



**UNIVERSITI PUTRA MALAYSIA**

***EFFICACY OF A PROTOTYPE VACCINE FOR CASEOUS  
LYMPHADENITIS DISEASE IN GOATS***

**WESSAM MONTHER MOHAMMED SALEH**

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LYMPHADENITIS DISEASE IN GOATS**

**By**

**WESSAM MONTER MOHAMMED SALEH**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra  
Malaysia in Fulfillment of the Requirements of the Degree of Doctor of  
Philosophy**

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## DEDICATION

To my parents: for them fearless spirits; thank you for unconditional support with my life, I am honored to have you as my parents, thank you for giving me a chance to prove and improve myself through all my walk of life. I love you.

To my beloved wife, wonderful children: thank you for believing in me, for allowing me; for allowing me to further my studies. Please do not ever doubt my dedication and love for you.

To my brothers and sisters: Hoping that with this research I have proven to you that there is no mountain higher as long as God is on our side. Hoping that, you will walk again and be able to fulfill your dreams.

To my teachers, instructors and mentors: my sincere thanks to make it reality, and utmost respect to all my teachers past, present and future, and to all teachers everywhere.

To my friends: I also dedicate this dissertation and give special thanks to my many friends who have supported me throughout this process.

I would like to conclude by again expressing my deepest gratitude and love to all.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

## **EFFICACY OF A PROTOTYPE VACCINE FOR CASEOUS LYMPHADENITIS DISEASE IN GOATS**

By

**WESSAM MONTHER MOHAMMED SALEH**

**November 2016**

**Chairman : Associate Professor Faez Firdaus Jesse Abdullah, PhD**  
**Faculty : Veterinary Medicine**

*Corynebacterium pseudotuberculosis* is bacterium responsible for caseous lymphadenitis (CLA), a disease characterized by the formation of suppurative abscesses, particularly in superficial and internal lymph nodes within almost all internal organs of sheep, goats and other species including human. Due to the chronic and subclinical nature of CLA, its control is difficult and hence records a high worldwide prevalence with significant economic impact. In Malaysia, CLA is a potential threat to the livestock industry, details of the immune responses against *C. pseudotuberculosis* infection are not completely understood and effective vaccine is still lacking.

This study was conducted using a local Malaysian *C. pseudotuberculosis* isolate, a murine and caprine experimental model testing the capacity of two concentrations of a prototype vaccine to protect mice and goats against experimental challenge with *C. pseudotuberculosis* were determined. Forty healthy female mice, aged between 3-4 weeks and weight between 15.5-20g were randomly divided in to four equal groups consisting of 10 mice each, designated groups A, B, C and D. Mice in groups A and B were inoculated intraperitoneally with 200µl of the prepared 0.5%, 1% formalin-killed vaccine of local *C. pseudotuberculosis* strain respectively. Groups C and D were kept as positive and negative control respectively, and inoculated intraperitoneally only with 200µl of PBS pH 7.4. Booster doses were inoculated to the mice in groups A and B after 21 days of the initial doses. Week 6<sup>th</sup> of post inoculation of the first vaccination dose; mice in all groups except negative control group were inoculated intraperitoneally with 200µl of virulent type of *C. pseudotuberculosis* 10<sup>6</sup> CFU per mice and were observed. All mice were euthanized at day 10 post challenged, and post mortem examination were carried on the liver, lungs, heart, kidneys, spleen and lymph nodes, while tissue samples of same organs were fixed for histopathological examination and further cultured on blood agar for bacterial isolation and identification.

For goat model, 24 adult female goats aged between 11-13 months, weighing 23-30 kg, with no history of vaccination against CLA were randomly divided into 4 groups consisting of 6 goats each as A, B, C and D. Goats in groups A and B were vaccinated, respectively, with 1 ml intramuscular injection of the 0.5% and 1% formalin-killed *C. pseudotuberculosis* vaccine. Groups C and D were kept, respectively, as positive and negative control, and inoculated intramuscularly only with 1 ml of oil-in-water emulsion adjuvant. Booster dose was inoculated after 21 of the initial treatment. 2 weeks of post-booster dose, the goats (except in group D) were challenged with 2ml of  $10^6$  CFU of wild isolate of *C. pseudotuberculosis* subcutaneously. They were monitored for the entire experiment for clinical signs, and blood samples were collected at predetermined intervals into plain and EDTA tubes for IgM, IgG, IgA, Haptoglobin, Serum Amyloid A and hematology analysis. At the end of the study, the goats were sacrificed and post mortem were carried out and samples such as external and internal lymph nodes, liver, lungs, heart, kidneys, spleen, intestine, spinal cord and brain were collected for histopathological examination and for bacterial isolation and identification.

