



Immunohistochemical Detection of *Brucella melitensis* Antigens in Lungs tissues of Sheep and Goats Diagnosed with Different Diseases at Necropsy

Nurul-Izzati, U.Z.¹; Tanko, P.N.^{2*}; Sabri, M.Y.¹ and Onilude, O.P.³

¹Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, ²Faculty of Veterinary Medicine, University of Jos. ³Department of Veterinary Services, Ministry of Agriculture and Natural Resources, Ogun State, Nigeria. *Corresponding author: Email: pntanko@gmail.com; Tel No: +2348141215527.

SUMMARY

Brucellosis is one of the world most common zoonotic diseases and infected animals are often the source of human infection. The prevalence of Brucellosis in cases presented to the necropsy unit of the Faculty of Veterinary Medicine, Universiti Putra Malaysia from 2007 to 2012 was unknown. In a retrospective study, the presence of *B. melitensis* antigen in 107 formalin-fixed, paraffin embedded lung tissue blocks of small ruminants were investigated using immunohistochemistry (IHC) technique. Sample blocks from year 2007 to 2012 were collected from postmortem archive in necropsy unit of the Faculty of Veterinary Medicine, Universiti Putra Malaysia. A total of 10 tissue samples with positive immunoreactivity to *B. melitensis* antigen were detected. Out of the 10 positive tissue samples, one was from sheep and the rest were from goats. The prevalence of *B. melitensis* in post-mortem cases for the six year period was 9.4%. Statistical analysis revealed there were no difference between breed, gender and age among the 10 positive samples. Based on the findings of this study, it was concluded that *Brucella* antigen could be present in small ruminant species which had died of other diseases and this could pose significant public health risk, especially among staff and students while conducting post-mortem examinations. Strict protective measures should be emphasized before carrying out postmortem examination

Keywords: *Brucella melitensis*, Immunohistochemistry, Post mortem, Small ruminant.

Running title: Prevalence of Brucellosis in necropsied ruminant

INTRODUCTION

Brucellosis is regarded by World Health Organization (OIE Terrestrial Manual, 2009) as one of the most important zoonosis. Brucellosis in small ruminants, especially goats is caused by *Brucella melitensis* and the rate of transmission to humans have substantially increased in countries that lack effective control based programmes (Megid *et al.*, 2010; Onilude *et al.*, 2017; Higgins *et al.*, 2017). Infected animals are often the main source of human infection (Tanko *et al.*, 2013; Higgins *et al.*, 2017). Human infection most frequently results from consumption of unpasteurized milk or contact with infected animals and their products (Nwunuji *et al.*, 2013; Higgins *et al.*, 2017). The *B. melitensis* is reported to be the most virulent *Brucella* sp. for humans as only 10 to 100 microorganisms are sufficient to cause a debilitating chronic infection (Fugier *et al.*, 2007).

In animals, the clinical signs vary and often not specific and they may include abortion in late pregnancy, orchitis and significant loss of production (Megid *et al.*, 2010). Most infected animal do not usually exhibit obvious clinical signs, thus facilitating the transmission of the disease to both non-infected animal and humans. Other factors that could facilitate *Brucella* transmission have also been reported to be subjection of infected animals to stress (Polycarp *et al.*, 2017). Currently, diagnosis of Brucellosis is based on serological and microbiological methods which are not without limitations. The limitations for serological tests include cross-reactivity with other antigenically related bacteria and inability of the serological techniques to detect early or latent infection (Nwunuji *et al.*, 2013; Onilude *et al.*, 2017). Bacteriological methods on the other hand are time consuming, cumbersome,

relatively expensive and with limited sensitivity (Ahmed *et al.*, 2010). Immunohistochemistry being a rapid, specific, and useful technique has been employed by several authors for the diagnosis of Brucellosis in formalin-fixed, paraffin-embedded (FFPE) tissues of ruminants (Ilhan and Yener, 2008; Emikpe *et al.*, 2013). Indeed, immunohistochemistry (IHC) technique is possibly one of the best tools that permits retrospective study to be conducted on formalin-fixed, paraffin embedded tissues for the detection of *Brucella* antigens. In view of the fact that *Brucella*-infected animals which are often the main source of human infection may not manifest clinical signs, the need to investigate small ruminants that died of other diseases becomes imperative. Thus our study was to evaluate the prevalence of *B. melitensis* antigen in formalin-fixed tissue samples of small ruminants from the post-mortem archive at Faculty of Veterinary Medicine, UPM, from 2007 to 2012 using IHC technique.

