UNIVERSITI PUTRA MALAYSIA

PATHOLOGY AND PATHOGENESIS OF Brucella melitensis INFECTION IN BUCKS

NURRUL SHAQINAH BINTI NASRUDDIN

FPV 2014 23

PATHOLOGY AND PATHOGENESIS OF *Brucella melitensis* INFECTION IN BUCKS

By

NURRUL SHAQINAH BINTI NASRUDDIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

September, 2014

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

PATHOLOGY AND PATHOGENESIS OF *Brucella melitensis* INFECTION IN BUCKS

By

NURRUL SHAQINAH BINTI NASRUDDIN
September 2014

Chairman : Mohd Zamri Saad, DVM, Ph.D.
Faculty : Veterinary Medicine

Brucellosis is an important disease of ruminants in many countries, including Malaysia. It is caused by *Brucella melitensis* leading to serious economic impact to goat farmers following abortions and stillbirths. The infection has not been thoroughly studied in bucks, particularly on the pathological changes and distribution of the organisms in the host. Furthermore, the efficacy of intracellular killing of *B. melitensis* by exposed bucks and the effectiveness of commonly used serological tests in identifying infected bucks need to be clarified. This study was conducted to observe the pathological changes and pathogenesis in bucks following experimental infection by *B. melitensis*.

Nine clinically healthy crossbred Jamnapari bucks of approximately 12 months old were used. The animals were confirmed as sero-negative for brucellosis following Rose Bengal Precipitation Test (RBPT) and Complement Fixation Test (CFT) tests. The selected bucks were divided into 3 equal groups. Groups 1 and 2 were infected intraconjunctival with 50 µl of an inoculum containing 10^9 cells/ml live local strain of *B. melitensis* and were sacrificed on days 7 and 14, respectively. Group 3 were similarly exposed to 50 µl normal saline before they were sacrificed on day 14. Serum samples for RBPT, CFT and ELISA, and conjunctival and prepuce swabs for bacterial isolation were collected at 3-day intervals. During post-mortem examination, the prescapular, submandibular and supramammary lymph nodes, the testis, epididymis, prepuce, seminal vesicle, bulbourethral gland, liver, spleen, conjunctiva and synovial membrane were collected for bacterial isolation and histopathology assessment.

Infected bucks developed mild pathological changes at 7 and 14 days post-infection (P.I) but did not demonstrate any clinical sign. There was no significant different ($p>0.05$) in the severity of pathological changes at days 7 and 14 post-infection. The histopathological lesions included necrotizing orchitis, epididymitis, seminal vesiculitis, hepatitis and phostitis. Nevertheless, immunoperoxidase positive reactions were observed in almost all organs that were sampled. The pathological findings proved that acute brucellosis led to mild histopathological changes even though the antigen was disseminated to all organs. *Brucella*

melitensis was not isolated from prepucial swabs that were collected between days 0 and 9 P.I. Later, isolations were successfully made from 66% of prepucial swabs on day 12 P.I and from 33% of the swabs on day 14 P.I. Isolations from the conjunctival swabs were successful on days 3, 12 and 14 P.I. Approximately 33% and 50% of the synovial membrane samples collected between days 7 and 14 P.I revealed positive isolations, and the synovial membrane was found to be the most suitable sample for isolations of *B. melitensis* in acutely infected bucks. Nevertheless, polymerase chain reaction (PCR) resulted in highest frequency of detection of *B. melitensis* and the most consistent results were observed in the testis (100% positive).

The *in vitro* assessments of phagocytosis and intracellular killing of *B. melitensis* were carried out using 6 healthy crossbred Jamnapari bucks of approximately 12 months of age. They were divided into 2 groups after the animals were tested with RBPT and CFT to ensure the brucellosis free status. The bucks of Group 1 were exposed subcutaneously with 2 ml inoculums containing 10^9 cells/ml of formalin-killed *B. melitensis.* The bucks of Group 2 were given 2ml sterile PBS as unexposed control group. Both groups were kept for 14 days before the neutrophil, macrophages and lymphocytes were harvested. The cells were then prepared as cell suspension containing 10^6 cells/ml in 200 µl in each individual chamber before 200 µl of an inoculum containing 10^7 cells/ml of live *B. melitensis* was introduced into the chambers. The extracellular Gram-positive bacterium, *Streptococcus agalactiae* and Gram-negative bacterium, *Pasteurella multocida* were used for comparison. The cells were then harvested at 0, 30, 60 and 120 minutes post-incubation and stained with Acridine orange and Crystal violet for viewing under fluorescent microscope to determine the phagocytosis index rate and intracellular killing index.

Phagocytosis activity by the neutrophils revealed no significant difference ($p>0.05$) between 30 and 60 min of incubation as well as between the two animal groups. However, rate of phagocytosis by neutrophils that were derived from exposed bucks was significantly ($p<0.05$) higher at 120 min. Subsequently, the neutrophils were able to kill 68% of the phagocytosed *B. melitensis*, which was significantly ($p<0.05$) lower than the two other extracellular bacteria. Similarly, macrophages from both groups showed no significant difference ($p>0.05$) in the phagocytosis activities at 30 and 60 min of incubation. However, at 120 min, macrophages that were derived from the exposed group demonstrated significantly ($p<0.05$) higher rate of phagocytosis. On the other hand, penetration of *B. melitensis* into lymphocytes of bucks revealed that *B. melitensis* was able to penetrate but was unable to survive long in the cells. The study proved the capability of *B. melitensis* to invade the lymphocytic cells, which enhanced movement of the organism within the body without triggering immunological response. Nevertheless, *B. melitensis* lacked replication capabilities in the lymphocytes.

In this study, sera from infected and uninfected bucks were processed to determine the antibody levels using ELISA and the two standard screening tests; the RBPT and the CFT. The RBPT and CFT assays provided negative results for all sera collected throughout the 14-day experiment. Meanwhile, ELISA revealed significantly ($p<0.05$) increased IgG level post-infection. However, the IgA levels in conjunctiva and prepuce showed fluctuating patterns

and peaked on day 6 P.I. Therefore, RBPT and CFT were found to be less useful for detection of acute brucellosis while ELISA would be a better test to be used for acute caprine brucellosis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan untuk Ijazah Doktor Falsafah

PATOLOGI DAN PATOGENESIS JANGKITAN *Brucella melitensis* DALAM KAMBING JANTAN

Oleh

NURRUL SHAQINAH BINTI NASRUDDIN
September 2014

Pengerusi : Mohd Zamri Saad, DVM, Ph.D.
FaKulti : Perubatan Veterinar

Bruselosis merupakan penyakit ruminan yang penting di kebanyakan negara termasuk Malaysia. Masalah utama penyakit ini adalah keguguran pada kambing betina dan kematian anak kambing yang pasti memberi impak ekonomi yang serius kepada penternak. Selain itu, kambing jantan juga akan menghadapi masalah seperti radang sendi dan radang zakar yang boleh menyebabkan kemandulan jika tidak dirawat. Di Malaysia, masalah bruselosis kambing adalah disebabkan oleh *Brucella melitensis* dan dipercayai penyakit ini tersebar melalui makanan yang tercemar, transmisi melalui membran mukus dan hubungan seksual. Oleh itu, kajian ini dijalankan adalah untuk memahami perubahan patologi ke atas kambing jantan yang dijangkiti *B. melitensis*, corak penyebaran bakteria tersebut di dalam badan, respon imuniti terhadap jangkitan terutama proses fagosit oleh neutrofil, makrofaj dan kadar penembusan ke dalam limfosit, serta mengkaji keberkesanan ujian serologi yang sedia ada iaitu RBPT, CFT dan ELISA.

Sembilan ekor kambing jantan yang sihat, baka kacukan Jamnapari, berusia dalam lingkungan 12 bulan dan dibahagi kepada 3 kumpulan telah digunakan di dalam eksperimen ini. Semua kambing diuji dengan ujian RBPT dan CFT untuk membuktikan status bruselosis. Kumpulan 1 dan 2 telah diberi inokulasi sebanyak 50 µl 10^9 sel/ml *B. melitensis* hidup yang diperoleh dari wabak tempatan ke dalam setiap selaput mata, manakala kambing-kambing di dalam Kumpulan 3 diberi 50 µl salin normal ke dalam setiap mata sebagai kumpulan kawalan negatif. Sampel darah, sampel calitan selaput prepus dan selaput mata diambil pada setiap 3 hari untuk tujuan ujian RBPT, CFT dan ELISA manakala sampel calitan digunakan untuk ujian ELISA dan ujian pemencilan organism. Kumpulan 1 dikorbankan pada hari ke-7 selepas inokulasi manakala Kumpulan 2 dan 3 dikorbankan pada hari ke-14 selepas inokulasi. Semasa pos mortem, sampel nodus limfa dari preskapular, submandibular dan supramamari, testis, epididimis, selaput prepus, hati, limpa, selaput sinovial, selaput mata, kelenjar bulbouretral dan kelenjar seminal vesikel diambil untuk tujuan ujian pemencilan organisma dan pemeriksaan histologi.

Berikutan inokulasi *B. melitensis* ke mata kambing jantan, semua kambing tidak menunjukkan sebarang petanda klinikal walaupun pemeriksaan histopatologi menunjukkan

terdapat kesan sederhana. Jangkamasa jangkitan selama 7 dan 14 hari tidak menunjukkan sebarang perbezaan ketara ($p>0.05$). Lesi histopatologi termasuklah radang pada selaput prepus, zakar, kelenjar seminal dan hati. Pewarnaan imuno-peroksidas memberikan keputusan positif yang membuktikan kehadiran patogen tersebut di dalam semua organ yang diambil. Penemuan ini membuktikan bruselosis akut menyebabkan lesi histopatologi yang sederhana walaupun organism tersebut ditemui dalam setiap organ. Tambahan lagi, salur darah memberi pewarnaan imuno-peroksidas positif telah membuktikan bahawa *B. melitensis* merebak ke seluruh badan melalui saluran darah.

Pemencilan organisma daripada calitan selaput prepus yang diambil dari hari 0 hingga 9 menunjukkan keputusan negatif. Namun, calitan selaput prepus yang diambil pada hari ke 12 menunjukkan keputusan positif tertinggi iaitu sebanyak 66% dan pada hari ke 14 sebanyak 33%. Calitan mata memberi keputusan yang tidak konsisten di mana hanya pada hari ke 3, 12 dan 14 sahaja menunjukkan keputusan positif. Oleh itu, calitan selaput prepus merupakan sampel paling sesuai untuk diguna bagi pemencilan *B. melitensis*. Selaput sinovial pula menunjukkan keputusan positif tertinggi bagi kultur langsung dan terbukti sesuai untuk digunakan bagi proses pemencilan *B. melitensis*. Sementara itu, ekstrak DNA dan PCR menghasilkan keputusan positif bagi semua sampel. Ini sekali gus menunjukkan teknik tersebut sangat berguna untuk mengenalpasti patogen penyebab penyakit berikutan kaedah tersebut di dapati mempamerkan spesifikasi dan sensitiviti yang tinggi.