Mice in group C which were inoculated only with *C. pseudotuberculosis* showed significant ( $P < 0.05$ ) changes of clinical signs. Moderate to severe ruffled fur, reduced rate of movement, decreased responsiveness and ocular discharge were observed. Signs such as huddling together, dejection, loss of appetite, pasty feces and respiratory distress were also recorded. In post mortem examination, congestion and abscesses in the site of inoculation, spleen, liver and kidneys were recorded in group C only. Microscopic examination of infected group showed significant ( $P < 0.05$ ) moderate to severe hemorrhage and congestion in all organs, mild to moderate inflammatory cell infiltration and degeneration, multiple abscesses with higher scoring of necrosis and less to mild edema were observed in all organs. In contrast, the results of the vaccinated mice with CLA vaccine showed normal tissues with non-significant histopathological changes. *Corynebacterium pseudotuberculosis* was detected by PCR from most visceral organs and lymph nodes of 9 mice (90%) in control positive group; one mouse was found clear of infection, while it was detected only from 30% and 20% of mice in groups A and B respectively.

For the goat experiment, positive control animals (group C) showed significant clinical symptoms compared to vaccinated groups that observed one week post-bacterial inoculation such as significant raise in body temperature, respiratory and heart rates and sharply decreased in rumen motility. Poor hair coat condition, decrease in body condition scoring and significant enlargement and ruptured of injection sites after 2 to 3 weeks of challenges were also observed. Moreover, a marked enlargement and occasional rupture of the affected lymph nodes were recorded in positive control group 5 to 6 weeks post challenged with *C. pseudotuberculosis*. Slight enlargement of regional lymph nodes near the site bacterial challenged of only 1 goat (16%) in each group A and B was observed.

Both groups exposed to our formulated vaccines (groups A and B) induced significant (for both vaccines) elevations in serum Haptoglobin (Hp) and plasma serum amyloid A (SAA) concentrations which increased after 2, 5, 6 and 7 weeks when compared to non-vaccinated healthy animals of the same conditions. Hp and SAA levels in goats, which had never been exposed to vaccine, were elevated significantly one week after exposure to wild bacteria and maintained sharp increase for the interval of the evaluation. The concentration of serum IgM was found to be significantly increased at week 3 of the exposure to the first dose of vaccination, where 7 and 9-fold increases were observed in groups A and B respectively. Sharp increase up to 18 and 17-fold were observed one week after challenged with viable bacteria in groups A and B respectively.

The levels for the IgM response in group C were significantly higher than the uninfected control goats (group D). The highest concentrations of IgG in group A and B ( $156.63 \pm 2.17$  ng/mL) and ( $164.03 \pm 2.92$  ng/mL) were observed after two weeks post-exposure to whole viable bacteria, respectively. For Group C, IgG mean concentration reached the maximum level ( $138.58 \pm 1.34$  ng/mL) one month after the exposure to whole viable bacteria. However the mean concentration of IgG in the vaccinated groups continued to rise following the primary and secondary vaccination. The goats vaccinated by both the formulated vaccines had no significant elevation of IgA concentration throughout the study period. In contrast, a marked increase of serum IgA levels was observed in group C during the period between 7 to 14 days post infection with viable *C. pseudotuberculosis*. However, only 1.2 and 1.3-fold increases were recorded in group C respectively at weeks 6 and 7.

Post mortem examination of vaccinated groups revealed no significant gross pathological changes in visceral organs and also in the internal and external lymph nodes. On the other hand, high rate of gross pathological changes were recorded in all goats of positive control group. No significant gross pathological changes were observed in the brain and spinal cord of goats in group C. In the visceral organs such as lungs, liver, kidneys, spleen and heart the main gross pathological lesions observed were congestion, hemorrhage, focal areas of necrosis, fatty atrophy and abscessation. Abscess formation was rarely observed in group C except in the few organs such as the liver and lungs. In the case of lymph nodes, high percentages of abscess formation of different sizes were observed in all goats in group C. In contrast, both vaccinated groups did not develop any abscess in the lymph nodes or the visceral organs even though slight enlargements of the lymph nodes were recorded in two goats (16%) only. The histopathological changes which were observed in the positive group include edema, congestion, infiltration of inflammatory cells mainly lymphocytes and macrophages, hemorrhages, degeneration, granulomatous inflammation and caseous necrosis unlike vaccinated goats which showed significant functional hyperplasia in lymphoid tissues without histopathological changes.