MATERIALS AND METHODS

Samples Retrieved from Post Mortem Archive

The number of postmortem cases examined and samples collected in sheep and goats in the Department of Veterinary Pathology, Universiti Putra, from 2007 to 2012 were retrieved from postmortem record. A total of 195 animals, comprising of 189 goats and 6 sheep were examined during the study period. Out of the 195 cases, 107 samples of formalin-fixed paraffin embedded lung tissues were retrieved from postmortem archive. Lung tissue was chose based on earlier reports (Ilhan and Yener, 2008; Emikpe *et al.*, 2013). Moreso, lung tissue is the most common

organ collected for histopathology during postmortem examination, when compared to liver, placenta and testis. Tissue blocks from animals experimentally infected with *B. melitensis* were used as positive control.

Preparation of hyperimmune serum

The primary antibody that was used was prepared following the guide lines stated in OIE (OIE Terrestrial Manual, 2009) as described in our earlier study (Onilude *et al.*, 2017).

Procedure for Immunohistochemistry

Immunohistochemistry was done according to the procedure described by Emikpe *et al.*, (2013). Briefly, serial 4 µm thick sections were cut from the paraffin blocks onto silane-coated glass slides. The slides were dried and incubated at 37°C overnight in an incubator or on a heat-plate. The slides were kept in a hot oven for 15 min at 56-60°C prior to dewax in organic solvent xylene. Then slides were rehydrated through a graded alcohol series. After rinsing with flowing filtered water for 30 s, the slides were washed with PBS for 10 min. Endogenous peroxidase activity was blocked with freshly prepared 3% hydrogen peroxide for 5 min at room temperature, then rinsed and washed with PBS for 2 min. To enhance tissue immunoreactivity, heat-mediated antigen retrieval method was used by placing the slides in citrate buffer solution and incubated for 10 min in microwave oven. Blocking buffer of 1% normal serum (BSA-PBS) was used to dilute the primary antibody. This was to promote uniform distribution of antibody on tissue sections. Primary antibody was derived from serum of *B. melitensis*-infected goat which was tested seropositive by RBPT (Onilude *et al.*, 2017). Sections were incubated with primary antibody at the dilution of 1:50 in BSA-PBS for at least one

hour at 37°C in an incubator. The slides were rinsed and washed with PBS for five min to remove unbound or weakly bound antibodies. Sections were incubated again at 37°C for 30 min with secondary antibody (rabbit anti-goat) conjugated with horseradish peroxidase enzyme (SIGMA, USA) at dilution rate of 1:100 in BSA-PBS. The slides were rinsed and washed with PBS for five min and enzymatic substrate called DAB was applied (one ml diluents to one drop DAB) (VECTOR LABORATORIES, USA) for colour change. Once the sections became brown, the slides were immediately rinsed with distilled water. The slides were counter-stained using Harris's haematoxylin solution. Finally, the slides were mounted with DPX and cover slip. The sections were then visualized under the microscope (image analyzer NIS-Elements D 3.2 Nikon, Japan) for detection of immunoreaction.

