



UNIVERSITI PUTRA MALAYSIA

**EVALUATIONS OF THE PATHOPHYSIOLOGY AND REPRODUCTIVE
PATHOLOGY OF BUCKS INOCULATED WITH
Corynebacterium pseudotuberculosis AND ITS IMMUNOGEN MYCOLIC
ACID EXTRACT**

NUR FAEZA MOHAMAD NOR

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By

NUR FAEZA BINTI MOHAMAD NOR

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

March 2019

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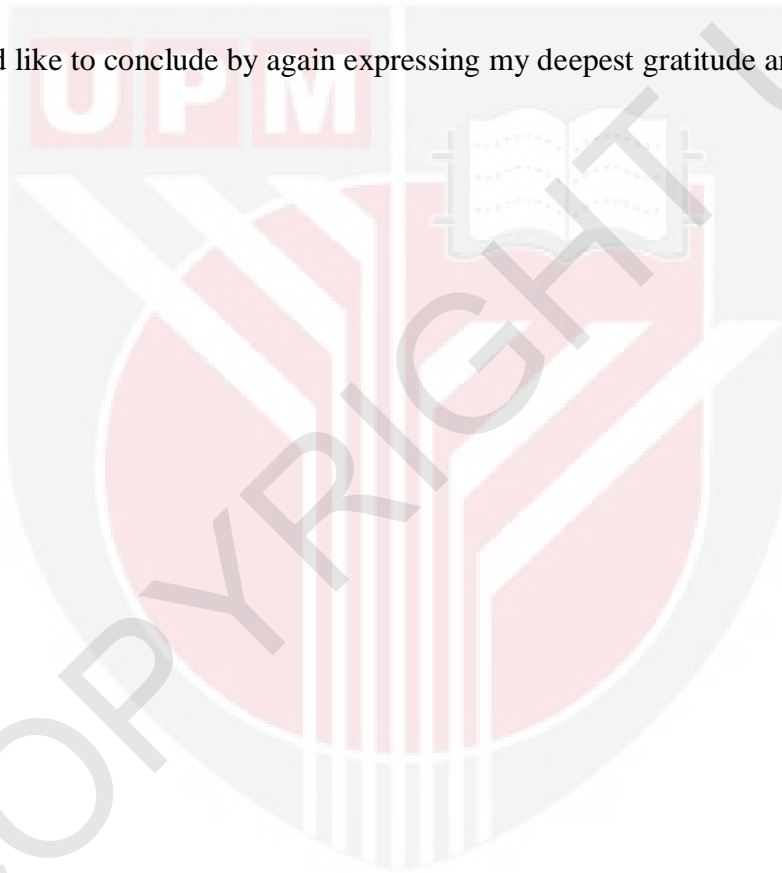
DEDICATION

To my beloved husband: Thank you for allowing me to further my studies, thank you for your support and guidance, without you, this journey will be harder.

To my beloved families, especially my beloved mother: Thank you for the kind words and encouragement throughout this process.

To my supervisory committees: my sincere thanks for the guidance and support.

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



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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Veterinary Science

EVALUATIONS OF THE PATHOPHYSIOLOGY AND REPRODUCTIVE PATHOLOGY OF BUCKS INOCULATED WITH *Corynebacterium pseudotuberculosis* AND ITS IMMUNOGEN MYCOLIC ACID EXTRACT

By

NUR FAEZA BINTI MOHAMAD NOR

March 2019

Chairman : Professor Abdul Wahid Haron, PhD
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Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis, a chronic disease of sheep and goats, characterized by the formation of suppurative abscesses in superficial and visceral lymph nodes and internal organs of small ruminants. Little is known about the male goat's reproductive pathophysiology inoculated with *Corynebacterium pseudotuberculosis* and its immunogen mycolic acid extract. Hence, this study was designed to determine the concentration of testosterone hormone and proinflammatory cytokines of the experimental animals and to determine the histopathological effect of male reproductive organs and the associated lymph nodes. A total of 12 bucks, divided into 3 treatment groups of equal numbers (4 goats per group) were selected in this study.

Group 1 (Negative control group) was inoculated intradermally with 2ml of sterile phosphate buffered saline (PBS) pH7. Group 2 (Positive control group) was inoculated intradermally with 2ml of 1×10^9 colony forming unit (cfu) of *C. pseudotuberculosis*. Group 3 (Mycolic acid group) was inoculated intradermally with 2ml of mycolic acid extract. During the post inoculation period of 60 days, the goats were observed for clinical signs. Body temperature was taken daily. Heart rate, respiratory rate and body score were measured on weekly basis. Lymph nodes were checked daily and findings were recorded.