Kajian *in vitro* ke atas aktiviti fagositosis dan pembunuhan di dalam sel terhadap *B. melitensis* telah dijalankan dengan menggunakan 6 ekor kambing jantan yang sihat, baka kacukan Jamnapari, berusia dalam lingkungan 12 bulan kepada dibahagi kepada 2 kumpulan. Semua kambing diuji dengan ujian RBPT dan CFT untuk membuktikan status bruselosis. Kumpulan 1 telah diberi inokulasi 10^9 sel/ml *B. melitensis* mati yang dibunuh dengan formalin sebanyak 2 ml di bawah kulit. Kumpulan 2 diberi 2 ml PBS secara bawah kulit sebagai kumpulan kawalan negatif. Kedua-dua kumpulan dbiarkan selama 14 hari sebelum neutrofil, makrofaj dan limfosit diambil. Semasa ujian fagosit, 200 µl yang mengandungi 10^6 sel/ml neutrofil, makrofaj dan limfosit dicampurkan dengan 200 µl 10^7 sel/ml organisma *B. melitensis,* dan bakteria yang diguna sebagai kawalan negatif, *Streptococcus agalactiae* dan *Pasteurella multocida* secara berasingan. Proses inkubasi dijalankan mengikut tempoh waktu yang pelbagai iaitu 0, 30, 60 dan 120 minit. Kemudian, sel-sel tersebut diwarnakan dengan menggunakan Acridine orange and Crystal violet sebelum divisualisasikan melalui mikroskop fluresen. Indek fagosit dikira secara peratus sel yang mengandungi satu atau lebih bacteria daripada 100 sel yang dikira. Indek pembunuhan di dalam sel pula dikira secara peratus bakteria yang telah mati di dalam sel daripada jumlah keseluruhan bakteria yang difagosit oleh sel.

Kajian *in vitro* ke atas aktiviti fagosit oleh neutrofil, mendapati bahawa tiada perbezaan ketara ($p>0.05$) setelah inkubasi selama 30 dan 60 minit. Walaubagaimana pun, neutrofil yang diambil dari kumpulan terdedah menunjukkan perbezaan nyata ($p<0.05$) selepas 120 minit tempoh inkubasi. Keputusan ini adalah berikutan proses opsinisasi oleh antibodi yang terdapat di dalam serum haiwan terdedah. Aktiviti pembunuhan di dalam sel, tidak menunjukkan perbezaan nyata ($p>0.05$) di antara kedua-dua kumpulan bagi semua tempoh

inkubasi. Neutrofil hanya mampu membunuh sehingga 68% daripada *B. melitensis* yang difagositnya. Ini adalah kerana *B. melitensis* rintang dan boleh mengelak aktiviti bakteriasidal oleh neutrofil. Aktiviti fagosit *B. melitensis* oleh makrofaj juga tidak menunjukkan perbezaan ketara ($p>0.05$) di antara kedua-dua kumpulan setelah inkubasi selama 30 dan 60 minit. Walaubagaimana pun, pada minit ke 120, makrofaj dari kambing terdedah menunjukkan keupayaan fagositosis *B. melitensis* yang signifikan ($p<0.05$) berbanding kumpulan tidak terdedah. Hal ini adalah kerana proses opsinisasi yang dijalankan oleh serum yang membantu proses fagositosis. Kajian terhadap kadar penembusan oleh *B. melitensis* ke dalam sel limfosit mendapati bahawa patogen berjaya menembusi sel limfosit tetapi tidak boleh hidup lama di dalam sel. Ini membuktikan keupayaan *B. melitensis* untuk menjangkiti sel tanpa mencetus gerakbalas keimunan. Ketidakupayaan *B. melitensis* untuk hidup di dalam sel limfosit adalah disebakan kekurangan faktor replikasi yang diperlukan.

Sepanjang tempoh ujikaji, sampel serum diambil dan diproses menggunakan kaedah RBPT, CFT dan ELISA. Secara keseluruhannya, ujian konvensional; RBPT dan CFT menghasilkan keputusan negatif bagi semua sampel yang diambil sepanjang tempoh eksperimen. Manakala ujian ELISA menunjukkan paras IgG yang meningkat secara ketara ($p<0.05$) selepas terjangkit. Paras IgA dari selaput mata dan selaput prepus adalah tidak konsisten dan hanya berada di paras tertinggi pada hari ke 6 selepas terjangkit. Oleh itu, ujian RBPT dan CFT adalah kurang sesuai untuk digunakan dalam mengenalpasti jangkitan bruselosis akut, manakala ujian ELISA adalah berguna kerana keputusan yang diberikan adalah lebih spesifik.

ACKNOWLEDGEMENTS

In the Name of Allah, The Most Gracious, The Most Merciful and
Prophet Mohammad S.A.W

I would like to express the deepest appreciation to committee chair, Prof. Dr. Mohd Zamri Saad, who continually and convincingly conveyed a spirit of adventure and excitement in regard to research. Without his guidance and persistent help this thesis would not have been possible.

I would like to thank my committee members, Prof. Dr. Jasni Sabri and Dr. Hazilawati Hamzah for their guidance and supports. Their expertise in veterinary studies inspired and prepared me for future challenges.

It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

Above all, I would like to thank my husband Dr. Mohammad Noor Amal Azmai for his personal support and great patience at all times. You were there cheering me up and stood by me through the good times and bad. To my lovely Hanna, please keep Mama strong and make my world wonderful every day and night. To my parents, Nasruddin Husin and Norizan Hamid, thank you so much for your unequivocal support throughout, as always, for which my mere expression of thanks likewise does not suffice. The patience and understandings shown by my siblings, Norhidayah, Hajidah, Nasirah and Shafiqah during the honour years is greatly appreciated.

A major research project like this is never the work of anyone alone. The contributions of many different people, in their different ways, have made this possible. I would like to extend my appreciation especially to the following:

- Dr. Mazlina Mazlan: who as a good friend was always willing to help and bearing the peaks and valleys throughout our journey.
- Dr. Mohd Hezmee Mohd Noor: thanks so much for taking the time to help with our cell line.
- Members of the Histopathology Laboratory, Post Mortem Unit, Large Animal Experimental House Unit and Virology Laboratory, Faculty of Veterinary Medicine (UPM), Assoc. Prof. Dr. Md. Sabri Mohd Yusoff, Dr. Shahirudin Shamsudin, Dr. Muhd Taqiyudin Zainal Ulum, Mrs. Jamilah Jahari, Mrs. Latifah Hanan, Mr. Mohd Jamil Samad, Mr. Ghazali Md Yusoff, Mr. Apparau Somanaidu, Mr. Mohd Najib Yahya, Mr.

Noraziman Sulaiman, Mr. Ariff Ahmad, Mrs. Nurzatul Iffah Ahmad and Mr. Kamaruddin Awang Isa. My special thanks are extended to these great persons who have assisted me and making the facilities available during the research works.

- Post-graduate friends; Dr. Nur Adzarina Md Radzi, Dr. Annas Salleh, Mr. Mohd. Firdaus Nawi, Dr. Nur Nazifah Mansor, Ms. Noraini Omar, Mrs. Illazuwa Mohd Yusoff, Ms. Shafiqah Adilah Saadun, Ms. Roslindawani Md Nor, Ms. Nur Hazwani Oslan, Dr. Didik Handijatno, Dr. Sriyanto, Dr. Yulianna Puspitasari, Dr. Hani Plumeriastuti, Dr. Mohd Shahrom Salisi, Dr. Rafidah Othman and Dr. Abu Bakar Salisu. Their support during the present work is gratefully acknowledged.
- My in laws families: Mr. Khairuddin Abd. Manap. Mrs. Kaushar Harun, , Mr. Azmai Mohamad, Mrs. Siti Zubaidah Ahmad Zuhari, Mr. Mohamad Azraz Azmai, Ms. Nur Izzati Khairuddin, Mr. Mohammad Ihsan Khairuddin, Mrs. Nurhasanah Bustami, Mr. Rafiq Danial Mohd. Azraz, Ms. Nisa Qaisara Mohd. Azraz and Mr. Rifqi Haqeem Mohd Azraz. I warmly appreciate the kindness and understanding of the families.
- My friends: Dr. Noraini Warzukni, Dr. Siti Suzana Selamat, Dr. Noor Idzatul Khairiah Ithnin, Dr. Fauziah Mohd Said, Dr. Nurazreen Zulaidi, and Kitty's Cottage Staffs: Dr. Azlan Shah Abdul Ghani, Dr. Farhana, Dr. Naim, Nas, Farah and Syuhada. I wish to thank the support and encouragement given.

Finally, I acknowledge the Ministry of Education, Malaysia (MyPhD) and Universiti Putra Malaysia (Graduate Research fellowship) for providing me the necessary financial support.

I certify that a Thesis Examination Committee has met 9 September 2014 to conduct the final examination of Nurrul Shaqinah Nasruddin on her thesis entitle "**Pathology and Pathogenesis of *Brucella melitensis* Infection in Bucks**" 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 Doctor of Philosophy.

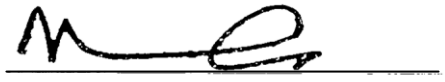
Member of the Thesis Examination Committee were as follows:

Md. Zuki Abu Bakar @ Zakaria, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Siti Khairani Bejo, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Md Sabri Mohd Yusoff, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Corrie Brown, PhD
Professor
College of Veterinary Medicine
The University of Georgia
UNITED STATES
(External Examiner)



NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 September 2014

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Mohd Zamri Saad, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Jasni Sabri, PhD
Professor
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Member)

Hazilawati Hamzah, PhD
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- This thesis is my original work;
- Quotations, illustrations and citations have been duly referenced;
- This thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- Intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- Written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- There is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rule 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

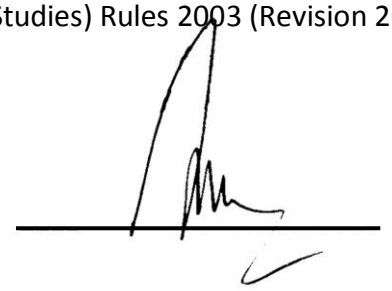
Signature: _____ Date: _____

Name and Matric No.: Nurrul Shaqinah Nasruddin GS 29489

Declaration by Members of Supervisory Committee

This is to confirm that:

- The research conducted and the writing of the thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: 
Name of Chairman of Supervisory Committee: PROF. DR. MOHD ZAMRI SAAD

Signature: 
Name of Member of Supervisory Committee: PROF. DR. JASNI SABRI

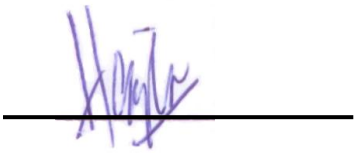
Signature: 
Name of Member of Supervisory Committee: DR. HAZILAWATI HAMZAH

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µl	Micro liter
APC	Antigen presenting cell
BCV	*Brucella*-containing vacuoles
Bp	Base pair
CFT	Complement Fixation Test
CFU	Colony Forming Units (bacteria)
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
Dntp	Deoxynucleotide triphosphate
EDTA	Ethyenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal bovine serum
g	Gravitational force
H&E	Hematoxylin and Eosin
H_2S	Hydrogen sulfide
ICI	Inflammatory cells infiltration
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
In vitro	In an experimental situation outside the organism. Biological or chemical work done in the test tube rather than in living systems
IP	Immuno peroxidase staining
LAMP	Lysosomal-associated membrane protein
LN	lymph node
LPS	Lipopolysaccharide
M	Molar
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
NET	Neutrophilic extracellular trap
OD	Optical density
OMP	Outer membrane protein
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Post infection
RBPT	Rose Bengal Plate Test
RM	Malaysian Ringgit
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute Medium
TBE	Tris-boric EDTA
Tc	T cytotoxic

Th	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor
U	unit
USD	United States Dollar
v/v	Volume per volume
Y	Gamma
α	Alpha

CHAPTER 1

INTRODUCTION

Caprine brucellosis is caused by *Brucella melitensis*, the most virulent *Brucella* species (Barbier *et al*., 2011). The disease is one of the major causes of reproductive related problems in goats and is becoming an important zoonotic infection in Malaysia (Bamaiyi *et al*., 2010). The disease is proved to be well distributed throughout the country and the trend of seroprevalence among animals is increasing every year (Bamaiyi *et al*., 2010). Although *B. melitensis* is known to infect goats and sheep, it can also infect other animal species such as cows, camels and buffalo (Blasco and Moriyon, 2010). Furthermore, *B. melitensis* is highly zoonotic and responsible for most of human brucellosis (Franco *et al*., 2007), which is presented with undulant fever, arthralgia, back pain and in chronic cases, abscess may developed in any organs such as liver, spinal cord, meninges and others (Hartady *et al.,* 2014).