Data Analysis

Data on age, breed and gender for every samples collected were retrieved from post mortem record. Statistical analysis was performed using IBM Statistical Package for the Social Sciences (SPSS) Statistics 19. The prevalence of *B. melitensis* antigens in the lung tissue of sheep and goats presented for postmortem examination was calculated as percentage of positive samples among the total number that were tested. Statistical analysis was performed using non-parametric tests Kruskal-Wallis and Mann-Whitney. Prevalence of *B. melitensis* antigens detected in post-mortem cases of small ruminant was computed accordingly

RESULTS

Out of the 107 samples retrieved from the post mortem archive, 10 samples showed positive reaction. The prevalence of *B.*

melitensis antigen detected in post mortem cases from year 2007 to 2012 was 9.4%. Intensity and distribution of antigen were recorded as in TABLE 1.

The formalin-fixed paraffin-embedded (FFPE) lung tissue sections with positive

immunoreactivity showed that *B. melitensis* antigens were localized in the alveolar macrophages in both the positive control (figure 1) and test lung samples figures 2 & 3. Figure 4 shows a negative immunoreactivity to *B. melitensis* antibody in the lung.

TABLE I: Immunohistochemical reaction of *Brucella melitensis* antigen in lung tissue of sheep and goats.

Sample Number	Amount of antigen	Intensity of Staining
1	+++	+++
2	+++	++
3	++	+++
4	++	+++
5	++	++
6	++	++
7	+	++
8	+	+
9	+	+
10	+	+

+ = Mild; ++ = Moderate; +++ = Abundant/intense

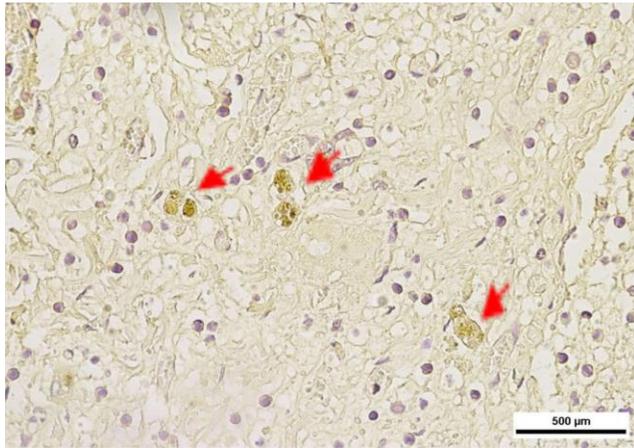


Figure 1: Photomicrograph of positive control lungs section showing positive immunoreactivity to anti-*B. melitensis* polyclonal antibody by alveolar macrophages in lung tissue (red arrows). Counter stain with H and E.

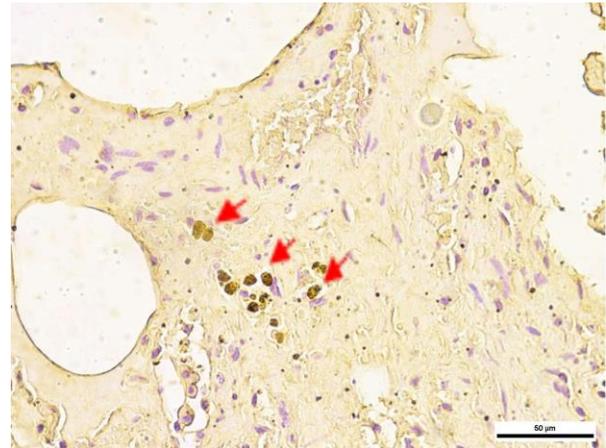


Figure 2: Representative photomicrograph of lungs section showing positive immunoreactivity to anti-*B. melitensis* polyclonal antibody by alveolar macrophages in lung tissue (red arrows). Counter stain with H and E.