In the hematology, there was a statistical significant decline ( $P < 0.05$ ) in Hb concentration, RBC count, MCV value and PCV in group C after exposure to viable bacteria cells. Parallel to that, statistically non-significant elevations ( $P > 0.05$ ) have been observed MCHC in the same group. The WBC counts were the highest during the experimental infection of non-vaccinated goats (group C) compared with those pre-vaccinated groups (A and B). The highest stimulation in segmented neutrophil was reached 7 days post-challenge to live bacteria, when the numbers were ( $12.55 \pm 1.05 \times 10^9/L$ ) in positive control group, ( $10.93 \pm 1.55 \times 10^9/L$ ) and ( $10.14 \pm 1.38 \times 10^9/L$ ) in groups A and B respectively, when compared to ( $8.23 \pm 0.46 \times 10^9$ ) the untreated group (group D). A significant increase ( $p < 0.05$ ) of bands neutrophils count ( $0.83 \pm 0.17 \times 10^9/L$ ) was detected in positive control group after inoculation of *C. pseudotuberculosis*, whereas no significant change was recorded in groups A ( $0.49 \pm 0.09 \times 10^9/L$ ), B ( $0.51 \pm 0.13 \times 10^9/L$ ) and D ( $0.30 \pm 0.01 \times 10^9/L$ ) in the same period. The monocytes, eosinophil and basophiles counts and plasma proteins levels in all blood samples were within the normal range. Nevertheless, no significant change was recorded on the CBC values of both vaccinated groups.

The findings of the PCR showed that five goats (84%) of the six animals in groups A and B pre-exposed to 0.5 % and 1.0% formalin-killed *C. pseudotuberculosis* remained negative. Significantly lower spreading of *C. pseudotuberculosis* DNA was detected in the organs and lymph nodes of the vaccinated groups. The percentage of detection in investigated tissues was found to be 100% in group C.

In conclusion, following experimental challenge with wild type *C. pseudotuberculosis* local isolate, the formalin-killed vaccines were observed to confer statistically significant protection against infection and appeared to significantly restrict the dissemination of challenged bacteria beyond the inoculation site in the 84% of goats. Vaccinated mice (groups A, B) developed humoral immunity and produced 70% and 80% protection respectively against the disease. Moreover, only 16% of the goats immunized with this vaccine manifested mild clinical and pathological infection thus, a potentially important route of disease transmission was eliminated. The results of this study provide information pertinent to the development of an effective caseous lymphadenitis eradication vaccination strategy in Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**KEBERKESANAN VAKSIN PROTOTAIP UNTUK PENYALUT NODUS LIMFA PENYAKIT PADA KAMBING**

Oleh

**WEWSSAM MONTHER MOHAMMED SALEH**

**November 2016**

**Pengerusi : Profesor Madya Faez Firdaus Jesse Bin Abdullah, PhD**  
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*Corynebacterium pseudotuberculosis*, bakteria yang bertanggungjawab untuk penyalut nodus limfa (CLA), penyakit yang dicirikan oleh pembentukan abses bernanah, terutamanya dalam nodus limfa luar dan dalaman di dalam hampir semua organ dalaman kambing biri-biri, kambing dan spesies lain termasuk manusia. Oleh kerana sifat kronik dan subklinikal CLA, kawalan adalah sukar dan dengan itu merekodkan kelaziman di seluruh dunia yang tinggi dengan kesan ekonomi yang penting. Di Malaysia, CLA adalah satu ancaman yang berpotensi untuk industri ternakan, terperinci tindak balas imun terhadap jangkitan *C. pseudotuberculosis* tidak difahami sepenuhnya dan vaksin yang berkesan masih kurang.

Kajian ini dijalankan dengan menggunakan *C. pseudotuberculosis* isolate Malaysia yang getir, murine dan berhubung dgn kambing, model eksperimen menguji keupayaan dua vaksin prototaip untuk melindungi tikus dan kambing terhadap cabaran eksperimen dengan *C. pseudotuberculosis* ditentukan. Empat puluh tikus betina yang sihat, berumur antara 3-4 minggu dan berat badan antara 15.5-20g telah dibahagikan secara rawak kepada empat kumpulan yang sama yang terdiri daripada 10 tikus setiap satu, kumpulan ditetapkan A, B, C dan D. Tikus dalam kumpulan A dan B telah disuntik intraperitoneum dengan 200µl daripada bersedia 0.5%, 1% formalin dibunuh vaksin ketegangan *C. pseudotuberculosis* tempatan masing-masing. Kumpulan C dan D merupakan kumpulan kawalan positif dan negatif masing-masing, dan disuntik intraperitoneum hanya dengan 200µl PBS pH 7.4. Dos booster telah disuntik kepada tikus dalam kumpulan A dan B selepas 21 hari dari dos awal. Minggu ke-6 selepas inokulasi dos vaksin pertama; tikus dalam semua kumpulan kecuali kumpulan kawalan negatif telah disuntik intraperitoneally dengan 200µl jenis getir *C. pseudotuberculosis* 10<sup>6</sup> CFU setiap tikus dan diperhatikan. Semua tikus telah dieutanasia pada akhir kajian, dan pemeriksaan bedah siasat telah dijalankan ke atas hati, paru-paru, jantung, buah pinggang, limpa dan limfa nodus, manakala sampel tisu organ

yang sama telah ditetapkan untuk pemeriksaan histopatologi dan seterusnya diletakkan pada agar darah untuk pengasingan bakteria dan pengenalan.