Goat suffering from brucellosis will demonstrate a systemic clinical feature but prominently on the reproductive system. Infected does exhibit abortion, stillbirth, retained placenta, metritis or sub clinical mastitis. Infected bucks endure arthritis, orchitis and epididymitis (Eaglesome and Garcia, 1992; Xavier *et al.,* 2010). Most research activities were focussed on the reproductive organs of female animals infected with *B. abortus* rather than *B. melitensis* (Poester *et al*., 2006; Xavier *et al*., 2009). Infections are believed to occur by ingestion of particles contaminated by those animal excretion, consumption of contaminated colostrum or milk and the organism can be sexually transmitted although the rate of occurrence is low (SCAHAW, 2001). However, lesser histopathological studies have been conducted in bucks infected with brucellosis. Only several investigations were done exclusively to describe the lesions in male goats (Izadjoo *et al.*, 2008; Carvalho *et al*., 2012). In addition, immunoperoxidase (IP) technique is known to be an important tool to show the presence of antigen and its localization, thus it is recognized as the sensitive and specific test to detect *Brucella* antigen (Ilhan and Yener, 2008). Indeed, the technique has the capability to reveal the relationship of severity of the histopathology lesions and the antigen distribution in the tissues (Haritani *et al*., 1989).

The predilection sites of *B. melitensis* in female animals have been well documented (Keppie *et al*., 1965; SCAHAW, 2001). Nevertheless, few studies proved the predilection site of *B. melitensis* in male animals. Study by SCAHAW (2001) reported the localization of *B. melitensis* in the testis and epididymis, while *B. ovis* infection in sheep revealed localization in the epididymis rather in testis (Júnior *et al*., 2012). Isolation of the *Brucella* from any clinical specimens or post mortem samples is the gold standard for diagnosis of brucellosis (Lang *et al*., 1995). Blood culture is one of the suggestive methods to isolate the *Brucella* in canine (Carmichael and Kenney, 1970) and human cases (Colmenero *et al*., 2002). However, the procedure is unlikely to be used in other animals since the disease induces shorter bacteraemia as opposed to the canine and human brucellosis (Xavier *et al*., 2010). The

organs collected during post mortem should be handled with extra care to prevent exposure of the organism to the personnel involved. Direct isolation of *B. melitensis* is performed on selective media to enhance the growth and to ensure enough nutrient supply to the colonies (OIE, 2009). The polymerase chain reaction (PCR) technique is a powerful technique to be used since it is a specific and sensitive tool for identification. Furthermore, PCR technique is useful in identification of causative agent especially for any tedious microorganism such as *B. melitensis,* which required long incubation time. Thus, application of PCR in diagnosis of brucellosis may enhance the efficiency of the national control and eradication program.

The ability to cause persistent infection animals and humans is the unique characteristic of intracellular bacteria such as *B. melitensis* (Sangari and Aguero, 1996). The professional phagocytes, which comprised of neutrophils and macrophages are functioned to engulf, kill and disposal of pathogens (Lee *et al*., 2003). The capabilities of *Brucella* to replicate, to transmit to new host cells in intracellular environment and to avoid the immune detection make this pathogen to often be referred as 'Mr. Hides' (Gorvel, 2008). The main survival criteria in phagocytic cells is that *Brucella* incorporates itself into phagosomes after being engulfed and remains in the cells as a hiding site as well as a mechanism of transportation. In non-professional phagocyte cells, *Brucella* changes its method by residing itself in the endoplasmic reticulum (Arenas *et al*., 2000).

Neutrophils are the essential innate immune cell that quickly gathered at the site of infection with an important purpose; to ingest microbes and eventually kill them (Appelberg, 2006). Classically, it was thought that the main role of neutrophils in defensive system is to fight mainly the extracellular pathogens but recent study showed that the neutrophils also important in controlling the intracellular pathogens by initiate an adaptive immune system and bridging the neutrophil and macrophage cooperation to kill the intracellular bacteria (Appelberg, 2006). Correspondingly, it has been proved that the neutrophils are capable to response rapidly in order to phagocytize *Brucella* (Gallego and Lapena, 1990). It is important to realize that neutrophils are able to serve as transport medium for the engulfed pathogen to the lymphatic circulation with the purpose of enhancing the adaptive immune response in order to kill them (Abadie *et al.,* 2005; Maletto *et al.,* 2006). So far, however, there has been little discussion on the quantification of phagocytosis activity of neutrophils derived from bucks against *B. melitensis* except for the study done by Gallego and Lapena (1990).

The macrophages, also known as scavenger cells play an important role in defence mechanism. This professional phagocytic cell is known to be an important character in cellular immune system during battling the intracellular bacteria such as *Brucella*. Following ingestion, the activated macrophages induce bactericidal properties such as degradative hydrolytic enzyme, phagolysosomes acidification, cationic peptide and oxidative burst to kill *Brucella* (Gross *et al.,* 2004; Baldwin and Goenka, 2006). However, *Brucella* has a unique mechanism to prevent and resist the attack of these phagocytic cells (Köhler *et al*., 2002). Consequently, when these bactericidal properties failed to be executed, the macrophages trigger its own apoptosis process to prevent any intracellular replication, but unfortunately, *Brucella* is capable to prevent the host cell apoptosis, which resulted in persistence infection (Monack *et al*., 1997; Weinrauch and Zychlinsky, 1999). The preventive mechanism has been

demonstrated in *B. suis* infection in human macrophages (Gross *et al.,* 2000). Fortunately, *Brucella* also has its own weakness, which was proven by Dornand *et al.* (2002) who revealed that the survived *Brucella* was sensitive to the macrophages killing activity that was activated through Th1, and with the incorporation of IFN-α and cytotoxic T cells.

Besides phagocytic cells, *Brucella* can also invade other immune cells such as lymphocytes (Velásquez *et al*., 2012). It has been known that the only interaction between *Brucella* and T lymphocytes is through expression of Major Histocompatibility Complex Class II (MHC II), on toll-like receptor 2 (TLR2) (Barrionuevo *et al*., 2008). However, recent study proved the ability of *Brucella* to directly interfere with lymphocytes (Velásquez *et al.,* 2012). Back in 1981, Bratescu *et al*. (1981) provided evidence on the capability of *Brucella* to bind to the surface of B lymphocytes and suggested that the process might have some influence in pathogenesis of human brucellosis. The vast selection of invasion and preventive mode of *Brucella* make the eradication process by immune system harder than usual.

Caprine brucellosis remains endemic in many parts of the world except a few countries such as Canada, Australia, Cyprus, Finland, Denmark, United Kingdom, The Netherland, Norway, Sweden and New Zealand (Bamaiyani *et al*., 2012). In Malaysia, the caprine brucellosis was considered a re-emerging disease following extensive importation of goats into the country (Ibrahim *et al*., 1988; Zamri-Saad and Shafarin, 2007). Thus, the government has implemented a 'test and slaughter' policy in order to eradicate the disease. A comprehensive surveillance program was carried out to detect and monitor cases using the Rose Bengal Plate Test (RBPT) as screening test and the Complement Fixation Test (CFT) as the gold standard protocol for confirmation. Combination of these serological tests was required to reach a final diagnostic evaluation for brucellosis (de Oliveira *et al*., 2011).

Enzyme linked imunosorbent assay (ELISA) was considered as a meaningful tool to comprehend the current diagnostic tools in performing diagnostic activity for caprine brucellosis (García-Bocanegra *et al*., 2014). Although the gold standard for diagnosis of brucellosis is isolation and identification, serological test such as ELISA is an important routine serological test for brucellosis control and eradication program (EFSA, 2006). Efforts have been made to develop tests such as indirect ELISA, blocking ELISA and competitive ELISA that functioned in improving serological detection assay for caprine brucellosis (Minas *et al*., 2005; Garin-Bastuji *et al.,* 2006). According to OIE (2009), ELISA that used high content of smooth lipopolysaccharide as antigen produces better diagnostic result. In addition, the indirect ELISA produces more sensitive results, while competitive ELISA demonstrated similar sensitivity as other conventional serology tests including RBPT and CFT (OIE, 2009). On top of that, ELISA can be used to test desired immunoglobulin titration such as IgG, IgM and IgA. Ig G is an important antibody isotype found in the serum, which is widely used as indicative of immune status towards specific pathogen. Thus, elevation of IgG level with a combination of other immunoglobulin may help to indicate the chronicity of brucellosis (Lulu *et al*., 1988). On the other hand, IgM becomes one of the highest concentrations after IgG. It plays vital role in complement activation and opsonisation process, which play important roles in immunity response against brucellosis (Tizard, 2000). The IgA is an important defence mechanism at the mucosal surface against invading pathogens. Because of *B. melitensis* can

be transmitted through ingestion and mating activity, it is believed that the IgA level at the genital tract may reduce the presence of microorganism during shading (SCAHAW, 2001).

Problem statements

Generally, study and understanding on the pathology and disease development following *B. melitensis* infection in bucks are still lacking, particularly on the distribution of the organism within the host. Similarly, *B. melitensis* has been recognised as an intracellular bacterium that can survive intracellular killing. However, the phagocytosis and killing efficiencies by the neutrophils, macrophages and lymphocytes of exposed and unexposed bucks had never been studies and compared. Thus, understanding of these unique capabilities may improve the knowledge of the disease. Furthermore, the RBPT and CFT have been used in identifying goats naturally infected with *B. melitensis,* which usually chronic in nature. Their effectiveness in detecting acute infection must be studied to help in disease control.

Hypothesis

Consequently, the hypothesis for the study are:

1. bucks experimentally infected with *B. melitensis* at 14 days show significantly more severe lesions and more generalised distribution of the organism than at 7 days of infection
2. phagocytosis and intracellular killing activities by the phagocytic cells derived from exposed group are significantly higher than the non-exposed group
3. Rose Bengal Plate Test and Complement Fixation Test are capable to detect acute brucellosis

Objectives

Thus, this study was conducted with the following objectives:

1. to observe the pathological changes in bucks following experimental infection by *B. melitensis*
2. to determine the distribution of *B. melitensis* in the organs and tissues of bucks following experimental infection
3. to evaluate the phagocytosis and intracellular killing capability of neutrophils and macrophages derived from exposed and non-exposed bucks
4. to determine the penetration and survival capability of *B. melitensis* in lymphocytes of bucks
5. to evaluate the efficacy of commonly available diagnostic tests in detecting experimental caprine brucellosis

REFERENCES

Abadie, V., Badell, E., Douillard, P., Ensergueix, D., Leenen, P.J.M., Tanguy, M., Fiette, L., Saeland, S., Gicquel, B. and Winter, N. (2005). Neutrophils rapidly migrate via lymphatics after *Mycobacterium bovis* BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood* 106: 1843–1850.

Abdoel, T., Dias, I.T., Cardoso, R. and Smits, H.L. (2008). Simple and rapid field tests for brucellosis in livestock. *Veterinary Microbiology* 130: 312-319.