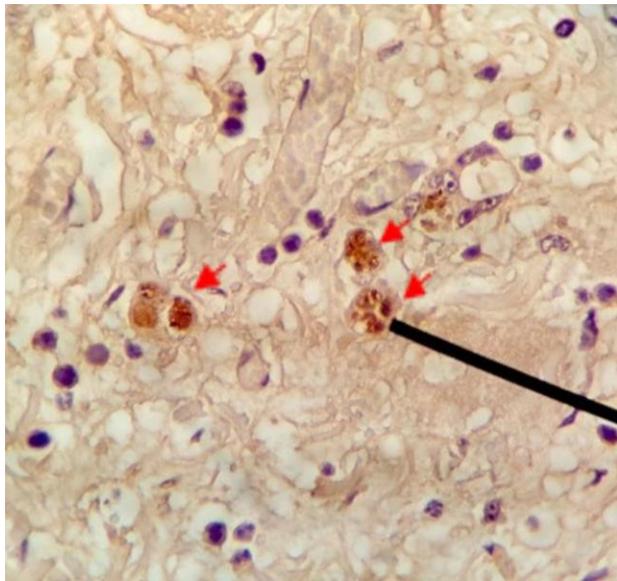


Figure 3: Representative photomicrograph of lungs section showing positive immunoreactivity to anti-*B. melitensis* polyclonal antibody by alveolar macrophages in lung tissue (red arrows). Counter stain with H and E, Mag. X1000.

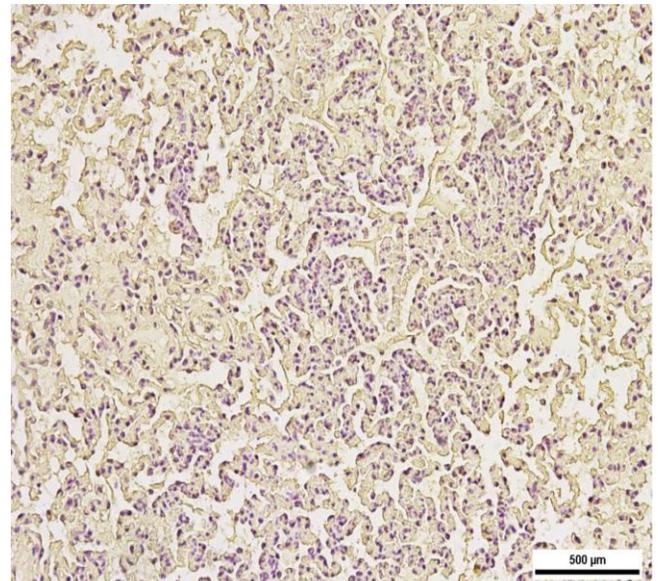


Figure 4: Photomicrograph of negative control lungs section showing absence of immunoreactivity in lungs tissue.

The bacteria isolated from *B. melitensis*-positive samples were obtained from records and presented in Table II. The predominant

bacteria isolated from the cases was *Escherichia coli*

TABLE II: Record of bacteria isolated from the lungs of *Brucella melitensis*-positive samples (n=10)

Sample ID	Bacteria isolated
P07/63	<i>Bacillus cereus, E. coli, Pseudomonas aeruginosa</i>
P0793*	<i>E. coli & Salmonella sp.</i>
P07/121	<i>E. coli</i>
P09/160	<i>Pure growth of enterococcus</i>
P09/187	<i>Not done</i>
P10/170	<i>Pasteurella multocida, Pasteurella hemolytica, E. coli</i>
P11/21	<i>E. coli, P. multocida, Klebsiella pneumonia</i>
P11/214	No record
P11/229	No growth
P12/66	<i>E. coli</i>