Untuk model kambing, 24 ekor kambing betina baka yang berusia antara 11-13 bulan, berat 23-30 kg, yang tidak mempunyai sejarah pelalian CLA secara rawak dibahagikan kepada 4 kumpulan yang terdiri 6 ekor kambing setiap kumpulan terdiri daripada A, B, C dan D. Kambing dalam kumpulan A dan B telah disuntik masing-masing dengan 1 ml suntikan intramuskular vaksin *C. pseudotuberculosis* 0.5% dan 1% formalin-membunuh. Kumpulan C dan D disimpan masing-masing sebagai kawalan positif dan negatif, dan disuntik secara intraotot hanya dengan 1 ml minyak dalam air emulsi. Booster dos yang disuntik selepas 21 rawatan awal. 2 minggu selepas booster dos, kambing (kecuali dalam kumpulan D) telah dicabar dengan 2ml  $10^6$  CFU mengasingkan liar *C. pseudotuberculosis* suntikan secara subcutaneously. Kesemua kambing telah dipantau untuk keseluruhan eksperimen tanda-tanda klinikal, dan sampel darah telah diambil pada jangka masa yang telah ditetapkan ke dalam tiub kosong dan EDTA untuk analisis IgM, IgG, IgA, Haptoglobin, Serum Amyloid A dan analisis hematologi. Pada akhir kajian, kambing dikorbankan dan bedah siasat telah dijalankan dan sampel seperti nodus limfa dalaman dan luaran, hati, paru-paru, jantung, buah pinggang, limpa, usus, saraf tunjang dan otak telah dikumpulkan untuk pemeriksaan histopatologi dan untuk pengasingan bakteria dan pengenalan.

Tikus dalam kumpulan C yang telah disuntik hanya dengan *C. pseudotuberculosis* menunjukkan signifikan perubahan tanda-tanda klinikal. Sederhana kepada bulu bergolak teruk, kadar pergerakan dikurangkan, mengurangkan responsif dan pelepasan okular diperhatikan. Tanda-tanda seperti "huddling" bersama-sama, hilang selera makan, najis pucat dan masalah pernafasan juga telah direkodkan. Dalam pemeriksaan bedah siasat, kesesakan dan abses di tapak inokulasi, limpa, hati dan buah pinggang telah direkodkan dalam kumpulan C sahaja. Pemeriksaan mikroskopik kumpulan dijangkiti menunjukkan signifikan sederhana kepada pendarahan yang teruk dan kesesakan di semua organ, ringan kepada sederhana penyusutan sel radang dan degenerasi, pelbagai abses dengan pemarkahan yang lebih tinggi nekrosis dan kurang kepada edema ringan diperhatikan dalam semua organ-organ. Sebaliknya, keputusan tikus disuntik dengan vaksin CLA menunjukkan tisu normal dengan perubahan histopatologi bukan ketara. *Corynebacterium pseudotuberculosis* dikesan oleh PCR daripada organ-organ dan nodus limfa 9 ekor tikus (90%) dalam kumpulan positif kawalan yang paling mendalam, satu tikus ditemui jelas jangkitan, semasa ia dikesan hanya dari 30% dan 20% daripada tikus dalam kumpulan A dan B masing-masing.

Untuk percubaan kambing, haiwan kawalan positif (Kumpulan C) menunjukkan signifikan gejala klinikal berbanding kumpulan vaksin yang diperhatikan inokulasi satu minggu selepas bakteria seperti kenaikan besar nodus limfa; suhu badan, pernafasan dan kadar jantung dan mendadak berkurangan dalam pergerakan rumen. Teruk keadaan kot rambut, penurunan dalam keadaan

badan pemarkahan dan pembesaran yang ketara dan pecah nodus limfa suntikan selepas 2 hingga 3 minggu cabaran juga diperhatikan. Selain itu, pembesaran ketara dan pecah sesekali nodus limfa yang terjejas telah direkodkan dalam kumpulan kawalan positif 5 hingga 6 minggu selepas cabaran dengan *C. pseudotuberculosis*. Walaupun sedikit pembesaran nodus limfa berhampiran tapak cabaran bakteria hanya sekor kambing (16%) dalam setiap kumpulan A dan B diperhatikan.