Abdullah, F.F.J., Adamu, L., Hazirah, N., Osman, A.Y., Rozaihan, Y., Harun, A.W., Zamri-Saad, M., Omar, A.R. and Saharee, A.Z. (2013). Clinical and reproductive pathological changes associated with *Brucella melitensis* and its lipopolysaccharides in female mice via oral inoculation. *American Journal of Animal and Veterinary Science* 8: 104-111.

Abraham, S.N. and Beachey, E.H. (1985). Host defenses against adhesion of bacteria to mucosal surfaces. In: Gallin, J.F. and Fauci, A.S. (Eds.) Advances in Host Defense Mechanisms. New York, Raven Press. Pp: 63–88.

Acha, N.P. and Szyfres, B. (2003). Zoonoses and communicable diseases common to man and animals, 3rd ed., Washington, DC, Pan American Health Organization (PAHO).

Akhtar, M. (2001). Histopathological features. In: Madkour, M.M. (Ed.) Madkour's Brucellosis, 2nd ed., Berlin, Springer-Verlag. Pp: 65-73.

Al-Garadi, M.A., Khairani-Bejo, S., Zunita, Z. and Omar, A.R. (2011). Detection of *Brucella melitensis* in blood samples collected from goats. *Journal of Animal and Veterinary Advances* 10(11): 1437-1444.

Alton, G.G. (1990). *Brucella melitensis*. In: Nielsen, K. and Duncan, J.R. (Eds.) Animal Brucellosis. Boca Raton, CRS Press Inc. Pp: 383-409.

Alton, G.G., Jones, L.M., Angus, R.D. and Verger, J.M. (1988). Techniques for the Brucellosis Laboratory. Paris, INRA.

Andreesen, R., Scheibenbogen, C., Brugger, W., Krause, S., Meerpohl, H.G., Leser, H.G. and Engler, G.W. (1990). Adoptive transfer of tumor cytotoxic macrophages generated *in vitro* from circulating blood monocytes: a new approach to cancer immunotherapy. *Cancer Research* 50: 7450.

Appelberg, R. (2006). Neutrophils and intracellular pathogens: beyond phagocytosis and killing. *TRENDS in Microbiology* 15(2):87-92.

Araj, G.F., Lulu, A.R., Mustafa, M.Y. and Khateeb, M.I. (1986). Evaluation of ELISA in the diagnosis of acute and chronic brucellosis in human beings. *The Journal of Hygiene* 97:457–469.

Arenas, G.N., Staskevich, A.S., Aballay, A. and Mayorga L.S. (2000). Intracellular trafficking of *Brucella abortus* in J774 macrophages. *American Society for Microbiology* 68: 4255–4263.

Ariza, J., Pellicer, T., Pallarés, R. and Gudiol, F. (1992). Specific antibody profile in human brucellosis. *Clinical infection diseases* 14: 131-140.

Armstrong, J.A. and Hart, P.D. (1971). Response of cultured macrophages to *Mycobacterium tuberculosis* with observations on fusion of lysosomes with phagosomes. *The Journal of Experimental Medicine* 134: 713-740.

Asmare, K., Sibhat, B., Molla, W., Ayelet, G., Shiferaw, J., Martin, A.D., Skjerve, E. and Godfroid, J. (2013). The status of bovine brucellosis in Ethiopia with special emphasis on exotic and cross bred cattle in dairy and breeding farms. *Acta Tropica* 126: 186–192.

Bahaman, A.R., Joseph, P.G. and Siti-Khairani, B. (2007). A review of the epidemiology and control of brucellosis in Malaysia. *The Malaysian Journal of Veterinary Research* 19(1):1-6.

Baily, G.G., Krahn, J.B., Drasar, B.S. and Stocker, N.G. (1992). Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *The American Journal of Tropical Medicine and Hygiene* 95: 271–275.

Baldi, P.C., Wanke, M.M., Loza, M.E. and Fossati, C.A. (1994). *Brucella abortus* cytoplasmic proteins used as antigens in an ELISA potentially useful for the diagnosis of canine brucellosis. *Veterinary Microbiology* 41: 127–134.

Baldwin, C.L. and Goenka, R. (2006). Host immune responses to the intracellular bacteria *Brucella:* does the bacteria instruct the host to facilitate chronic infection?. *Critical Reviews in Immunology* 26: 407-442.

Bamaiyi, P.H., Hassan, L., Khairani-Bejo, S., Zainal Abidin, M., Ramlan, M., Krishnan, N., Adzhar, A., Abdullah, N., Hamidah, N.H.M., Norsuhanna, M.M. and Hashim, S.N. (2012). Isolation and molecular characterization of *Brucella melitensis* from seropositive goats in Peninsular Malaysia. *Tropical Biomedicine* 29(4): 513–518.

Bamaiyi, P.H., Hassan, L., Siti-Khairani, B., Adzhar, A. and Rachmat, R.F.N. (2010). The seroprevalence of *Brucella melitensis* in goats of Malaysia from year 2000 to 2008 In: 22nd Veterinary Association of Malaysia Congress and 4th Wildlife Society of Zoo and Wildlife Medicine International Meeting.

Barbier, T., Nicolas, C. and Letesson, J.J. (2011). *Brucella* adaptation and survival at the crossroad of metabolism and virulence. *Federation of European Biochemical Societies Letters* 585: 2929–2934.

Barquero-Calvo, E., Chaves-Olarte, E., Weiss, D.S., Guzman-Verri, C., Chacon-Diaz, C., Rucavado, A., Moriyon, I. and Moreno, E. (2007). *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. *PLoS One* 2(7): e631.

Barrionuevo, P., Cassataro, J., Delpino, M.V., Zwerdling, A., Pasquevich, K.A., Garcia Samartino, C., Wallach, J.C., Fossati, C.A. and Giambartolomei, G.H. (2008). *Brucella abortus* inhibits major histocompatibility complex class II expression and antigen

processing through interleukin-6 secretion via Toll-like receptor 2. *Infection and Immunity* 76: 250-262.

Biancifiori, F., Garrido, F., Nielsen, K., Moscati, L., Durán, M. and Gall, D. (2000). Assessment of a monoclonal antibody-based competitive enzyme linked immunosorbent assay (cELISA) for diagnosis of brucellosis in infected and Rev. 1 vaccinated sheep and goats. *New Microbiology* 23: 399–406.

Billard, E., Cazevieille, C. and Dornand, J. (2005). High susceptibility of human dendritic cells to invasion by the intracellular pathogens *Brucella suis, B. abortus,* and *B. melitensis*. *Infection and Immunity* 73: 8418-8424.

Billard, E., Dornand, J. and Gross, A. (2007). *Brucella suis* prevents human dendritic cell maturation and antigen presentation through regulation of tumor necrosis factor alpha secretion. *Infection and Immunity* 75: 4980-4989.

Birmingham, J.R. and Jeska, E.L. (1981). Characterization of macrophage functions in mice infected with *Brucella abortus. Infection and Immunity* 32(3): 1079-1083.

Blasco, J.M. (1997). A review of the use of *B. melitensis* Rev.1 vaccine in adult sheep and goats. *Preventive Veterinary Medicine* 31: 275-281.

Blasco, J.M. and Moriyon, I. (2010). Eradication of bovine brucellosis in the Azores, Portugal-outcome of a 5-year programme (2002–2007) based on test-and-slaughter and RB51 vaccination. *Preventive Veterinary Medicine* 94: 154–157.

Blasco, J.M., Garin-Bastuji, B., Marín, C. M., Gerbier, G., Fanlo, J., Jiménez de Bagüés, M. P. and Cau, C. (1994). Efficacy of different rose bengal and complementof fixation antigens for the diagnosis of *Brucella melitensis* in sheep and goats. *Veterinary Record* 134: 415-420.

Boom, R., Sol, C.J. and Salimans, M.M. (1990). Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology* 28: 495–503.

Bounaadja, L., Albert, D., Chénais, B., Hénault, S., Zygmunt, M.S., Poliak, S. and Garin-Bastuji, B. (2009). Real-time PCR for identification of *Brucella* spp.: a comparative study of IS711, BCSP31 and per target genes. *Veterinary Microbiology* 137: 156–164.

Bratescu, A., Mayer, E. and Teodorescu, M. (1981). Binding of bacteria from the genus *Brucella* to human B lymphocytes. *Infection and Immunity* 31: 816–821.

Bricker, B.J. (2002). PCR as a diagnostic tool for brucellosis. *Veterinary Microbiology* 90: 435-446.

Bricker, B.J. and Halling, S.M. (1994). Differentiation of *B. abortus* bv. 1, 2, and 4, *B. melitensis, B. ovis,* and *B. suis* bv. 1 by PCR. *Journal of Clinical Microbiology* 32: 2660-2666.

Brown, S.A., Palmer, K.L. and Whiteley, M. (2008). Revisiting the host as a growth medium. *Nature Reviews Microbiology* 6: 657–666.

Bukharie, H.A. (2009). Clinical features, complications and treatment outcome of *Brucella* infection: ten years' experience in an endemic area. *Tropical Journal of Pharmaceutical Research* 4: 303-310.

Campbell, G.A., Adams, L.G. and Sowa, B.A. (1994). Mechanism of binding of *Brucella abortus* to mononuclear phagocytes from cows naturally resistant or susceptible to brucellosis. *Veterinary Immunology and Immunopathology* 41: 295-306.

Campos, M.A., Rosinha, G.M., Almeida, I.C., Salgueiro, X.S., Jarvis, B.W., Splitter, G.A., Canning, P.C., Roth, J.A., Tabatabai, L.B. and Deyoe, B.L. (1985). Isolation of components of *Brucella abortus* responsible for inhibition of function in bovine neutrophils. *Journal of Infection* 152: 913-921.

Canning, P.C., Roth, J.A., Tabatabai, L.B. and Deyoe, B.L. (1985). Isolation of components of *Brucella abortus* responsible for inhibition of function in bovine neutrophils. The Journal of Infectious Diseases 152(2): 913-921.

Carbonare, S.B., Silva, M.L.M., Trabulsi, L.R. and Carneiro-Sampaio, M.M.S. (1995). Inhibition of HEP-2 cell invasion by entero invasive *Escherichia coli* by human colostrum IgA. *International Archives of Allergy and Immunology* 108: 13–118.

Cardoso, P.G., Macedo, G.C., Azevedo, V. and Oliveira, S.C. (2006). *Brucella* spp noncanonical LPS: structure, biosynthesis, and interaction with host immune system. *Microbial Cell Factories* 5: 13.

Carmichael, L.E. (1990) *Brucella canis*. In: Nielsen, K.H. and Duncan, J.R. (Eds.) Animal Brucellosis. Boca Raton, CRC Press. Pp. 335-350.

Carmichael, L.E. and Kenney, R.M. (1970). Canine brucellosis: the clinical disease, pathogenesis and immune response. *Journal of American Veterinary Medicine Association* 156: 1726-1734.

Carmichael, L.E. and Shin, S.J. (1996). Canine brucellosis: a diagnostician's dilemma. *Seminars in Veterinary Medical and Surgery (Small Animals*) 1: 161–165.

Caron, E., Gross, A. and Liautard, J.P. (1996). *Brucella* species release a specific, protease sensitive inhibitor of TNF expression, active on human macrophage-like cells. *Journal of Immunology* 156: 2885-2893.

Caron, E., Peyrard, T. and Kohler, S. (1994). Live *Brucella* spp fail to induce tumor necrosis factor alpha excretion upon infection of U937-derived phagocytes. *Infection and Immunity* 62: 5267–5274.