**Type of sample sent not stated*

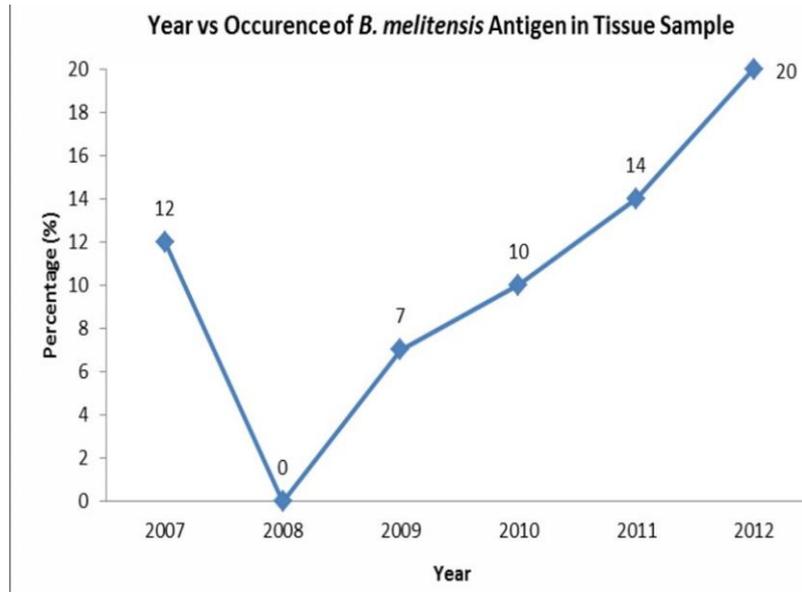


Figure 5: Number of positive and negative *Brucella melitensis* antigen in lung tissue of sheep and goats based on year.

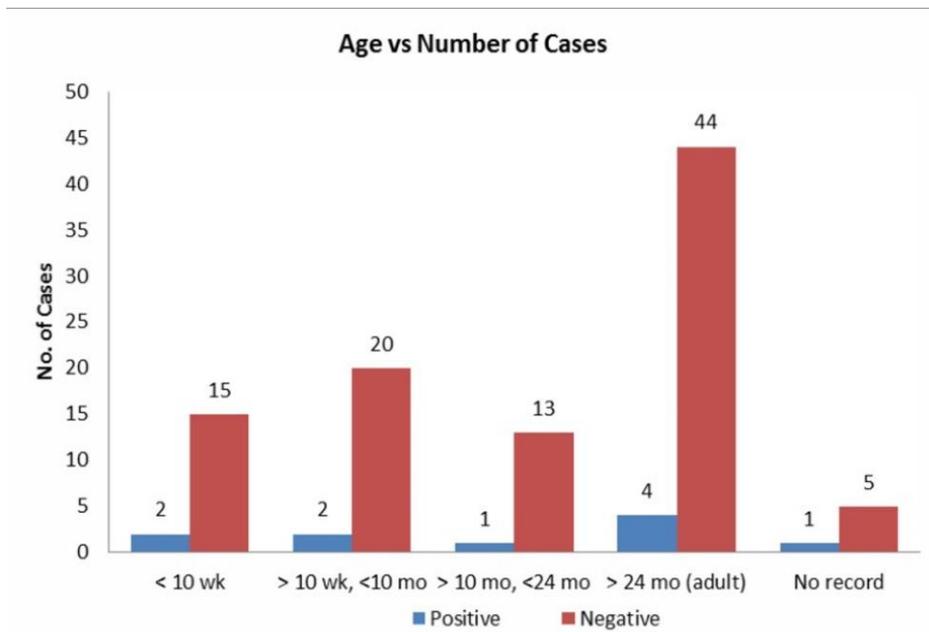


Figure 6: Number of positive and negative *Brucella melitensis* antigen in lung tissue of sheep and goats based on age group.

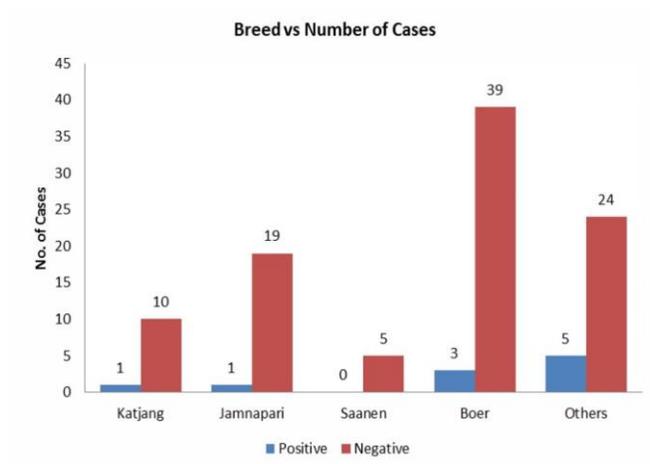


Figure 7: Number of positive and negative *Brucella melitensis* antigen in lung tissue of sheep and goats based on breed.