All experimental animals in Group 1 survived throughout the course of study period with no evidence of clinical manifestation. There was an increment in body temperature observed from week 0 to week 3 for group 2 (PC) compared to group 1 (NC). In Group 3 (MA), there was an increased in body temperature observed

during week 0- 3 but not as severe as group 2. However, the heart rate and respiratory rate of both groups 2 and 3 were normal.

The concentration of testosterone hormone in group 2 increased significantly ($p < 0.05$) in week 4, 5 and 8 but decreased in weeks 1 and 6 post inoculation. In group 3, the mean concentration of this hormone was increased significantly ($p < 0.05$) in weeks 4, 5, 6 and 8 post inoculation but decreased in week 1.

The concentration of interleukin 6 (IL 6) in group 2 showed no significant difference ($p > 0.05$) but increased significantly ($p < 0.05$) in group 3 at week 1 post inoculation compared to group 1. For concentration of interleukin 1 β (IL1 β), both groups 2 and 3 showed significant difference ($p < 0.05$) in weeks 1 and 2 post inoculation. For tumor necrosis factor-alpha (TNF- α) concentration, both groups 2 and 3 did not show any significant difference ($p > 0.05$) compared to group 1. The concentration of Interferon- γ (IFN γ) of group 2 was significantly increased ($p < 0.05$) in weeks 1, 2, 3 and 4 where else for group 3 was not significantly different ($p > 0.05$) compared to group 1.

Both groups 2 and group 3 showed a reduction in semen qualities as compared to group 1 but the severity were more intense in group 2 if compared to group 3. The cellular changes were observed in both group 2 and group 3 as compared to group 1. The cellular changes of the epididymis in both groups 2 and 3 represented by degeneration and necrosis, haemorrhage and congestion, and also the presence of abnormal sperms in the epididymal tubules. The cellular changes of the testes in groups 2 and 3 represented by an irregular shape and shrinkage of the seminiferous tubules, less spermatid cells in the seminiferous tubules, degeneration and necrosis, and also haemorrhage and congestion. However, the severity was more intense in group 2 if compared to group 3.

Histopathologically, both group 2 and group 3 showed the cellular changes of the lymph nodes represented by infiltration of the inflammatory cells, oedema and haemorrhage and congestions but in lesser extent of severity in group 3 if compared to group 2.

Conclusively, this study demonstrated some clinical manifestations, changes in concentration of testosterone hormone, changes in the cytokines concentrations, with histopathology cellular changes and deterioration of semen quality in both groups 2 and 3. However, for group 3, the changes were less severe compared to group 2. Hence, concluded that Mycolic acid is not a good immunogen.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Veterinar

PENILAIAN PATHOFISIOLOGI DAN PATOLOGI PEMBIAKAN PADA KAMBING JANTAN TERHADAP INOKULASI DENGAN *Corynebacterium pseudotuberculosis* DAN EKSTRAK IMMUNOGEN ASID MYCOLIC NYA

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Corynebacterium pseudotuberculosis adalah agen penyebab penyakit caseous lymphadenitis, iaitu penyakit kronik kambing dan bebiri yang dicirikan dengan pembentukan bisul yang bernanah pada nodus limfa luaran dan dalaman juga pada organ-organ dalaman haiwan ruminan kecil. Tidak banyak diketahui tentang kesan inokulasi *Corynebacterium pseudotuberculosis* dan ekstrak immunogen mycolic asid nya terhadap patofisiologi sistem pembiakan kambing jantan. Oleh itu, kajian ini dirancang untuk menentukan konsentrasi hormon testosterone dan juga sitokin proinflamasi pada haiwan eksperimen dan juga untuk mengetahui kesan histopatologi pada organ pembiakan jantan beserta nodus limfa yang berkaitan. Jumlah keseluruhan 12 ekor kambing jantan, dibahagikan kepada 3 kumpulan dengan bilangan 4 ekor kambing untuk setiap kumpulan digunakan dalam eksperimen ini.

Kumpulan 1 (Kumpulan kawalan negatif) diinokulasi dengan 2ml saline buffer fosfat steril (PBS) pH7 secara intradermal. Kumpulan 2 (Kumpulan kawalan positif) diinokulasi dengan 2ml 1×10^9 CFU *C.pseudotuberculosis* secara intradermal dan kumpulan 3 (Kumpulan asid mycolic) diinokulasi dengan 2ml ekstrak asid mycolic daripada *C.pseudotuberculosis*. Dalam tempoh 60 selepas inokulasi, tanda-tanda klinikal pada semua kambing-kambing tersebut akan diperhatikan. Suhu badan diambil setiap hari. Kadar denyutan jantung, kadar pernafasan dan skor keadaan badan diambil sekali setiap minggu. Perubahan pada nodus limfa juga akan diperhatikan dan setiap pemerhatian akan direkod.