Carvalho, C.A.C., Moustacas, V.S., Xavier, M.N., Costa, E.A., Costa, L.F., Silva, T.M.A., Paixão, T.A., Borges, A.M., Gouveia, A.M.G. and Santos, R.L. (2012). Andrological, pathologic, morphometric, and ultrasonographic findings in rams experimentally infected with *Brucella ovis. Small Ruminant Research* 102: 213– 222.

Cassataro, J., Velikovsky, C.A., de la Barrera, S., Estein, S.M., Bruno, L. and Bowden, R. (2005). A DNA vaccine coding for the *Brucella* outer membrane protein 31 confers

protection against *B. melitensis* and *B. ovis* infection by eliciting a specific cytotoxic response. *Infection and Immunity* 73(10): 6537–6546.

Celli, J. (2006). Surviving inside a macrophage: the many ways of *Brucella. Research in Microbiology* 157: 93–98.

Celli, J., De Chastellier, C., Franchini, D.M., Pizarro-Cerda, J., Moreno, E. and Gorvel, J.P. (2003). *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. *The Journal of Experimental Medicine* 198: 545–556.

Cesta, M.F. (2006). Normal structure, function, and histology of mucosa-associated lymphoid tissue. *Toxicologic Pathology* 34(5): 599-608.

Chand, P., Sadana, J.R., Batra, H.V. and Chauhan, R.S. (1989). Comparison of the dot-immunobinding assay with the complement fixation test for the detection of *Brucella* antibodies in heep. *Veterinary Microbiology* 20: 281-287.

Cheers, C. (1984). Pathogenesis and cellular immunity in experimental murine brucellosis. *Developments in Biological Standardization* 56: 237–246.

Chertov, O., Ueda, H., Xu, L.L., Tani, K., Murphy, W.J. and Wang, J.M. (1997). Identification of human neutrophil derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *The Journal of Experimental Medicine* 186: 739-747.

Clavareau, C., Wellemans, V. and Walravens, K. (1998). Phenotypic and molecular characterization of a *Brucella* strain isolated from a minke whale (*Balaenoptera acutorostrata*). *Microbiology* 144: 3267-3273.

Cloeckaert, A., Verger, J.M., Grayon, M. and Vizcano, N. (1996). Molecular and immunological characterization of the major outer membrane proteins of *Brucella. Federation of European Microbiological Society Microbiology Letters* 145: 1–8.

Coboa, E.R., Corbeila, L.B., Gershwinc, L.J. and Bon Durant, R.H. (2010). Preputial cellular and antibody responses of bulls vaccinated and/or challenged with *Tritrichomonas foetus. Vaccine* 28: 361–370.

Cohen, J.J., Duke, R.C., Fadok, V.A. and Sellins, K.S. (1992). Apoptosis and programmed cell death in immunity. *Annual Review of Immunology* 10: 267-293.

Colmenero, D., Queipo-Ortunõ, M.I., Maria Reguera, J., Angel Suarez-Munõz, M., Martiń-Carballino, S. and Morata, P. (2002). Chronic hepatosplenic abscesses in brucellosis. Clinico-therapeutic features and molecular diagnostic approach. *Diagostic Microbiology and Infectious Diseases* 42:159–167.

Comerci, D.J., Altabe, S. and de Mendoza, D. (2006). *Brucella abortus* synthesizes phosphatidylcholine from choline provided by the host. *Journal of Bacteriology* 188: 1929-1934.

Conde-Alvarez, R., Grilló, M.J. and Salcedo, S.P. (2006). Synthesis of phosphatidylcholine, a typical eukaryotic phospholipid, is necessary for full virulence of the intracellular bacterial parasite *Brucella abortus. Cellular Microbiology* 8: 1322-1335.

Confer, A.W., Hall, S.M. and Faulkner, C.B. (1985). Effects of challenge dose on the clinical and immune responses of cattle vaccinated with reduced doses of *Brucella abortus* strain 19. *Veterinary Microbiology* 10: 561–575.

Conlan, J.W. and North, R.J. (1992). Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. *Infection and Immunity* 60: 5164-5171.

Corbeil, L.B., Blau, K., Inzana, T.J., Nielsen, K.H., Jacobson, R.H., Corbeil, R.R. and Winter, A.J. (1988). Killing of *Brucella abort*us by bovine serum. *Infection and Immunity* 56: 3251–3261.

Corbel, M.J. (1997). Brucellosis: an overview. *Emerging Infectioud Disease* 3(2): 213-221.

Corbel, M.J. (2006). Brucellosis: an overview. *Emerging Infectious Diseases* 3: 213-221.

Cravioto, A.,Tello, A., Villafan, H., Ruiz, J., del Vedovo, S., and Neeser, J.R. (1991). Inhibition of localized adhesion of enteropathogenic *Escherichia coli* to HEP-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. *The Journal of Infectious Disesaes* 163: 1247–1255.

Cutler, S.J., Whatmore, A.M. and Commander, N.J. (2005). Brucellosis-new aspects of an old disease. *Journal of Applied Microbiology* 98: 1270-1281.

Czuprynski, C.J. and Brown, J.F. (1990). Effects of purified anti-Lyt-2 mAb treatment on murine listeriosis: comparative roles of Lyt-2 and L3T4 cells in resistance to primary and secondary infection, delayed-type hypersensitivity and adoptive transfer for resistance. *Immunology* 71: 107-112.

de Oliveira, M.Z.D., Vale, V., Keid, L., Freire, S.M., Meyer, R., Portela, R.W. and Barrouin-Meloa, S.M. (2011). Validation of an ELISA method for the serological diagnosis of canine brucellosis due to *Brucella canis. Research in Veterinary Science* 90: 425–431.

Debbarh, H.S.A., Zygmunt, M., Dubray, G. and Cloeckaert, A. (1996). Competitive enzyme-linked immunosorbent assay using monoclonal antibodies to the *B. melitensis* BP26 protein to evaluate antibody response in infected and *B. melitensis* Rev-1 vaccinated sheep. *Veterinary Microbiology* 53: 325-337.

Delpino, M.V., Barrionuevo, P., Scian, R., Fossati, C.A. and Baldi, P.C. (2010). *Brucella*-infected hepatocytes mediate potentially tissue-damaging immune responses. *Journal of Hepatology* 53: 145-154.

Delvecchio, V.G., Kapatral, V. and Elzer, P. (2002). The genome of *Brucella melitensis. Veterinary Microbiology* 90: 587-592.

Department of Veterinary Sevices (DVS) (2011). Malaysia: Livestock Population 2005-2011. 2012, from http://www.dvs.gov.my/c/ document_library / get_file? uuid=2ddf02a7 (Retrieved: 18-07-2012).

Díaz, R., Jones, L.M., Leong, D. and Wilson, J.B. (1968). Surface antigens of smooth *Brucellae. Journal of Bacteriology* 96: 893-901.

Dornand, J., Gross, A., Lafont, V., Liautard, J. and Liautard, J.P. (2002). The innate immune response against *Brucella* in humans. *Veterinary Microbiology* 90: 383.

Eaglesome, M.D. and Garcia, M.M. (1992). Microbial agents associated with bovine genital tract infection and semen. Part I. *Brucella abortus, Leptospira, Campylobacter fetus* and T*ritrichomonas foetus. Veterinary Bulletin* 62: 743-775.

Edmonds, M.D., Samartino, L.E., Hoyt, P.G., Hagius, S.D., Walker, J.V., Enright, F.M., Schurig, G.G. and Elzer, P. (2001). Oral vaccination of sexually mature pigs with *Brucella abortus* vaccine strain RB51. *American Journal of Veterinary Research* 62: 1328–1331.

Elberg, S.S. (1996). Rev 1 *B. melitensis* vaccine. Part III 1981-1995. *Veterinary Bulletin* 66: 1193-1200.

Elmore, S.A. (2006). Enhanced histopathology of the spleen. *Toxicologic Pathology* 34: 648–655.

Emikpe, B.O., Tanko, P.N., Onilude O.M. and Sabri, M.Y. (2013). The influence of dexamethasone treatment and successive road transport stress on the occurrence of caprine pneumonia in a hot humid tropical environment. *Veterinary World* 6: 497-501.

Enright, F. and Duncan, J. (1990). The pathogenesis and pathobiology of *Brucella* infection in domestic animals. In: Nielsen, K. (Ed.) Animal Brucellosis, Boca Raton, CRC Press. Pp: 301-320.

Escande, A. and Serre, A. (1982). IgE anti-*Brucella* antibodies in the course of human brucellosis and after specific vaccination. *International archive of allergy and immunology* 68:172–175.

European Food Safety Authority (EFSA). (2006). Scientific opinion on performance of brucellosis diagnostic methods for bovines, sheep, and goats. *The EFSA Journal* 432: 1–44.

Ewalt, D.R., Payeur, J.B. and Rhyan, J.C. (1997). *Brucella suis* biovar 1 in naturally infected cattle: a bacteriological, serological and histological study. *Journal of Veterinary Diagnostic Investigation* 9: 417-420.

Ewalt, D.R., Payeur, J.B., Martin, B.M., Cummins, D.R. and Miller, W.G. (1994). Characteristics of a *Brucella* species from a bottlenose dolphin *(Tursiops truncatus). Journal of Veterinary Diagnostic Investigation* 6:448–452.

Fensterbank, R. (1987). Some aspects of experimental bovine brucellosis. *Annales De Recherches Veterinaires* 18: 421-428.

Fernandes, D.M., Jiang, X., Jung, J.H. and Baldwin, C.L. (1996). Comparison of T cells in resistant and susceptible mice infected with virulent *Brucella abortus* strain 2308. *Federation of European Microbiological Society Immunology and Medical Microbiology* 16: 193-203.

Ferrero, M.C., Bregante, J., Delpino, M.V., Barrionuevo, P., Fossati, C.A., Giambartolomei, G.H. and Baldi, P.C. (2011). Proinflammatory response of human endothelial cells to *Brucella i*nfection. *Microbes and Infection* 13: 852-861.

Forestier, C., Deleuil, F., Lapaque, N., Moreno, E. and Gorvel, J.P. (2000). *Brucella abortus* lipopolysaccharide in murine peritoneal macrophages acts as a down-regulator of T cell activation. *The Journal of Immunology* 165: 5202-5210.

Forsgren, A. and Quie, P.G. (1974). Influence of the alternate complement pathway in opsonization of several bacterial species. *Infection and Immunity* 10(2): 402–404.

Foster, G., Osterman, B.S. and Godfroid, J. (2007). *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *International Journal of Systematic and Evolutionary Microbiology* 257: 2688-2693.

Franco, M.P., Mulder, M., Gilman, R.H. and Smits, H.L. (2007). Human brucellosis. *Lancet Infection Disease* 7: 775–786.

Gallego, M.C and Lapena, M.A. (1990). The interaction of *Brucella melitensis* 16-M and caprine polymorphonuclear leukocytes. *Comparative Immunology, Microbiology and Infectious Diseases* 13: 59-65.

García-Bocanegra, I., Allepuz, A., José Pérez, J., Alba, A., Giovannini, A., Arenas, A., Candeloro, L., Pacios, A., Saez, J.L. and González, M.A. (2014). Evaluation of different enzyme-linked immunosorbent assays for the diagnosis of brucellosis due to *Brucella melitensis* in sheep. *The Veterinary Journal* 199: 439–445.

Garcia-Yoldi, D., Marin, C.M., De Miguel, P.M., Munoz, P.M., Vizmanos, J.L. and Lopez-Goni, I. (2006). Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clinical Chemistry* 52: 779–781.

Garin-Bastuji, B., Blasco, J.M., Marin, C. and Albert, D. (2006). The diagnosis of brucellosis in sheep and goats, old and new tools. *Small Ruminant Research* 62: 63–70.