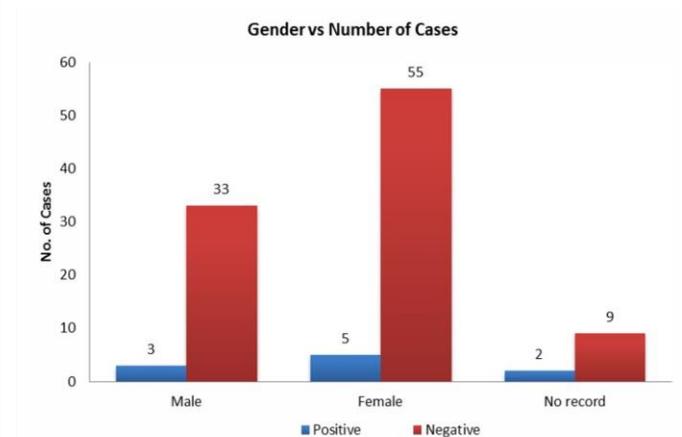


Figure 8: Number of positive and negative *Brucella melitensis* antigen in lung tissue of sheep and goats based on gender

The percentage of total cases on yearly bases is illustrated in Figure 7. The number of cases according to age, breed and gender were illustrated in Figures 8, 9 and 10 respectively. Statistically, there were no association between age, breed and gender among the positive *B. melitensis* antigens detected in tissue samples by IHC method.

DISCUSSION

Immunohistochemical techniques have been utilized in detecting the location of *Brucella* antigens in formalin-fixed, paraffin-embedded tissues of cow, goats (Meador *et al.*, 1986; Emikpe *et al.*, 2013), and sheep (Yazicioglu, 1997). Immunohistochemical examination of paraffin wax-embedded tissues for *Brucella* antigens is not only both sensitive and specific but also clearly shows antigen localisation; thereby revealing the distribution of antigen in the tissues, a valuable attribute for the study of pathogenesis of *Brucella* infection (Meador *et al.*, 1986; Pe´rez *et al.*, 1998; Emikpe *et al.*, 2013).

Even though Brucellosis has been reported as endemic in Malaysia through serological tests and PCR (Bamaiyi *et al.*, 2012), this study appears to be the first retrospective study of formalin fixed tissues of small ruminants that died of different pathogens other than *Brucella*. The localization of the *Brucella* antigen in the alveoli macrophages as seen in this study is in agreement with earlier studies (Ilhan and Yener, 2007; Emikpe *et al.*, 2013) where *B. melitensis* antigen was mainly detected in alveolar macrophages from aborted fetus and adult goats using IHC.

This localization of the antigen in alveoli macrophages of the sheep and goats not diagnosed of Brucellosis calls for serious concern as these animals might have been

shedding the bacterium through the nasal, ocular or vaginal route as reported in an earlier study (Nwunuji *et al.*, 2013). In another study, Paixao *et al.*, (2010) further buttressed the survival mechanisms employed by *Brucella* organisms in the alveoli macrophages which include its ability to survive intracellular acidification and inhibit apoptosis in the macrophages. Among the 10 positive cases, there was a case with history of abortion and because of the abortion; Brucellosis was considered as a differential diagnosis. However, bacterial isolation and Modified Ziehl Nielsen staining for *Brucella* antigen were found to be negative. Immunohistochemistry had been described to be useful in diagnosing suspected cases where bacterial culture is negative (Sozmen *et al.*, 2004). Immunohistochemistry has been reported to be a credible complimentary tool to bacteriology and serology examination for diagnosis of Brucellosis (Essmail *et al.*, 2002). Moreover, this method has the advantage of revealing the distribution and localization of the antigen in tissues.

