ISSN: 1511-3701
Pertanika J. Trop. Agric. Sci. **34 (1)**: 167 - 173 (2011)

Immunohistological Localisation of *Coxiella burnetii* in Various Organs of Naturally Q-Fever Infected Goats

Norina, L.[1], Sabri, Y.[1], Goh, M.Y.[1], Zamri-Saad, M.[1], Sarenasulastri, A.B.[2], Latifah, H.[1], Jamilah, J.[1] and Noordin, M.M.[1*]

[1]Department of Pathology and Microbiology,
Faculty of Veterinary Medicine, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[2]Regional Diagnostic Laboratory, Bukit Tengah,
14000 Bukit Mertajam, Penang, Malaysia
[*]E-mail: noordin@vet.upm.edu.my

ABSTRACT

The rather uncommonly reported Q-fever disease in Malaysia is currently demonstrating an increasing trend of outbreaks. A total of 197 goat carcasses during the period of July 2007 to December 2009 were submitted to Regional Diagnostic Laboratory (MVK), Bukit Tengah, Penang, for post-mortem examination. Morphological diagnosis of necrotic placentitis, interstitial pneumonia, hepatitis and nephritis were observed in majority of the cases. Likewise, Giemsa stained sections of selected tissues revealed *C.burnetii* trophoblast. Acute cases yielded lesions conforming to doughnut granuloma, while those of the chronic form exhibited chronic inflammation. In an attempt to further confirm the presence of the organism, these selected tissues were subjected to immunohistochemical confirmation. Out of the total suspected cases, 152 (77.2%) were confirmed as positive of Q-fever based on their IHC. Thus, this study demonstrated pertinent lesions of acute and chronic forms of Q-fever which might be beneficial to laboratories without IHC facilities.

Keywords: *Coxiella burnetii*, immunohistochemistry, goat, Peninsular Malaysia

INTRODUCTION

The incidences of Q-fever have been globally reported with the exception of New Zealand (Rodolakis, 2005). *Coxiella burnetii* is strictly an intracellular bacterium that inhabits monocytes and macrophages (Baca and Paretsky, 1983). The transmission to human originated from contaminated secretion or excreta from infected animals warranting importance of food safety when dealing with the consumption of contaminated raw milk and milk products.

Coxiellosis occurs during late pregnancy, i.e. about 15 days before term leading to abortion in small ruminants and stillbirth in cattle (Russo and Malo, 1981). The organism's *in-vivo* survival is largely dependent on its ability to with stand the acidic environment of macrophages (pH 4.0 to pH 5.5) without affecting the cell's viability (Seshadri *et al.*, 2003). The organism can be demonstrated within trophoblast and mononuclear cells in ruminant placenta (Bildfell, 2000). Janigan and Marrie (1983) discovered the presence of giants and plasma cells in a pulmonary pseudotumour induced by *C.burnetii*. Recently, Norina *et al*. (2008) reported the appearance of fibrin ring or

Received: 20 July 2010
Accepted: 23 September 2010
[*]Corresponding Author

"doughnut granuloma" in infected lung, liver, and spleen in typical *C.burnetii* infection.

Several conventional staining methods have been used in demonstrating the organism in impression smears, cell culture, and formalin-fixed tissues samples (Russo, 1997). Likewise, egg inoculation, as well as immunohistochemistry (Anon, 2008) and PCR (Henning, 2002) have been used as the methods of isolating the organism. It is believed that cases or outbreaks of caprine Q-fever have been under-reported in Malaysia, owing the lack of database on lesions pertaining to the disease. Thus, the aims of the study were to demonstrate and develop a set of database on caprine Q-fever with the emphasis on tissue tropism and pertinent lesions.

MATERIALS AND METHODS

Tissue Samples

The study was performed retrospectively on formalin-fixed paraffin embedded tissue samples obtained from 197 Boer goats that had died from the period of July 2007 to December 2009. The tissues examined included the placenta, lung, heart, liver, spleen, and kidney, which were available for examination. Smears of the cut surface of placentome from every placenta received were stained by Giemsa and examined for *C. burnetii* trophoblast. Other organs were fixed in 10% buffered formalin and processed in the routine manner stained with Haematoxylin and Eosin (H&E). These tissues were examined microscopically for the evidence of necrotizing placentitis with cytoplasm of trophoblast cells, granulomatous lesion called as "doughnut granuloma" and infiltration of PMNs and macrophages cells.