Ghosh, S.S., Sen, G.P. and Gajindar Singh. (1968). The use of three vaccines against *Brucella melitensis* in sheep. *Journal of Comparative Pathology* 78: 387-392.

Godfroid, J. and Kasbihrer, A. (2002). Brucellosis in the European Union and Norway at the turn of the twenty-first century. *Veterinary Microbiology* 90: 135-145.

Godfroid, J., Al Dahouk, S., Pappas, G., Roth, F., Matope, G., Mumah, J., Marcotty, T., Pfeiffer, D. and Skjerve, E. (2013). A "One Health" surveillance and control of brucellosis in developing countries: moving away from improvisation. *Comparative Immunology, Microbiology and Infectious Diseases* 36: 241– 248.

Godfroid, J., Scholz, H.C., Barbier, T., Nicolas, C., Wattiau, P. and Fretin, D. (2011). Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. *Preventive Veterinary Medicine* 102:118–131.

Golding, B., Scott, D.E., Scharf, O., Huang, L.Y., Zaitseva, M., Lapham, C., Eller, N. and Golding, H. (2001). Immunity and protection against *Brucella abortus. Microbes and Infection* 3: 43-48.

Gorvel, J.P. (2008). *Brucella*: a "Mr. Hide". *Microbes and Infection* 10(9): 1010-1013.

Gorvel, J.P. and Moreno E. (2002). *Brucella* intracellular life: from invasion to intracellular replication. *Veterinary Microbiology* 90: 281–297.

Gouletsou, P.G., Fthenakis, G.C., Cripps, P.J., Papaioannou, N., Lainas, T., Psalla, D. and Amiridis, G.S. (2004). Experimentally induced orchitis associated with *Arcanobacterium pyogenes*: clinical, ultrasonographic, seminological and pathological features. *Theriogenology* 62: 1307–1328.

Greenberg, S. and Grinstein, S. (2002). Phagocytosis and innate immunity. *Current Opinion in Immunology* 14:136–145.

Gross, A., Bertholet, S., Mauel, J. and Dornand, J. (2004). Impairment of *Brucella* growth in human macrophagic cells that produce nitric oxide. *Microbial Pathogenesis* 36: 75-82.

Gross, A., Terraza, A., Ouahrani-Bettache, S., Liautard, J.P. and Dornand, J. (2000). *In vitro Brucella suis* infection prevents the programmed cell death of human monocytic cells. *Infection and Immunity* 68: 342–351.

Gupta, V.K., Ranjeeta, K., Jyoti, V., Singh, S.V. and Vihan, V.S. (2010). Comparative evaluation of recombinant BP26 protein for serological diagnosis of *Brucella melitensis* infection in goats. *Small Ruminant Reseaarch* 93: 119–125.

Gwida, M., El-Gohary, A., Melzer, F., Khan, I., Rösler. U. and Neubauer, H. (2012). Brucellosis in camels. *Research in Veterinary Science* 92: 351–355.

Haritani, M., Ishino, S., Oka, M., Nakazawa, M., Kobayashi, M., Narita, M. and Takizawa, T. (1989). Immunoperoxidase evaluation of pneumonic lesions in calves naturally infected with *Pasteurella haemolytica. Nippon Juigaku Zasshi* 51(6): 1137–114.

Hartady, T., Zamri-Saad, M., Siti-Khairani, B. and Mohd-Shahrom, S. (2014). Clinical human brucellosis in Malaysia: a case report. *Asian Pacific Journal of Tropical Disease* 4(2): 150-153.

Hazilawati, H. (2000). The effect of benzo(a)pyrene (BAP) of the respiratory tract of dogs, Master of Veterinary Science Thesis, Universiti Putra Malaysia.

Heller, M.C., Watsona, J.L., Blanchard, M.T., Jackson, K.A., Stott, J.L. and Tsoli, R.M. (2012). Characterization of *Brucella abortus* infection of bovine monocyte-derived dendritic cells. *Veterinary Immunology and Immunopathology* 149: 255– 261.

Hernandez-Mora, G., González-Barrientos, R. and Morales, J.A. (2008). Neurobrucellosis in stranded dolphins, Costa Rica. *Emerging Infectious Diseases Journal* 14: 1430-1433.

Hoffmann, E.M. and Houle, J.J. (1995). Contradictory holes for antibody and complement in the interaction of *Brucella abortus* with its host. *Critical Reviews in Microbiology* 21: 153–163.

Ibrahim, A. S., Shetty, M.S. and Bilal, N. (1988). Genitourinary complications of brucellosis. *British Journal of Urology* 61: 294-298.

Ilhan, F. and Yener, Z. (2008). Immunohistochemical detection of *Brucella melitensis* antigens in cases of naturally occurring abortions in sheep. *Journal of Veterinary Diagnostic Investigation* 20: 803–806.

Izadjoo, M. J., Mense, M. G., Bhattacharjee, A. K., Hadfield, T. L., Crawford, R. M. and Hoover, D. L. (2008). A study on the use of male animal models for developing a live vaccine for brucellosis. *Transboundary and Emerging Diseases* 55: 145–151.

Jacques, I., Olivier-Bernardin, V. and Dubray, G. (1998). Efficacy of ELISA compared to conventional tests (RBPT and CFT) for the diagnosis of *Brucella melitensis* infection in sheep. *Veterinary Microbiology* 64: 61-73.

Jansen, B.C. (1980). The pathology of bacterial infection of the genitalia in bucks. *Onderstepoort Journal of Veterinary Research* 47: 263–267.

Jiang, X. and Baldwin, C.L. (1993). Effects of cytokines on the ability of macrophages to control intracellular *Brucella abortus. Infection and Immunity* 61: 124-134.

Johnson, C.A. and Walker, R.D. (1992). Clinical signs and diagnosis of *Brucella canis* infection. *Compendium on Continuing Education* 14: 763–772.

Jones, T.C. and Hirsch, J.G. (1972). The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *The Journal of Experimental Medicine* 136: 1173-1194.

Joo-eun Bae. (1980). Generation of Baculo virus-*Brucella abortus* heat shock protein recombinants; mice immune responses against the recombinants, and *B. abortus* superoxide dismutase and l7/l12 recombinant proteins. Doctor of Philosophy thesis, Virginia Polytechnic Institute and State University.

Joseph, P.G. (1971). Major bacterial diseases in Malaysia, their prevalence, detection and control. Paper presented at the 5th FAO Regional Conference on Animal Production and Health in the Far East, Kuala Lumpur. Pp: 56-58.

Jubier-Maurin, V., Boigegrain, R.A., Cloeckaert, A., Gross, A., Alvarez-Martinez, M.T., Terraza, A., Liautard, J., Kohler, S., Rouot, B., Dornand, J. and Liautard, J.P. (2001). Major outer membrane protein Omp25 of *Brucella suis* is involved in inhibition of tumor

necrosis factor alpha production during infection of human macrophages. *Infection and Immunity* 69: 4823–4830.

Junior, D.G.J., Rosinha, G.M.S., Carvalho, C.E.G., Oliveira, C.E., Sanches, C.C. and Lima-Ribeiro, A.M.C. (2012). Detection of *Brucella* spp. DNA in the semen of seronegative bulls by polymerase reaction. *Transboundary and Emerging Disease* 60(4): 376-377.

Kaufmann, S.H.E. (1988). CD8+ T lymphocytes in intracellular microbial infections. *Immunology Today* 9: 168.

Kaufmann, S.H.E. (1993). Immunity to intracellular bacteria. *Annual Review of Immunology* 11: 129–163.

Keppie, J., Williams, A.E., Witt, K. and Smith, H. (1965). The role of erythritol in the tissue localization of the *Brucellae*. *The British Journal of Experimental Pathology* 46(1): 104-108.

Ko, J. and Splitter, G.A. (2003). Molecular host pathogen interaction in brucellosis: Current understanding and future approaches to vaccine development for mice and humans. *American Society for Microbiology* 16(1): 65-78

Köhler, S., Foulongne, V., Ouahrani-Bettache, S., Bourg, G., Teyssier, J., Ramuz, M. and Liautard, J.P. (2002). The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. *Proceedings of the National Academy of Sciences of the United States of America* 99: 15711–15716.

Kretzer, H., Habs, M. and Schmähl, D. (1979). Limitations of *in vitro* short-term tests as prescreening models for carcinogenicity in industry: a theoretical approach. *Toxicology* 14(3): 283-289.

Kumar, V. and Sharma, A. (2010). Neutrophils: Cinderella of innate immune system. *International Immunopharmacology* 10: 1325–1334.

Lamontagne, J., Beland, M., Forest, A., Cote-Martin, A., Nassif, N., Tomaki, F., Moriyon, I., Moreno, E. and Paramithiotis, E. (2010). Proteomics-based confirmation of protein expression and correction of annotation errors in the *Brucella abortus* genome. *BMC Genomics* 11(1): 300.

Lang, R., Banai, M., Lishner, M. and Rubinstein, E. (1995). Brucellosis. *International Journal of Antimicrobial Agents* 5: 203-208.

Lapaque, N., Moriyon, I. and Moreno, E. (2005). *Brucella* lipopolysaccharide acts as a virulence factor. *Current Opinion in Microbiology* 8: 60-66.

Lee, W.L., Harrison, R.E. and Grinstein, S. (2003). Phagocytosis by neutrophils. *Microbes and Infection* 5: 1299–1306.

Letesson, J.J., Lestrate, P., Delrue, R.M., Danese, I., Bellefontaine, F., Fretin, D., Taminiau, B., Tibor, A., Dricot, A., Deschamps, C., Haine, V., Leonard, S., Laurent, T., Mertens, P.,

Vandenhaute, J. and De Bolle, X. (2002). Fun stories about *Brucella*: the "furtive nasty bug". *Veterinary Microbiology* 90: 317–328.

Lulu, A.R., Araj, F., Khateeb, M.L., Mustafa, M.Y., Yusuf, A.R. and Fenech, F.F. (1988). Human brucellosis in Kuwait: a prospective study of 400 cases. *Quarterly Journal of Medicine* 66(1): 39-54.

Macedo, G.C., Magnani, D.M., Carvalho, N.B., Bruna-Romero, O., Gazzinelli, R.T. and Oliveira, S.C. (2008). Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. *Journal of Immunology* 180: 1080-1087.

MacMillan, A.P. (1990). Conventional serological tests. In: Nielsen, K. and Duncan, J.R. (Eds.) Animal Brucellosis. Boca Raton, CRC Press. Pp: 153-197.

Maletto, B.A., Ropolo, A.S., Alignani, D.O., Liscovsky, M.V., Ranocchia, R.P., Moron, V.G. and Pistoresi-Palencia, M.C. (2006). Presence of neutrophil-bearing antigen in lymphoid organs of immune mice. *Blood* 108: 3094–3102.

Manterola, L., Tejero-Garces, A., Ficapal, A., Shopayeva, G., Blasco, J.M., Marín, C.M. and López-Goñi, I. (2003). Evaluation of a PCR test for the diagnosis of *Brucella ovis* infection in semen samples from rams. *Veterinary Microbiology* 92: 65–72.

Maria-Pilar, J.B., Dudal, S., Dornand, J. and Gross, A. (2005). Cellular bioterrorism: how *Brucella* corrupts macrophage physiology to promote invasion and proliferation. *Clinical Immunology* 114: 227– 238.

Marin, C.M., Moren, E., Mariyon, I., Diaz, R. and Blasco, J.M. (1999). Performance of competitive and indirect ELISAs, gel immunoprecipitation with native hapten polysaccharide and standard serological tests in diagnosis sheep brucellosis. *Clinical and Diagnostic Laboratory Immunology* 6: 269-272.