Kedua-dua kumpulan terdedah kepada vaksin kami rumuskan (kumpulan A dan B) disebabkan signifikan (untuk kedua-dua vaksin) ketinggian dalam serum Haptoglobin (Hp) dan tahap plasma serum amiloid A (SAA) yang meningkat dengan ketara selepas 2, 5, 6 dan 7 minggu berbanding dengan haiwan yang sihat bukan vaksin pada keadaan yang sama. Hp dan SAA peringkat dalam kambing, yang tidak pernah didedahkan kepada vaksin, telah dinaikkan dengan ketara seminggu selepas pendedahan kepada bakteria liar dan mengekalkan peningkatan mendadak bagi selang penilaian. Kepekatan serum IgM didapati signifikan meningkat pada minggu 3 daripada pendedahan kepada dos pertama suntikan, di mana 7 dan 9 kali ganda kenaikan diperhatikan masing-masing dalam kumpulan A dan B. Walaupun meningkat secara mendadak sehingga 18 dan 17 kali ganda diperhatikan seminggu selepas dicabar dengan bakteria berdaya maju dalam masing-masing kumpulan A dan B.

Tahap respons IgM dalam kumpulan C adalah lebih tinggi berbanding kambing kawalan yang tidak dijangkiti (kumpulan D). Kepekatan tertinggi IgG dalam kumpulan A dan B ( $156.63 \pm 2.17$  ng / mL) dan ( $164.03 \pm 2.92$  ng / mL) dikesan selepas dua minggu selepas pendedahan kepada bakteria berdaya maju keseluruhan, masing-masing. Bagi Kumpulan C, IgG bermakna kepekatan mencapai tahap maksimum ( $138.58 \pm 1.34$  ng / mL) satu bulan selepas pendedahan kepada bakteria berdaya maju keseluruhan. Walau bagaimanapun kepekatan minimum IgG dalam kumpulan vaksin kami terus meningkat berikutan vaksinasi dos rendah dan tinggi. Kambing-kambing yang telah divaksin oleh kedua-dua vaksin dirumuskan tidak mempunyai ketinggian yang signifikan kepekatan IgA sepanjang tempoh kajian. Sebaliknya, bertanda meningkat tahap IgA serum diperhatikan dalam kumpulan C dalam tempoh antara 7 hingga 14 hari jangkitan pos dengan berdaya maju *C. pseudotuberculosis*. Walau bagaimanapun, hanya 1.2 dan 1.3 kali ganda meningkat dicatatkan dalam kumpulan C masing-masing pada minggu 6 dan 7

Post mortem kumpulan vaksin menurunkan sebarang perubahan patologi kasar ketara dalam organ dalaman dan juga dalam nodus limfa dalaman dan luaran. Sebaliknya, kadar yang tinggi perubahan patologi kasar dicatatkan dalam semua kambing daripada kumpulan kawalan positif. Signifikan perubahan patologi kasar diperhatikan dalam saraf otak dan tulang belakang kambing dalam kumpulan C. Dalam organ dalaman seperti paru-paru, hati, buah pinggang, limpa dan hati; lesi patologi kasar utama diperhatikan adalah kesesakan, pendarahan, kawasan tumpuan nekrosis, atrofi lemak dan

abscessation. Pembentukan nanah jarang diperhatikan dalam kumpulan C kecuali dalam beberapa organ-organ seperti hati dan paru-paru. Sekiranya nodus limfa, peratusan yang tinggi pembentukan nanah dalam saiz yang berbeza diperhatikan dalam semua kambing dalam kumpulan C. Sebaliknya, kedua-dua kumpulan vaksin tidak membangunkan apa-apa abses dalam nodus limfa atau organ-organ dalaman walaupun pembesaran sedikit nodus limfa telah direkodkan dalam dua ekor kambing (16%) sahaja. Perubahan histopatologi yang diperhatikan dalam kumpulan positif termasuk edema, kesesakan, penyusupan sel-sel radang terutamanya limfosit dan makrofaj, pendarahan, degenerasi, keradangan granulomatous dan nekrosis caseous tidak seperti kambing vaksin yang menunjukkan hiperplasia berfungsi penting dalam tisu limfoid tanpa perubahan histopatologi.