Semua haiwan eksperimen kumpulan 1 bertahan hidup sehingga akhir eksperimen tanpa sebarang tanda klinikal. Terdapat kenaikan ketara pada suhu badan kumpulan 2 bermula minggu 0 hingga minggu ke-3 dibandingkan dengan kumpulan 1. Kumpulan 3 juga menunjukkan kenaikan suhu badan pada minggu 0 hingga minggu ke-3 tetapi tidak seteruk kumpulan 2. Walaubagaimanapun, kadar pernafasan dan degupan jantung adalah normal bagi kedua-dua kumpulan 2 dan 3.

Konsentrasi hormon testosterone pada kumpulan 2 meningkat dengan ketara ($p < 0.05$) pada minggu ke- 4, 5 dan 8 tetapi menurun pada minggu pertama dan ke-6 selepas inokulasi. Konsentrasi hormon testosterone pada kumpulan 3 meningkat dengan ketara ($p < 0.05$) pada minggu ke- 4, 5 dan 8 tetapi menurun pada minggu pertama.

Konsistensi interleukin 6 (IL 6) pada kumpulan 2 tidak menunjukkan sebarang perubahan ketara ($p > 0.05$) tetapi meningkat dengan ketara ($p < 0.05$) pada kumpulan 3 semasa minggu pertama selepas inokulasi jika dibandingkan dengan kumpulan 1. Konsentrasi Interleukin 1β (IL 1β) menunjukkan perubahan ketara ($p < 0.05$) pada kedua-dua kumpulan 2 dan 3 dalam minggu pertama dan ke-2 selepas inokulasi. Untuk kepekatan faktor-alpha nekrosis tumor (TNF- α), kedua-dua kumpulan 2 dan 3 tidak menunjukkan perubahan ketara ($p > 0.05$) jika dibandingkan dengan kumpulan 1. Kepekatan Interferon- γ (IFN γ) pula meningkat secara ketara ($p < 0.05$) pada kumpulan 2, dalam minggu pertama, ke- 2, 3 dan 4, manakala kumpulan 3 tidak menunjukkan sebarang perubahan ketara ($p > 0.05$) dibandingkan dengan kumpulan 1.

Kedua-dua kumpulan 2 dan 3 menunjukkan penurunan kualiti semen dibandingkan dengan kumpulan 1, tetapi pada kadar keterukan yang berbeza dimana kumpulan 2 menunjukkan kesan yang lebih teruk jika dibandingkan dengan kumpulan 3. Kedua-dua kumpulan 2 dan 3 menunjukkan perubahan selular pada testes dan epididymis dibandingkan dengan kumpulan 1, tetapi pada kadar keterukan yang berbeza dimana kumpulan 2 menunjukkan kesan yang lebih teruk jika dibandingkan dengan kumpulan 3. Perubahan selular epididymis pada kedua-dua kumpulan 2 dan 3 ditunjukkan oleh degenerasi dan nekrosis, hemoraj berserta kongesi, dan kehadiran sperm yang tidak normal di dalam tiub-tiub epididymal. Perubahan selular testis pada kedua-dua kumpulan 2 dan 3 ditunjukkan oleh bentuk yang tidak sekata dan pengecutan tiub-tiub seminiferous, kekurangan sel-sel spermatid di dalam tiub-tiub seminiferous degenerasi berserta nekrosis dan hemoraj berserta kongesi. Walaubagaimanapun, tahap keterukan adalah lebih teruk pada kumpulan 2 jika dibandingkan dengan kumpulan 3.

Secara histopatologi, kumpulan 2 dan 3 menunjukkan perubahan selular pada nodus limfa dengan diwakili oleh penyusupan sel-sel inflammasi, edema dan konjensi beserta hemoraj tetapi pada kadar keterukan yang kurang pada kumpulan 3 jika dibandingkan dengan kumpulan 2.

Secara kesimpulan, kajian ini menunjukkan beberapa tanda klinikal, perubahan pada kepekatan hormone tetosteron, perubahan pada kepekatan sitokin pro-inflamasi, beserta perubahan selular histopatologi (testis, epididymis dan nodus limfa) disertai oleh kemerosotan pada kualiti semen pada kedua-dua kumpulan 2 dan 3. Walaubagaimanapun, kumpulan 3 menunjukkan perubahan yang kurang teruk berbanding kumpulan 2. Oleh itu, disimpulkan bahawa, Mycolic acid merupakan immunogen yang kurang baik.