Mayer-Scholl, A., Draeger, A., Göllner, C., Scholz, H.C. and Nöckler, K. (2010). Advancement of a multiplex PCR for the differentiation of all currently described *Brucella* species. *Journal of Microbiological Methods* 80: 112–114.

McFarland, J. (1907). An instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and vaccines. *Journal of American Medical Information Association* 14: 1176-1178.

Mekonnen, H., Kalayou, S. and Kyule, M. (2010). Serological survey of bovine brucellosis in barka and arado breeds (*Bos indicus*) of Western Tigray, Ethiopia. *Preventive Veterinary Medicine* 94: 28–35.

Mense, M.G., Richard, H., Borschel, R.H., Wilhelmsen, C.L., Louise Pitt, M.L. and Hoover, D.L. (2004). Pathologic changes associated with brucellosis experimentally induced by aerosol exposure in rhesus macaques *(Macaca mulatta). American Journal of Animal and Veterinary Science* 65: 644-652.

Mense, M.G., Van De Verg, L.L., Bhattacharjee, A.K., Garrett, J.L., Hart, J.A., Lindler, L.E., Hadfield, T.L. and Hoover, D.L. (2001). Bacteriologic and histologic features in mice

after intranasal inoculation of *Brucella melitensis*. *American Journal of Veterinary Research* 62: 398–405.

Minas, A., Stournara, A., Christodoulopoulos, G. and Katsoulos, P.D. (2008). Validation of a competitive ELISA for diagnosis of *Brucella melitensis* infection in sheep and goats. *The Veterinary Journal* 177: 411–417.

Minas, A., Stournara, A., Minas, M., Papaioannou, A., Krikelis, V. and Tselepidis, S. (2005). Validation of fluorescence polarization assay (FPA) and comparison with other tests used for diagnosis of *B. melitensis* infection in sheep. *Veterinary Microbiology* 111: 211–221.

Minas, A., Stournara, A., Minas, M., Stack, J., Petridou, E., Christodoulopoulos, G. and Krikelis, V. (2007). Validation of fluorescence polarization assay (FPA) performed in microplates and comparison with other tests used for diagnosing *B. melitensis* infection in sheep and goats. *Journal of Immunological Methods* 320: 94–103.

Mitka, S., Anetakis, S. and Souliou, E. (2007). Evaluation of different PCR assays for early detection of acute and relapsing brucellosis in comparison with conventional methods. *Journal of Clinical Microbiology* 45: 1211–1218.

Monack, D.M., Mecsas, J., Ghori, N. and Falkow, S. (1997). *Yersinia* signals macrophages to undergo apoptosis and YopJ is necessary for this cell death. *Proceedings of the National Academy of Sciences of the United States of America* 94: 10385–10390.

Moreno, E., Berman, D.T. and Boettcher, L.A. (1981). Biological activities of *Brucella abortus* lipopolysaccharides. *Infection and Immunity* 31: 362–369.

Muñoz, P.M., Marín, C.M., Monreal, D., González, D., Garin-Bastuji, B., Díaz, R., Mainar-Jaime, R.C., Moriyón, I. and Blasco, J.M. (2005). Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false positive serological results due to *Yersinia enterocolitica* O:9. *Clinical and Diagnostic Laboratory Immunology* 12: 141–151.

Neta, A.V.C., Mol, J.P.S., Xavier, M.N., Paixão, T.A., Lage, A.P. and Santos, R.L. (2010). Pathogenesis of bovine brucellosis. *The Veterinary Journal* 184: 146–155.

Nicoletti, P. (1969). Further evaluation of serologic test procedures used to diagnose brucellosis. *American Journal of Veterinary Research* 30: 1811–1816.

Nielsen, K. (2002). Diagnosis of brucellosis by serology. *Veterinary Microbiology* 90: 447–459.

Nielsen, K. and Gall, D. (2001). Fluorescence polarization assay for the diagnosis of brucellosis: a review. *Journal of Immunoassay and Immunochemistry* 22: 183–201.

Nielsen, K., Cherwonogrodzky, J.W., Duncan, J.R. and Bundle, D.R. (1989). Enzyme-immunoassay for differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19. *American Journal of Veterinary Research* 50: 5–9.

Nielsen, K., Kelly, L., Gall, D., Nicoletti, P. and Kelly, W. (1995). Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. *Veterinary Immunology and Immunopathology* 46: 285–291.

North, R.J. (1974). T cell dependence of macrophage activation and mobilization during infection with *Mycobacterium tuberculosis. Infection and Immunity* 10(1): 66-71.

Nuzzi, P.A., Lokuta, M.A. and Huttenlocher, A. (2007). Analysis of neutrophil chemotaxis. *Methods in Molecular Biology* 370: 23-35.

Oliveira, S.C., Soeurt, N. and Splitter, G. (2002). Molecular and cellular interactions between *Brucella abortus* antigens and host immune responses. *Veterinary Microbiology* 90: 417-424.

Orduña, A., Almaraz, A., Prado, A., Gutiérrez, M.P., García-Pascual, A., Dueñas, A., Cuervo, M., Abad, R., Hernández, B., Lorenzo, B., Bratos, M.A. and Rodríguez- Torres, A. (2000). Evaluation of an immunocapture-agglutination test 84 (Brucellacapt) for the seodiagnosis of human brucellosis. *Journal of Clinical Microbiology* 38: 4000-4005.

Paixão, T.A., Costa, E.A. and Xavier, M.N. (2009). Innate immunity in brucellosis. *Infection and Immunity* 1: 21-37.

Pandit, D. (2011). *Brucella* arthritis- an update. *Indian Journal of Rheumatology* 6(1): 75-79.

Pappas, G. (2010). The changing *Brucella* ecology: novel reservoirs, new threats. *International Journal of Antimicrobial Agents* 36S: S8–S11.

Pappas, G., Panagopoulou, P., Christou, L. and Akritidis, N. (2006). *Brucella* as a biological weapon. *Cellular and Molecular Life Sciences* 63: 2229–2236.

Pearce, J. H., Williams, A. E., Harris-Smith, P. W., Fitzgeorge, R. B. and Smith, H. (1962). The chemical basis of the virulence of *Brucella abortus*. II. Erythritol, a constituent of bovine foetal fluids which stimulates the growth of *Brucella abortus* in bovine phagocytes. *British Journal of Experimental Pathology* 43: 31-36.

Pei, J., Turse, J.E. and Ficht, T.A. (2008). Evidence of *Brucella abortus* OPS dictating uptake and restricting NF-kappa B activation in murine macrophages. *Microbes Infection* 10(6): 582–590.

Phillips, H.J. (1973) Dye exclusion tests for cell viability. In: Kruse, P.F. Jr. and Patterson, M.J. Jr. (Eds.) Tissue Culture: Methods and Applications. New York, Academic Press. Pp: 406-408.

Pizarro-Cerdá, J., Méresse, S. and Parton, R.G. (1998). *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. *Infection and Immunity* 66: 5711-5724.

Pizarro-Cerdá, J., Moreno, E. and Gorvel, J.P. (2000). Invasion and intracellular trafficking of *Brucella abortus* in non phagocytic cells. *Microbes and Infection* 2: 829-883.

Pizzaro-Cerdá, J., Desjardins, M., Moreno, E., Akira, S. and Gorvel, J.P. (1999). Modulation of endocytosis in nuclear factor IL-6 (-/-) macrophages is responsible for a high

susceptibility to intracellular bacterial infection. *Journal of Immunology* 162: 3519-3526.

Plumeriastuti, H. and Zamri-Saad, M. (2012). Detection of *Brucella melitensis* in seropositive goats. *Online Journal of Veterinary Research* 16(1): 1-7.

Poester, F.P., Gonçalves, V.S.P., Paixão, T.A., Santos, R.L., Olsen, L.C., Schurig, G.G. and Lage, A.P. (2006). Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. *Vaccine* 24: 5327-5334.

Poester, F.P., Nielsen, K., Samartino, L.E. and Yu, W.L. (2010). Diagnosis of Brucellosis. *Open Veterinary Science Journal* 4: 46-60.

Pomales-Lebron, A. and Stinebring, W.R. (1957). Intracellular multiplication of *Brucella abortus* in normal and immune mononuclear phagocytes. *Proceedings of the Society for Experimental Biology and Medicine* 94: 78-81.

Porte, F., Naroeni, A., Ouahrani-Bettache, S. and Liautard, J.P. (2003). Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome–lysosome fusion in murine macrophages. *Infection and Immunity* 71: 1481–1490.

Price, R.E., Templeton, J.W., Smith, R. and Adams, L.G. (1990). Ability of mononuclear phagocytes from cattle naturally resistant or susceptible to brucellosis to control *in vitro* intracellular survival of *Brucella abortus*. *Infection and Immunity* 58: 879–886.

Queipo-Ortuńo, M., Morata, P., Ocón, P., Manchado, P. and Colmenero, J.D. (1997). Rapid diagnosis of human brucellosis by peripheral blood PCR-assay. *Journal of Clinical Microbiology* 35: 2927–2930.

Qureshi, N., Bruna-Romero, O., Gazzinelli, R.T. and Oliveira, S.C. (2004). Role of Toll like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. *Infection and Immunity* 72: 176–186.

Rambow-Larsen, A.A., Rajashekara, G., Petersen, E. and Splitter, G. (2008). Putative quorum-sensing regulator BlxR of *Brucella melitensis* regulates virulence factors including the type IV secretion system and flagella. *Journal of Bacteriology* 190: 3274-3282.

Repnik, U., Knezevic, M. and Jeras, M. (2003). Simple and cost effective isolation of monocytes from buffy coats. *Journal of Immunological Methods* 278(1-2): 283-292.

Riley L. K. and Robertson D. C. (1984). Ingestion and intracellular survival of *Brucella* polymorphonuclear leucocytes. *Infection and Immunity* 46: 224-230.

Rittig, M.G., Alvarez-Martinez, M.T., Porte, F., Liautard, J.P. and Rouot, B. (2001). Intracellular survival of *Brucell*a spp. in human monocytes involves conventional uptake but special phagosomes. *Infection and Immunity* 69: 3995-4006.

Rittig, M.G., Kaufmann, A., Robins, A., Shaw, B., Sprenger, H., Gemsa, D., Foulongne, V., Rouot, B. and Dornand, J. (2003). Smooth and rough lipopolysaccharide phenotypes

of *Brucella* induce different intracellular trafficking and cytokine/chemokine release in human monocytes. *Journal of Leukocyte Biology* 74: 1045–1055.

Roberts, P.J. and Ford, J.M. (1982). A new combined assay of phagocytosis and intracellular killing of *Escherichia coli* by polymorphonuclear leukocytes. *Journal of lmmunological Methods* 49:193—207.

Robertson, M.J., Cochran, K.J., Cameron, C., Le, J.M., Tantravahi, R. and Ritz, J. (1996). Characterization of a cell line, NKL, derived from aggressive human natural killer cell leukemia. *Experimental Hematology* 24: 406– 415.

Roitt, I.M., Delves, P., Martin, S. and Burton, D. (2006). Roitt's Essential Immunology. New York, Wiley-Blackwell. Pp: 38.

Ross, H.M., Foster, G. and Reid, R.J. (1994). *Brucella* species infection in sea-mammals. *Veterinary Record* 134: 359.

Rossetti, C.A., Galindo, C.L., Everts, R.E., Lewin, H.A., Garner, H.R. and Adams, L.G. (2011). Comparative analysis of the early transcriptome of *Brucella abortus*-infected monocyte-derived macrophages from cattle naturally resistant or susceptible to brucellosis. *Research in Veterinary Science* 91: 40–51.