Dalam hematologi, terdapat penurunan statistik yang signifikan dalam kepekatan Hb, kiraan RBC, nilai MCV dan PCV dalam kumpulan C selepas terdedah kepada sel-sel bakteria berdaya maju. Selari dengan itu, ketinggian statistik tidak signifikan telah diperhatikan MCHC dalam kumpulan yang sama. Kiraan WBC adalah yang tertinggi semasa uji kaji jangkitan kambing bukan vaksin (Kumpulan C) berbanding dengan kumpulan-kumpulan pra-vaksin (A dan B). Rangsangan tertinggi di neutrophil berseghmen telah mencapai 7 hari selepas cabaran untuk hidup bakteria, apabila nombor itu ( $12.55 \pm 1.05 \times 10^9/L$ ) dalam kumpulan kawalan positif, ( $10.93 \pm 1.55 \times 10^9/L$ ) dan ( $10.14 \pm 1.38 \times 10^9/L$ ) dalam kumpulan A dan B masing-masing, jika dibandingkan dengan ( $8.23 \pm 0.46 \times 10^9/L$ ) yang dilihat dalam kumpulan yang tidak dirawat (kumpulan D). Peniingkatan ketara band neutrofil mengira ( $0.83 \pm 0.17 \times 10^9 /L$ ) dikesan dalam kumpulan kawalan positif selepas disuntik *C. pseudotuberculosis*, manakala tiada perubahan ketara dicatatkan dalam kumpulan A ( $0.49 \pm 0.09 \times 10^9 /L$ ), B ( $0.51 \pm 0.13 \times 10^9 /L$ ) dan D ( $0.30 \pm 0.01 \times 10^9 /L$ ) dalam tempoh yang sama. The monosit, eosonofil basofil tuduhan dan protein plasma berada dalam julat yang normal. Walau bagaimanapun, perubahan tidak signifikan telah direkodkan pada nilai CBC pada kedua-dua kumpulan vaksin.

Hasil PCR menunjukkan lima kambing (84% daripada jumlah) daripada enam haiwan dalam kumpulan A dan B sebelum terdedah kepada 0.5% dan 1.0% formalin-membunuh *C. pseudotuberculosis* kekal negatif. Jauh lebih rendah menyebarkan ( $P < 0.05$ ) *C. pseudotuberculosis* DNA dikesan pada organ-organ dan kelenjar limfa kumpulan vaksin. Peratusan pengesanan dalam tisu disiasat didapati 100% dalam kumpulan C.

Kesimpulannya, berikutan cabaran eksperimen dengan liar jenis *C. pseudotuberculosis* isolat tempatan, vaksin formalin dibunuh diperhatikan untuk memberikan perlindungan yang signifikan terhadap jangkitan dan kelihatan ketara menyekat penyebaran bakteria dicabar di luar tapak inokulasi dalam kebanyakan haiwan. Tikus disuntik (kumpulan A, B) membentuk imuniti dan masing-masing menghasilkan 70% dan perlindungan 80% terhadap penyakit ini. Walau bagaimanapun. Selain itu, hanya 16% kambing imunisasi dengan vaksin ini dimanifestasikan jangkitan klinikal dan patologi ringan

dengan itu, laluan penting yang berpotensi untuk penghantaran penyakit telah dihapuskan. Hasil kajian ini memberi maklumat penting kepada pembangunan caseous strategi suntikan pembasmian lymphadenitis yang berkesan di Malaysia.



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I certify that a Thesis Examination Committee has met on 11 November 2016 to conduct the final examination of Wessam Monther Mohammed Saleh on his thesis entitled "Efficacy of a Prototype Vaccine for Caseous Lymphadenitis Disease in Goats" 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 Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

**Mohd Azmi bin Mohd Lila, PhD**

Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
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Universiti Putra Malaysia

Date: 27 December 2016



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## LIST OF ABBREVIATIONS

AGID	Agar gel immunodiffusion
APP	Acute phase proteins
CBC	Complete Blood Count
CFU	Colony forming unit
CLA	Caseous lymphadenitis
DVS	Department of Veterinary Service
ELISA	Enzyme Linked Immunosorbent Assay
H&E	Hematoxylin and Eosin
Hb	Hemoglobin
Hp	Haptoglobin
I.V	Intravenous
IACUC	Institutional Animal Care and Use Committee
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Intraperitoneal
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
µg	Microgram
mg	Milligram
ng	Nanogram
ml	Milliliter
OD	Optical Density
OIE	Office of International Epizootic
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PLD	Phospholipase D
RBC	Red Blood Cell
S.C	Subcutaneous

SAA	Serum Amyloid A
TPU	Taman Pertanian Universiti
UPM	Universiti Putra Malaysia
VLSU	Veterinary Laboratories Service Unit
WBC	White Blood Cell



## CHAPTER 1

### INTRODUCTION

#### 1.1 Background of the Study

*Corynebacterium pseudotuberculosis* is the causative agent of caseous lymphadenitis (CLA) or cheesy gland disease which has been found in all major global areas of goats and sheep production (Williamson, 2001; Paton et al., 2003). Caseous lymphadenitis is characterized by the formation of suppurative abscesses particularly in superficial and internal lymph nodes, and also in internal organs of small ruminants. The disease is associated with considerable economic losses to sheep and goat farmers worldwide, mainly due to the reduction of wool, meat and milk yields, decreased reproductive efficiencies of affected animals and condemnation of carcasses and skins (Paton et al., 1994; Arsenault et al., 2003). Due to the chronic and generally subclinical nature of the disease, control is usually difficult and prevalence in animals and herds is high (de Sá Guimarães et al., 2011).