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LIST OF ABBREVIATIONS/SYMBOLS

µg	Microgram
pg	pictogram
mg	Milligram
ml	Milliliter
ng	Nanogram
°C	Degree celsius
BHI	Brain Heart Infusion Broth
CFU	Colony forming unit
CLA	Caseous lymphadenitis
DVS	Department of Veterinary Service
ELISA	Enzyme Linked Immunosorbent Assay
H&E	Hematoxylin and Eosin
IACUC	Institutional Animal Care and Use Committee
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IFNγ	Interferon-γ
IM	Intradermally
OD	Optical Density
PBS	Phosphate Buffered Saline
PLD	Phospholipase D
RIA	Radioimmunoassay
TNF-α	Tumor necrosis factor-α
TPU	Taman Pertanian Universiti
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

1.1 Background

Corynebacterium pseudotuberculosis (*C. pseudotuberculosis*) is the etiological agent that causes caseous lymphadenitis (CLA), a well-known disease of sheep and goats which mainly affects populations of small ruminants throughout the world and causes significant economic losses (Arsenault *et al.*, 2003). The CLA disease is described by formation of one or many abscesses in lymph nodes either external or internal and can be both lymph nodes (Paton, 2010). Ruptured abscesses are the main source of *C. Pseudotuberculosis* contaminating the environment. Animal exposure to the bacteria can be either by direct physical contact with the infected animal or indirectly via contaminated fomite (Stoops *et al.*, 1984; Collet *et al.*, 1994; Jesse *et al.*, 2008). *C. pseudotuberculosis* is an intracellular, gram positive, facultative anaerobe and a small curved rod non-spore forming bacterium. This bacterium is a facultative anaerobe and grows best at 37 °C, at a pH of 7.0 to 7.2. It grows sparse initially on the agar surface and then becomes organized in clumps or in palisades, taking on a cream to orange coloration; colonies are dry, opaque and concentrically ringed (Buxton *et al.*, 1962; Dorella *et al.*, 2006). However, *C. pseudotuberculosis* is a demanding organism from a nutritional standpoint, growing well on enriched media such as blood agar, brain heart infusion (BHI) agar or broth or enriched medium with animal serum (Batey, 1986).

Corynebacterium pseudotuberculosis is a pathogenic bacteria species that have cell membrane covered with waxy substances known as mycolic acids (MAs). Mycolic acids are the main and specific constituents of the bacteria cell envelope. It is a long chain fatty acid with a complex, structural design that ensures the impermeability of this bacteria cell membrane (Damien *et al.*, 2004; Jesse *et al.*, 2013). Mycolic acids, a 2-alkyl, 3-hydroxy long-chain fatty acids, are part of the cell envelope of *Mycobacterium tubercular* (Daffé & Draper, 1997). MAs are found extractable using organic solvents or terminal esterification of the penta-arabinofuranosyl units of arabinogalactan (McNeil *et al.*, 1991).

Corynebacterium strains have been reported to be viable in the absence of MAs (Portevin *et al.*, 2004; Portevin *et al.*, 2005). It has been proven that the outer membrane was no longer observed in other mutant strains devoid of MAs (Zuber *et al.*, 2008). However there is still lack of information on the mechanism of action and host cell responses towards MAs (Carne, 1939; Onon, 1979). Therefore, this study was conducted to fill the gap of the research of MAs in *C. pseudotuberculosis*.

The insidious nature of some infectious disease conditions can be associated with the pathology of the reproductive system and some evidences suggested that infectious disease-related reproductive abnormality reduces the reproductive efficiency in farm

animals (Grooms *et al.*, 2004; Robertand Walter, 2007). Caseous Lymphadenitis (CLA) infection in non-pregnant does may lead to infertility resulting from pathological changes in the reproductive organs as well as an imbalance in reproductive hormonal levels (Othman *et al.*, 2016). The pathogenesis of *C. pseudotuberculosis* in the reproductive organs is complex and not well understood even though it is believed that the bacteria disseminates through the afferent lymphatic system to the lymph node and internal organs, before multiplying within the macrophages as it survives the action of phagolysosomal enzymes (Dorella *et al.*, 2006; Baird *et al.*, 2007). From the study done by Zaid *et al.*, 2016 concluded *C. pseudotuberculosis* and its immunogen PLD exotoxin had direct detrimental effects on testosterone concentration, decreased the scrotal circumference, lowered semen pH and semen quality. All the said detrimental effects above could potentially implicate and affect the reproduction and fertility of male goat. The effect of CLA on buck's fertility can be said due to the nature of the disease as chronic disease (wasting and debilitating in nature) on the body systems resulting in collateral damages of reproductive system. However, there is still lack of information on the effects of its immunogen Mycolic acid extract towards the male reproductive systems. Hence, this study was designed and conducted to fill the gap of the research of MAs in *C. pseudotuberculosis*.