Sadiq, M.A., Tijjani, A.N., Auwal, M.S., Mustapha, A.R. and Gulani, I. (2013). Serological prevalence of brucellosis among donkeys (*Equus asinus*) in some local government areas of Yobe State, Nigeria. *Journal of Equine Veterinary Science* 33(3): 150–154.

Salcedo, S.P., Maechesini, M.I. and Lelouard, H. (2008). *Brucella* control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. *PLoS Pathogens* 4: e21.

Samartino, L.E. (2002). Brucellosis in Argentina. *Veterinary Microbiology* 90: 71-80.

Sangari, F.J. and Aguero, J. (1996). Molecular basis of *Brucella* pathogenicity: an update. *Microbiologia* 12: 207-218.

Santos, R.L. and Baumler, A.J. (2004). Cell tropism of *Salmonella enterica*. *International Journal of Medical Microbiology* 294: 225–233.

Sarram, M., Feiz, J., Foruzandeh, M. and Gazanfrpour, P. (1974). Intrauterine fetal infection with *Brucella melitensis* as a possible cause of second trimester abortion. *American Journal of Obstetrics and Gynecology* 119:657.

Saunders, V.F., Reddacliff, L.A., Berg, T. and Hornitzky, M. (2007). Multiplex PCR for the detection of *Brucella ovis, Actinobacillus seminis* and *Histophilus somni* in ram semen. *Australian Veterinary Journal* 85: 72–77.

Scholz, H.C., Hubalek, Z., Sedlácek, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H., Cloeckaert, A., Maquart, M., Zygmunt, M.S., Whatmore, A.M., Falsen, E., Bahn, P., Göllner, C., Pfeffer, M., Huber, B., Busse, H.J. and Nöckler, K. (2008). *Brucella microti* sp. nov. isolated from the common vole *Microtus arvalis*. *International Journal of Systematic and Evolutionary Microbiology* 58: 375–382.

Schurig, G.G., Jones, L.M., Speth, S.L. and Berman, D.T. (1978). Antibody response to antigens distinct from smooth lipopolysaccharide complex in *Brucella* infection. *Infection and Immunity* 21(3): 994-1002.

Scientific Committee on Animal Health and Animal Welfare. (SCAHAW) (2001). Brucellosis in sheeps and goats (*Brucella melitensis*) report of the Scientific Committee on Animal Health and Animal Welfare. Health and Consumer Protection Directorate General, European Commission. http://ec.europa.eu/food/fs/sc/scah/out59_en.pdf. (Retrieved: 10-10-2013).

Seleem, M. N., Stephan, M. B. and Sriranganathan, N. (2010). Brucellosis: a re-emerging zoonosis. *Veterinary Microbiology* 140(3-4): 392-398.

Shaqinah, N., Mazlina, M., Zamri-Saad, M., Hazilawati, H. and Jasni, S. (2012). *In vitro* penetration and survival of *Brucella melitens*is in lymphocytic cells of goats. *Online Journal of Veterinary Research* 16(3): 104-110.

Silva, M.T. and Pestana, N.T.S. (2013). The *in vivo* extracellular life of facultative intracellular bacterial parasites: role in pathogenesis. *Immunobiology* 218: 325– 337.

Skendros, P., Pappas, G. and Boura, P. (2011). Cell-mediated immunity in human brucellosis. *Microbes and Infection* 13: 134-142.

Smith, H. and Fitzgeorge, R.B. (1964). The chemical basis of the virulence of *Brucella abortus*. V. The basis of intracellular survival and growth in bovine phagocytosis. *British Journal of Experimental Pathology* 45: 174-176.

Smith, H., Williams, A.E., Pearce, J.H., Keppie, J., Harris-Smith, P.W., Fitz-George, R.B. and Witt, K. (1962). Foetal erythritol: a cause of the localization of *Brucella abortus* in bovine contagious abortion. *Nature* 193: 47-49.

Sohn, A.H., Probert, W.S., Glaser, C.A., Gupta, N., Bollen, A.W., Wong, J.D., Grace, E.M. and McDonald, W.C. (2003). Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. *Emerging Infectious Diseases* 9: 485–488.

Sriranganathan, N., Seleem, M.N., Olsen, S.C., Samartino, L.E., Whatmore, A.M., Bricker, B., O'Callaghan, D., Halling, S.M., Crasta, O.R., Wattam, R.A., Purkayastha, A., Sobral, B.W., Snyder, E.E., Williams, K.P., Yu G.X., Fitch, T.A., Roop, R.M., de Figueiredo, P., Boyle, S.M., He, Y. and Tsolis, R.M. (2009). *Brucella*. In: Nene, V and Kole, C. (Eds.) Genome Mapping and Genomics in Animal-associated Microbes. Berlin, Springer-Verlag. Pp: 1-64.

Suraud, V., Jacques, I., Olivier, M. and Guilloteau, L.A. (2008). Acute infection by conjunctival route with *Brucella melitensis* induces IgG+ cells and IFN-Y producing cells in peripheral and mucosal lymph nodes in sheep. *Microbes and Infection* 10: 1370-1378.

Suraud, V., Olivier, M., Bodier, C.C. and Guilloteau, L.A. (2007). Differential expression of homing receptors and vascular addressins in tonsils and draining lymph nodes: effect of *Brucella* infection in sheep. *Veterinary Immunology and Immunopathology* 115: 239-250.

Sutherland, S.S. (1985). Comparison of enzyme-linked immunosorbent assay and complement fixation test for the detection of specific antibody in cattle vaccinated and challenged with *Brucella abortus. Journal of Clinical Microbiology* 44-47.

Tabatabai, L.B. and Deyoe, B.L. (1984). Specific enzyme-linked immunosorbent assay for detection of bovine antibody to *Brucella abortus. Journal of Clinical Microbiology* 20: 209-213.

Tabatabai, L.B., Pugh Jr. and Smith, G.W. (1994). Modulation of immune responses in Balb/c mice vaccinated with *Brucella abortus* Cu–Zn superoxide dismutase synthetic peptide vaccine. *Vaccine* 12: 919–924.

Takeuchi, O. and Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell* 140 (6): 805–820.

Tizard, Ian R. (2000). Veterinary Immunology: An introduction. Pennsylvania, Saunders Company. Pp: 26.

Underhill, D.M., Ozinsky, A., Smith, K.D. and Adereem, A. (1999). Toll-like receptor-2 mediates *Mycobacteri*a-induced proinflammatory signalling in macrophages. *Proceedings of the National Academy of Sciences of the United States of America* 96: 14459-14463.

Van Bastelaere, E., Lambrecht, M., Vermeiren, H., Van Dommelen, A., Keijers, V., Proost, P. and Vanderleyden, J. (1999). Characterization of a sugar-binding protein from *Azospirillum brasilense* mediating chemotaxis to and uptake of sugars. *Molecular Microbiology* 32: 703–714.

Velásquez, L.N., Victoria Delpino, M., Andrés, E., Lorena, I., Coria, M., Cruz Miraglia, M., Romina, S., Cassataro, J., Guillermo, H.G. and Paulo, B. (2012). *Brucella abortus* induces apoptosis of human T lymphocytes. *Microbes and Infection* 14: 639-650.

Veselský, L. (1981). Immunological properties of seminal vesicle fluid. *Systems Biology in Reproductive Medicine* 7(1): 1-7.

Weinrauch, Y. and Zychlinsky, A. (1999). The induction of apoptosis by bacterial pathogens. *The Annual Review of Microbiology* 53: 155-187.

Williams, R.C. and Gibbons, R.J. (1972). Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science* 177: 697–699.

Willumeit, R., Kumpugdee, M., Funari, S.S., Lohner, K., Navas, B.P. and Brandenburg, K. (2005). Structural rearrangement of model membranes by the peptide antibiotic NK-2. *Biochimica et Biophysica Acta* 1669: 125–134.

World Organisation for Animal Health (OIE) (2009). Caprine and ovine brucellosis (excluding *Brucella ovis*). Manual of diagnostic tests and vaccines for terrestrial animals. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.02_caprine_Ovine_bruc.pdf. (Retrieved: 07-10-13).

Wyckoff, J.H. 3rd and Potts, R.D. (2007). Killing of *Brucella* antigen-sensitized macrophages by T lymphocytes in bovine brucellosis. *Veterinary Immunology and Immunopathology* 36: 45-64.

Wyckoff, J.H. 3rd, Howland, J.L. and Confer, A.W. (1993). Comparison of *Brucella abortus* antigen preparation for in vitro stimulation of immune bovine T-lymphocyte cell lines. *Veterinary Immunology Immunopathology* 36: 45-64.

Wyckoff, J.H. 3rd. (2002). Bovine T lymphocyte responses to *Brucella abortus. Veterinary Microbiology* 90: 395–415.

Xavier, M.N., Paixão, T.A., den Hartigh, A.B., Tsolis, R.M. and Santos, R.L. (2010). Pathogenesis of *Brucella* spp. *The Open Veterinary Science Journal* 4: 109-118.

Xavier, M.N., Paixão, T.A., Poester, F.P., Lage, A.P. and Santos, R.L. (2009). Pathological, immunohistochemical and bacteriological study of tissues and milk of cows and foetuses experimentally infected with *Brucella abortus. Journal of Comparative Pathology* 140: 149-157.

Young, E. J., Borchert, M., Kreutzer, F. L. and Musher, D. M. (1985). Phagocytosis and killing of *Brucella* by human polymorphonuclear leukocytes. The Journal of Infectious Diseases 151:682-690.

Young, E.J. (1989). Clinical manifestations of human brucellosis. In: Young, E.J. and Corbel, M.J. (Eds.) Brucellosis: Clinical and Laboratory Aspects. Boca Raton, CRC Press. Pp: 97-126.

Young, E.J. (1995). An overview of human brucellosis. *Clinical Infectious Diseases* 21: 283-289.

Yulianna Puspitasari. (2011). Development of a recombinant vaccine expressing the gene encoding 34-kilodalton outer membrane protein of *Brucella melitensis*. Master of Veterinary Science thesis, Universiti Putra Malaysia.

Zamri-Saad, M. and Shafarin, M.S. (2007). Response of goats to the different routes of infection by *Pasteurella multocida* B:2. *Journal of Animal and Veterinary Advances* 6(3): 340-343.

Zhan, Y., Liu, Z. and Cheers, C. (1996). Tumor necrosis factor alpha and interleukin 12 contribute to resistance to the intracellular bacterium *Brucella abortus* by different mechanisms. *Infection and Immunity* 64: 2782–2786.

Zundel, E., Verger, J.M., Grayon, M. and Michel, R. (1992). Conjunctival vaccination of pregnant ewes and goats with *Brucella melitensis* Rev.1 vaccine: safety and serological responses. *Annales De Recherches Veterinaires* 23: 177-188.

Zwerdling, A., Delpino, M.V., Barrionuevo, P., Cassataro, J., Pasquevich, K.A., Garcia Samartino, C., Fossati, C.A. and Giambartolomei, G.H. (2008). *Brucella* lipoproteins mimic dendritic cell maturation induced by *Brucella abortus. Microbes and Infection* 10: 1346-1354.

Zwerdling, A., Delpino, M.V., Pasquevich, K.A., Barrionuevo, P., Cassataro, J., Samartino, C.G. and Giambartolomei, G.H. (2009). *Brucella abortus* activates human neutrophils. *Microbes and Infection* 11: 689-697.

Zygmunt, M.S., Hagius, S.D. and Walker, J.V. (2006). Identification of *Brucella melitensis* 16M genes required for bacterial survival in the caprine host. *Microbes and Infection* 8: 2849-2854.