Caseous lymphadenitis has a global distribution and it is endemic in countries such as North and South America, Australia, New Zealand, Europe, Asia (including Malaysia) and Africa (Stanford et al., 1998a; Arsenault et al., 2003; Paton et al., 2003).

Malaysia has a small ruminant population, with the estimated number of sheep and goats as at 2012 to be about 129,850 and 458,646 heads respectively (Khadijah et al., 2014). However, caseous lymphadenitis has been considered as one of the major diseases confronting small ruminants' productivity in Malaysian (Chandrawathani, 1998).

The average prevalence of CLA in Malaysian small ruminant (sheep and goat), was found to be 30% using two (AGPT and ELISA) combined diagnostics tests (Komala et al., 2008). In Malaysia, the disease has been identified as potential threat to the livestock industry causing several negative impacts on both productivity and fertility of small ruminant herds (Jesse et al., 2013).

Once established in a herd or flock; the eradication of CLA is pretty difficult, owing to the inefficacy of antimicrobial therapy (Piontkowski and Shivvers, 1998; Stanford et al., 1998a; Williamson, 2001). Therefore, the most reliable control strategy for this disease involves vaccinating livestock and identifying and removing infected animals from the flock (Brown et al., 1986b; Paton et al., 2003).

Despite the known significant economic effect of CLA, details of the immune responses against *C. pseudotuberculosis* infection are not completely understood and effective vaccine is still lacking (Dorella et al., 2009). It is generally agreed that the best strategy to control the disease is vaccination of healthy animals, along with the identification/removal of infected animals (Paton et al., 1995; Williamson, 2001; Menzies et al., 2004). However, the difficulties associated with the early clinical identification of infected animals have been a great tricky problem to such a strategy. Several serodiagnostic tests have been developed to overcome the problem of clinical identification of CLA, but most have been reported to lack either sensitivity or specificity (Brown and Olander, 1987; Menzies and Muckle, 1989; Williamson, 2001; Menzies et al., 2004).

The diagnosis of CLA is mainly based on the characteristic clinical symptoms and on isolation of the agent from discharging abscesses. Identification of the cultured organisms as *C. pseudotuberculosis* is usually achieved by biochemical tests but is often problematic due to extensive variability in biochemical characteristics of the pathogen (Muckle and Gyles, 1982; Songer et al., 1988). The disease could be detected directly and rapidly from infected sheep and goats with a high diagnostic sensitivity by PCR technique (Pacheco et al., 2007).

Serological diagnosis is also of importance in the diagnosis of this disease, since subclinically infected animals play major roles in introducing CLA into a healthy flock. Serological tests, such as ELISA (Schreuder et al., 1994; Dercksen et al., 2000), complement fixation test (Shigidi, 1979), immunodiffusion test (Burrell, 1980) and the hemolysis inhibition test (Kuria and Holstad, 1988) have been employed for the diagnosis of CLA, and though most of these tests lack either sensitivity or specificity, the ELISA particularly proved to be a versatile tool in control and eradication programs (Dercksen et al., 2000).

Acute phase proteins (APPs) are proteins synthesized during an acute phase response (APR) against several stimuli like infection, inflammation, stress, trauma or tissue change (Petersen et al., 2004; Ceron et al., 2005). The positive APP may show up to 100-1000-fold increase in serum concentration in 1-2 days post inducing of the stimuli; a moderate APP displays a 5-10 fold increase in 2-3 days; and a minor APP increase between 50% and 100%. Negative APPs are those which decrease after a specific stimulus (Petersen et al., 2004; Cray et al., 2009; Eckersall and Bell, 2010). Hp and SAA are considered as major APPs in both ovine and caprine APPs. (Bastos et al., 2011) stated that serum Haptoglobin concentration and monocyte count may be the potential markers for progression of CLA in sheep.

In studies involving sheep challenged subcutaneously, vast numbers of neutrophils were observed at the site of inoculation within the first few hours following infection and from there began to move to the local drainage lymph node within 24 hours. Neutrophils population was found to decrease 3 days following inoculation, while the numbers of macrophages at the inoculation site were reported to increase significantly (Pépin et al., 1992). A period of generalized inflammation of the lymph node has been demonstrated to follow infection and micro-abscesses developed within the cortical region within 24 hours of inoculation (Pépin et al., 1991).