Suspected C.burnetii *Positive H&E Samples*

A total of 152 cases for the last three year that were suggestive Q-fever through H&E staining were selected for immunohistochemical (IHC) confirmation.

Brief Description of the Immunohistochemical (IHC) Method

Briefly, the paraffin-embedded blocks were sectioned at a thickness of 2 µm. These sections were collected on coated silanized slides. The slides were placed in an oven at 60°C for 15 minutes. Then, they were deparrafinised using xylene and rehydrated. For antigen retrieval, rehydrated slides were immersed in commercially BIOCARE's peroxidized blocking buffer with goat serum for 10 minutes and washed in distilled water. The following steps were digested using the heat methods. The slides were placed in a pyrex beaker containing a Rodent Decloaker solution prepared by the manufacturer and heated to 80°C – 90°C for 30 minutes. After digestion and cooling down at room temperature for 15 minutes, they were washed by dipping them in TBS Tween 20, followed by immersing them in primary antibody using strongly positive goat serum at dilution 1:1000 and left at room temperature for 60 minutes. Then, they were washed again with TBS-Tween 20 and immersed for 15 more minutes in commercially secondary antibody Goat Probe as instructed by the manufacturer. It was washed in TBS wash buffer. Goat polymer HRP was applied for 15 minutes and later washed with TBS wash buffer. Later, it was immersed for 15 minutes in the DAB solution as recommended by the manufacturer. This was followed by washing with TBS wash buffer and rinsed again in distilled water. Counter-staining with Harris Hematoxylin solution for 10 seconds was later carried out and rinsed with distilled water, air-dried, and mounted with DPX and a cover slip.

Statistical Analysis

Statistical analyses were done using Cohen's Kappa test for reliability. The results were computed for HE positive and IHC positive samples. The associations between six organs samples for the HE positive and IHC positive in three year were assessed by comparing both the techniques, with the association being tested using the Cohen's Kappa test.

RESULTS

Tissue Samples

152 out of 197 samples were found to be positive for immunohistochemical test for the past three years (2007 – 2009), as illustrated in *Figs. 1-11* and Table 1.

On the HE stains, the histopahological lesions seen were:

- **Lung -** filled with interstitial edema and infiltrated with lymphocytes and macrophages. The alveolar spaces were filled with histiocytes, intra-alveolar focal necrosis, haemorrhages and necrotizing bronchitis. Giant cells and plasma cells were also observed to have been due to *C. burnetii,* syncthial-like cells in bronchiole, and severe interstitial pneumonia.
- **Liver** - hepatomegaly and speckled with dilated, congested central veins typical of nutmeg liver appearance indicative of right heart failure. Hepatic fatty degeneration, with accumulation of inflammatory cells and bizzarecells, granulomatous foci, were observed under a microscope. Granulomatous lesions containing the so-called doughnut granulomas consisting of dense fibrin rings or "doughnut" granulomas were also seen. These lesions were suggestive of acute Q-fever.
- **Spleen -** Congested and fragile spleen while palpating. Granulomatous spleenitis was observed at the early form of doughnut.
- **Heart -** hydropericardium with light yellow fluid accumulate in pericardium sac. Grossly looked normal. Myocarditis was revealed under histopathology.
- **Placenta -** with severe acute necrotizing placentitis and whitish 0.5 - 1.0 mm necrotic foci on cotyledons. Interestingly, numerous intracellular coccobacilli, that were indicatives of *C. burnetti*, were seen as bluish haze within cytoplasm of trophoblasts of *C. burnetii*. These organisms were detected as clearly revealed by green apple appearance seen under DIC microscopy in IFAT. Placenta vasculitis characterized by pleomorphic cellular infiltrations of mononuclear cells, neutrophils or eosinophils were also observed under microscopy.
- **Kidney -** tubulonephritis which leads to nephrosis. Miscroscopically, diffuse necrotic interstitial nephritis were shown with intra mononuclear cells that attempted to invade in the vessel, and inside they contained a lot of neurophils cells suggesting an acute inflammation.

