct contact between animals.

Control of CLA via vaccination has not been recommended so far. This was due to uncertain efficacy of the only commercially available vaccine against CLA, the Glanvac6™ vaccine. In fact, earlier study has revealed that the Glanvac6™ vaccine failed to provide significant protection to goats following challenge by live wild-strain of *Corynebacterium pseudotuberculosis* (Adza Rina *et al.*, 2013). This was believed to be due to difference of *C. pseudotuberculosis* strains. Therefore, research activities toward producing vaccine against CLA using local isolates are extremely necessary, and the first step toward achieving this is the isolation, identification and confirmation of different strains of *C. pseudotuberculosis* and ultimately identification of suitable protein or isolate for vaccine production. Following this, recombinant vaccines encoding the 31kDa and 40kDa outer membrane proteins were developed but proved to be ineffective in protecting goats against challenge

due to inability to stimulate adequate antibody and cell-mediated responses (Syafiqah Adilah, 2017).

Recombinant proteins or synthetic peptides are generally safer than crude inactivated micro-organism but are less immunogenic (Aucouturier *et al.*, 2001). Therefore, co-administration of an adjuvant with recombinant protein is important to stimulate high immune response as recombinant proteins are generally poor immunogens when administered alone (Aucouturier *et al.*, 2001). However, there is no universal adjuvant and their action is not yet clearly studied against the newly developed recombinant CLA vaccine. For this reason, the potential of palm based oil and lipopolysaccharide (LPS) from *Pasteurella multocida* B:2 as adjuvant was evaluated in this study. Hence, the first strategy was to identify the best concentration of adjuvant to be used leading to high immune responses. This was followed by evaluating the performance of the newly developed recombinant CLA vaccine. Therefore, the objectives of the study were:

1. To determine the effect of adjuvant (palm-based oil or Freund's adjuvants) on the efficacy of the newly developed recombinant CLA vaccine.
  - 1.1 To determine the best concentration of adjuvant for the newly developed recombinant CLA vaccine in rat model.
  - 1.2 To determine the effect of newly adjuvanted recombinant CLA vaccine in goats.

The hypotheses of this study were:

1. Addition of palm oil as adjuvant at 3% concentration enhanced the immune responses, humoral immunity and also cell-mediated immunity in rat model.
2. Addition of oil palm and Freund's adjuvant for recombinant CLA vaccine enhance the humoral immunity but not cell-mediated immunity in host model (goat).

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