The increased prevalence of the disease over the years from 2008 through 2012 as revealed by IHC technique used in this study could be an indication that the actual prevalence of the disease especially in animals diagnosed of other diseases is on the increase. This could possibly be due to the fact that Brucellosis shares common clinical signs such as abortion, retained placenta, stillbirth and arthritis with other several pathogens as earlier reported (Fernando *et al.*, 2010). The World Health Organization (Food and Agriculture Organization, 2006) has similarly reported that Brucellosis has been often unrecognized and frequently goes

unreported in many countries despite its endemicity.

The insignificant differences between age and sex in the prevalence of the disease as revealed by IHC in this study is in agreement with that reported by WHO (MacMillan, 1990), where it was reported that in places where Brucellosis is endemic, no age or sex predilection for the disease exist.

Since there is probability of serological cross reactivity between *Brucella* spp with other pathogens such as *Salmonella urbana*, several types of *E. coli*, *Pseudomonas maltophilia* and *Yersinia enterocolitica* (Food and Agriculture Organization, 2006), bacterial isolation records from lung sample of positive cases by immunohistochemistry method were reviewed. It revealed that *E. coli* was isolated from six positive cases such as listed in Table 3. The fact that all bacteria listed in Table 3 were isolated from lung tissue and yet tested negative with IHC shows that the positive samples could not have been as a result of cross reactivity with these bacteria antigens. In view of the zoonotic nature of Brucellosis, in conjunction with the fact that infected animals may not show any clinical signs, the findings reported in this study calls for

REFERENCES

AHMED F Y., SOKKAR S M., DESOUKY H M., GHAZI Y A., AMIN A S and MADBOLY A A. (2010): Pathological and molecular studies on mammary glands and supra-mammary lymph nodes of naturally *Brucella* infected Buffalo- cows. *Journal of Reproduction and Infertility*, 2:33-40.

concern and we suggest that small ruminants submitted for necropsy, especially those from endemic areas need to be handled with caution.

CONCLUSION

The prevalence of 9.4% *B. melitensis* antigen in post mortem cases of small ruminants from year 2007-2012 in necropsy unit, Faculty of Veterinary Medicine, University Putra Malaysia by IHC was relatively high. Based on the findings of this study, there is risk of *B. melitensis* infection of staff and students during post-mortem examination. This could be minimized by strict adherence to safety measures during post-mortem examination and carcass disposal.

ACKNOWLEDGEMENT

This project was funded by the Ministry of Science, Technology and Innovation, Malaysia (MOSTI) under the grant number 5450546. We express our appreciation to all staff of Pathology Laboratory, Department of Veterinary Pathology & Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia for the excellent assistance rendered.

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 Peninsula Malaysia. *Tropical Biomedicine* 29: 513–518