DISCUSSION

Histopathology, Immunohistochemistry

Coxiella burnetii induces endemic abortion which results in necrotic placentitis in small ruminant. The most important route of infection is inhalation, especially when the environment is

TABLE 1
Comparison between H&E and IHC stained tissues in the detection of *C. burnetii*

Organs	H&E stain			IHC stain[a]		
	Positive (n)	Negative (n)	%	Positive (n)	Negative (n)	%
Placenta	11	0	100.0	11	0	100.0
Lung	67	41	62.0	58	9	86.6
Liver	57	51	52.8	45	12	78.9
Spleen	16	92	14.5	10	6	62.5
Kidney	26	82	24.0	16	10	61.5
Heart	23	85	21.3	12	11	52.2

[a]Only tissues that were positive on H&E were subjected to IHC staining

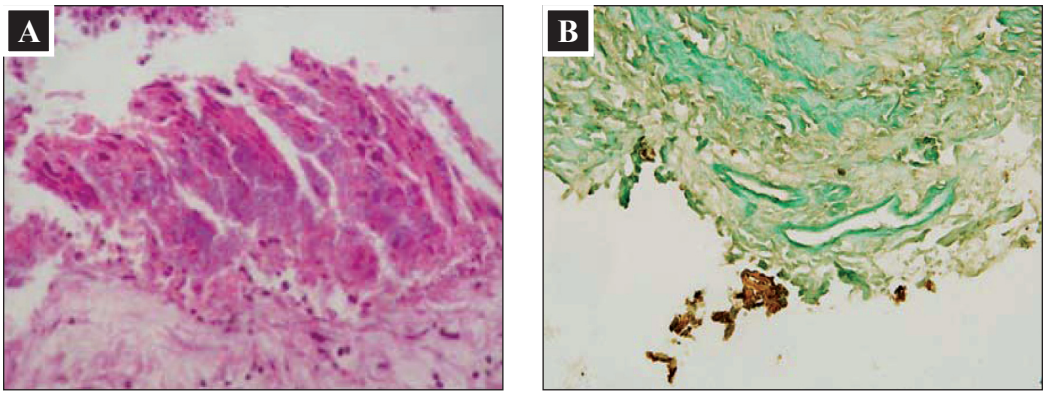

Fig. 1: (A) Photomicrograph demonstrating placentitis with trophoblast cell (H&E, X200); (B) which was later confirmed by to be C. burnetti *(IHC, X200)*

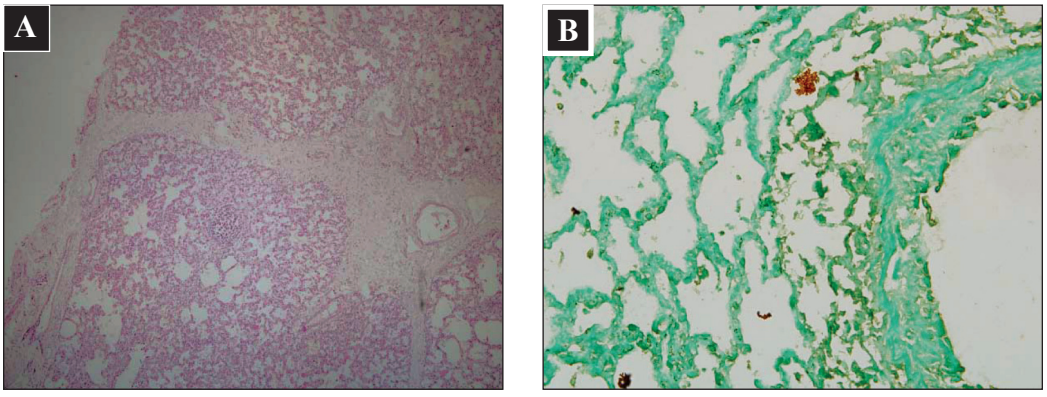

Fig. 2: (A) Photomicrograph of the lung demonstrating alveolar septa thickening and granuloma (H&E, X100); (B) and the presence of C. burnetii *(IHC, X200)*