Infection of small ruminants with *C. pseudotuberculosis* usually results in the development of pyogranulomatous lesions (Baird and Fontaine, 2007), which manifested in two different forms. The external which is also known as cutaneous or superficial, form disease is characterized by the development of abscesses within the superficial lymph nodes or within subcutaneous tissue while the visceral form involve the development of abscesses in the internal organs such as liver, lungs and kidneys. In either case, abscesses may develop over a protracted period, becoming swollen and encased within fibrous capsules, subsequently resulting to the loss of overlying hair and often rupturing of the abscess (Radostitis et al., 2000). The purulent contents of ruptured lesion are unfortunately a significant source of cross-infection between animals (Brown and Olander, 1987). In naturally observed infections, the main portal of bacterial entry has been generally accepted to be through the skin, normally due to the presence of minor wounds and abrasions (Batey, 1986b; Brown and Olander, 1987; Davis, 1990; Collett et al., 1994).

Vaccination is the primary means of prevention of the diseases in several countries, whereby immunization reduces the spread of infection, leading to a gradual decline in disease prevalence (Paton et al., 2003). Most of the currently-available commercial vaccines for caseous lymphadenitis are polyvalent vaccines (combined with vaccines against other pathogens). This includes polyvalent vaccines consisting of *Clostridium tetani*, *Clostridium perfringens*, *Clostridium septicum*, *Clostridium novyi* and *Clostridium chauvoei* antigens (Piontkowski and Shivvers, 1998; Paton et al., 2003). Formalin-killed vaccine of local isolate of *C. pseudotuberculosis* has been documented to induce up to 70% protection in sheep challenged with local isolate of *C. pseudotuberculosis* (Selim et al., 2010).

Although *C. pseudotuberculosis* infection has been documented to have worldwide distribution and the disease is associated with huge economic losses, there is currently no vaccine that induced 100% protection against the disease. Additionally, information on the detail clinical manifestation of the disease and identification of possible biomarkers of the disease are still scanty despite the economic significance of the disease. In view of the difficulties and challenges of eradicating CLA in endemic areas or farms through treatment, thrust for the development of an efficient CLA vaccine is a global necessity.



In this study, potential prototype vaccine candidate for CLA based on local Malaysian isolate was developed. It is the authors' view that the development of local isolate prototype vaccine may help in reducing the prevalence of this disease among small ruminants in Malaysia.

## 1.2 Problem Statement

1. Malaysian small ruminants (sheep and goat) have high average prevalence of CLA which was found to be about 30%. Currently in Malaysia, there is no any available local vaccine against CLA in small ruminant. Therefore this study is designed to develop prototype vaccine of *C. pseudotuberculosis* using local isolates.
2. Caseous lymphadenitis is a chronic disease and difficult to diagnose at an early stage. Acute phase proteins and hematological values have a significant, sensitive and valid role as diagnostic biomarkers for subclinical, acute and chronic conditions. APPs and hematological values were used as indicator of disease progression after vaccination.
3. Caseous lymphadenitis also has a cellular effect on all visceral organs and lymph nodes which eventually affects the activity of the goat. Histopathological changes of vital organs were used as an indicator of disease progression in challenged animals after vaccination.

## 1.3 Hypothesis

- The development of prototype vaccine from local isolates of *C. pseudotuberculosis* will help in reducing the prevalence of the disease among small ruminants in Malaysia with non-significant side effects on the host.
- Clinical response, complete blood count (CBC), biochemistry, acute phase proteins and histopathological changes of the tested animals will be used as an indicator for disease progression of challenged animals after vaccination in vitro work.

#### 1.4 The Objectives of this study were:

1. To develop a prototype vaccine of *C. pseudotuberculosis* from local Malaysian isolate and to study the efficacy of the vaccine in goats and mice models.
2. To evaluate the clinical response between the infected goat by *C. pseudotuberculosis* and the vaccinated ones.
3. To measure the changes in the haemogram between the infected goats by *C. pseudotuberculosis* and the vaccinated ones.
4. To assess the acute phase proteins responses between infected goats by *C. pseudotuberculosis* and the vaccinated ones.
5. To estimate the antibodies response (IgM, IgG, IgA) between the infected goats by *C. pseudotuberculosis* and the vaccinated ones.
6. To evaluate the histopathological changes of visceral organs and lymph nodes between the infected goats by *C. pseudotuberculosis* and the vaccinated ones.
7. To detect *C. pseudotuberculosis* from vital organs and lymph nodes using bacterial culture, biochemical tests and PCR techniques from the infected goats by *C. pseudotuberculosis* and the vaccinated ones.

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