- BENJAMIN O. EMIKPE, SABRI M. YUSSOUF, CHUKWUNONSO K. EZEASOR, POLYCAP N TANKO. (2013): Immunohistochemical detection of *Brucella Melitensis* and *Coxiella burnetii* antigens in formalin-fixed tissues of West African Dwarf goats. Archives Clin Microbiol, 4 No. 4:2 doi: 10.3823/270
- ESSMAIL, M.E., IBRAHIM, I.G., and YASSEN, M.H. (2002): Immunohistochemical detection of *Brucella* antigens in formalin-fixed, paraffin-embedded tissues of Buffalos. Journal of the Egyptian Veterinary Medical Association, 62:127-136
- FERNANDO PADILLA POESTER, KLAUS NIELSEN, LUIS ERNESTO SAMARTINO and WEI LING YU. (2010): Diagnosis of Brucellosis. The Open Veterinary Science Journal, 4, 46-60
- FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, WORLD ORGANISATION FOR ANIMAL HEALTH, AND WORLD HEALTH ORGANIZATION. (2006): Brucellosis in human and animals. Geneva: World Health Organization; 2006. WHO/CDS/EPR/2006.7
- FUGIER, E., PAPPAS, G., and GORVEL, J.P. (2007): Virulence factors in brucellosis: implication for aetiopathogenesis and treatment. Experts Review in Molecular Medicine, 9(35):1-10.
- HIGGINS, J. L., GONZALEZ-JUARRERO, M., and BOWEN, R. A. (2017): Evaluation of shedding, tissue burdens, and humoral immune response in goats after experimental challenge with the virulent *Brucella melitensis* strain 16M and the reduced virulence vaccine strain Rev. 1. PloS one, 12(10), e0185823.
- ILHAN, F., and YENER Z., (2008). Immunohistochemical detection of *Brucella melitensis* antigens in cases of naturally occurring abortions in sheep. J Vet Diag Investig, 6:803-806.
- MACMILLAN, A. (1990). Conventional serological tests in Animal Brucellosis, Nielsen, K. and Duncan, J. D., Eds., Boca Raton: CRC Press.
- MEADOR, VP, TABATABAI, LB, HAGEMOSER, WA, DEYOE, BL (1986): Identification of *Brucella abortus* in formalin-fixed, paraffin embedded tissues of cows, goats and mice with avidin-biotin-peroxidase complex immunoenzymatic staining technique. Amer J Vet Res 47: 2147-2150
- MEGID, J., MATHIAS, L.A., and ROBLES, C.A. (2010): Clinical Manifestation of Brucellosis in Domestic Animals and Humans, The Open Veterinary Science Journal, 4:199-126.
- NWUNUJI, P., EMIKPE, B. O., and SABRI, Y. M. (2013): Evaluation of the shedding routes and serological patterns in experimentally-induced *Brucella melitensis* infection in dexamethasone-treated and transport-stressed goats. Veterinary World, 6: 686-692.
- OFFICE INTERNATIONAL DES EPIZOOTIES TERRESTRIAL MANUAL (2009): Caprine and Ovine Brucellosis (excluding *Brucella ovis*). CHAPTER 2.7.2.

- ONILUDE, O. M., SABRI M. Y., EMIKPE, B. O., TANKO, P. N., SHAHROM, S. M., and EFFENDY, M. (2017): Development and application of dot-enzyme-linked immunosorbent (dot-ELISA) assay for detection of *Brucella melitensis* and evaluation of the shedding pattern in infected goats. *Journal of Immunoassay and Immunochemistry*, 38: 82-99.
- PAIXAO, T.A., COSTA, E.A., XAVIER, M.N., SILVA, T.M.A., and SANTOS, R.L. (2010): Natural resistance and innate immunity in brucellosis. *Veterinary Immunology and Immunopathology*. Brazil: Nova Science Publishers.
- PE´REZ, J., M. QUEZADA, J. LO´PEZ, O. CASQUET, M.A. SIERRA AND J. MARTIN DE LAS MULAS, (1998): Immunohistochemical detection of *Brucella abortus* antigens in tissues from aborted bovine fetuses using a commercially available polyclonal antibody. *Journal of Veterinary Diagnostic Investigation*, 10: 17-21
- POLYCARP, T. N., YUSOFF, S. M., BENJAMIN, E. O., SALISI, S. M., and BEJO, S. K. (2017): Influence of dexamethasone-induced stress on oxidative stress biomarkers in non-pregnant does experimentally infected with *Brucella melitensis*. *Comparative Clinical Pathology*, 26: 423-435.
- SOZMEN, M., ERGINSOY, S.D., GENC, O., BEYTUT, E., and OZCAN, K. (2004): Immunohistochemical and microbiological detection of *Brucella abortus* in aborted bovine fetuses. *Acta Veterinaria Brno*, 73:465-472.
- YAZICIOGLU, O (1997): Pathological and immunoperoxidase studies of the fetal lesions of ovine brucellosis. (Koyunlarda bruselloza baÆlı abortuslarda ftal lezyonlar üzerinde patolojik ve immunoperoksidaz alısmalar.) *Ankara niversitesi Veteriner Fakltesi Dergisi*, 44: 291-307