The presence of pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF- α), interleukin-1 alpha (IL-1 α) and interleukin 1 beta (IL-1 β) in the male reproductive tract (testis, epididymis and sperm) may have certain physiological functions. However, when the levels of these cytokines are higher than normal, as seen in conditions of inflammation, may give negative effects towards the sperm production. Inflammation is also associated with oxidative stress and the latter is well known to impair sperm function (Azenabor *et al.*, 2015). TNF- α concentration is also important to determine the semen quality (Azenabor *et al.*, 2015). Interferon- γ (IFN γ) concentration normally increased in chronic diseases or chronic inflammation that may lead to negative effect towards functional development and male reproductive capacity (Jeanine *et al.*, 1996). The cytokines that are involved in CLA infection are interleukin-1 β and interleukin-6, and tumor necrosis factor- α (TNF- α) (Pépin *et al.*, 1997; Jesse *et al.*, 2016). Recent study in female goats reported by Jesse *et al.* (2017), stated that *C. pseudotuberculosis* and its MAs showed significant changes in the cytokines concentration for IL-1 β and IL-6 and the study concluded that MAs plays a key role in pathogenesis of CLA in small ruminants and has immunogenic properties that may induce immunoglobulins production. The cellular changes in male reproductive system due to MAs infection had not been widely studied and understood till date. There is no study done on the effects of MAs in male goats thus, this study was designed and the results will be added knowledge in this study field.

1.2 Problem statement

The prevalence of CLA among small ruminants in Malaysia is estimated at 30% (Komala *et al.*, 2008) and it is an endemic disease in most of the small ruminant farms in Malaysia (Jesse *et al.*, 2015). Farmers have little concern or knowledge on the effect of CLA towards productivity of their farm as information on the effect of

CLA in relation to reproduction is limited. There is still no study that has been done to investigate the pathophysiology of *C. Pseudotuberculosis* and its immunogen Mycolic acid and their effects on the reproductive systems of bucks.

1.3 Hypothesis

The hypotheses of this study were as follows:

H_{A1}= both treated groups will show clinical responses after inoculation with *C. Pseudotuberculosis* and its immunogen Mycolic acid extract.

H_{A2}= there will be an alteration in the testosterone, and pro-inflammatory cytokines concentrations of the treated groups.

H_{A3}= semen quality of the treated groups will be reduced in quality compared to the negative control.

H_{A4}= reproductive system and its associated lymph nodes of the treated groups will exhibit cellular tissue changes.

1.4 Objectives

Thus, the objectives of this study are:

1. To determine the clinical response in male goats after inoculation with *Corynebacterium pseudotuberculosis* and its immunogen Mycolic acid extract.
2. To determine the concentration of testosterone hormone and concentrations of proinflammatory cytokines (IL-1 β , IL- 6, IFN γ and TNF alpha) in bucks experimentally infected with *Corynebacterium pseudotuberculosis* and its immunogen Mycolic acid extract.
3. To determine the semen quality of bucks experimentally infected with *Corynebacterium pseudotuberculosis* and its immunogen Mycolic acid extract.
4. To determine the severity of tissue changes in the reproductive system and its associated lymph nodes of buck infected with *Corynebacterium pseudotuberculosis* and its immunogen Mycolic acid extract.

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LIST OF PUBLICATIONS

Faeza, N.M.N; Jesse, F.F.A; I.U. Hambali; M.D. Odhah;; M. Umer; M.M.S. Wessam; M.S; M.L.Mohd-Azmi; A.H. Wahid. (2019). Responses of Testosterone Hormone Concentration, Semen Quality and its' related Pro-Inflammatory Cytokines in Bucks following Corynebacterium pseudotuberculosis and its Mycolic Acid infection. Tropical Animal Health and Production, <https://doi.org/10.1007/s11250-019-01878-2>. (Published).

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Determination of pathophysiology in male goats' reproductive system inoculated with corynebacterium pseudotuberculosis and its immunogen mycolic acid extract at the 9th Malaysian association of veterinary Pathology (MAVP), Scientific Conference, 2016. (Presented).



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