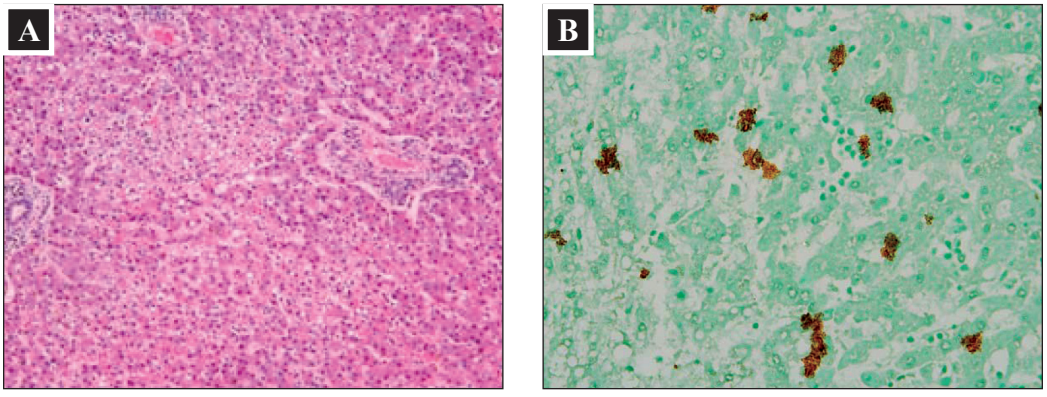

Fig. 3: (A) Photomicrograph of the liver showing fatty degeneration and early evidence of a doughnut granuloma (H&E, X100); (B) and the C. burnetii *positively-stained areas (IHC, X200)*

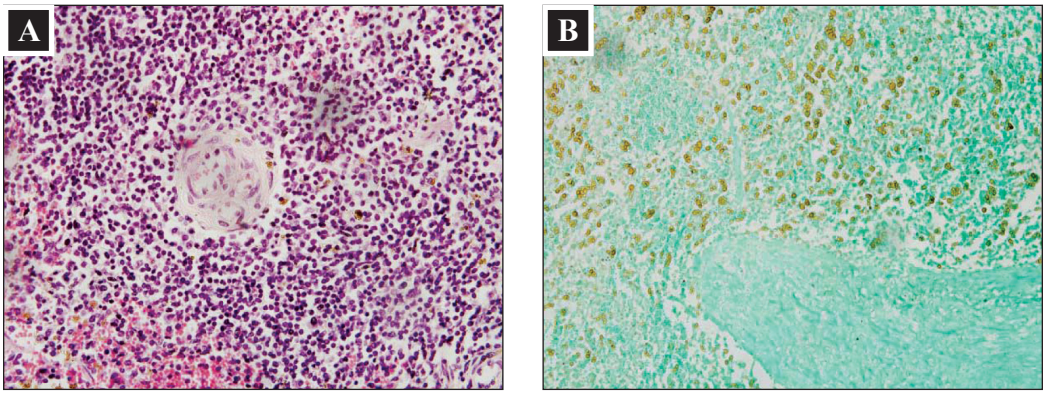

Fig. 4: (A) Photomicrograph of splenitis along with a doughnut granuloma (H&E, X100); (B) and strongly positive areas of C. burnetii *(IHC, X100)*

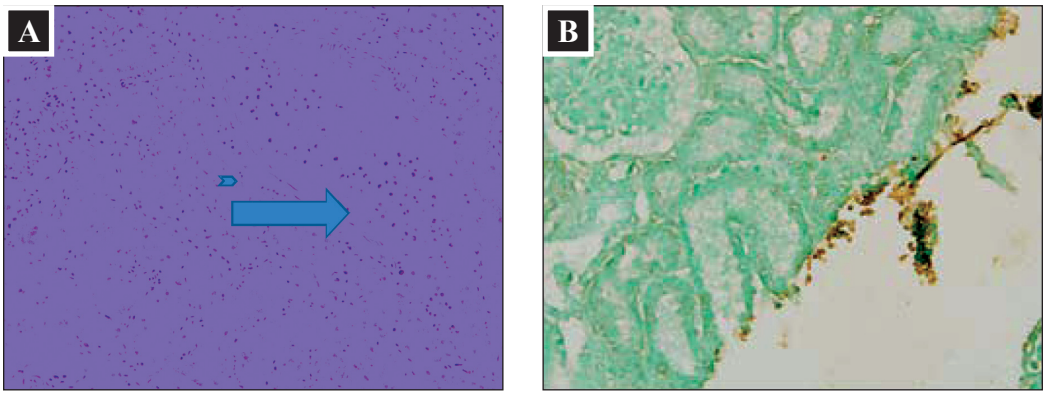

Fig. 5: (A) Photomicrograph of the kidney showing areas of interstitial nephritis, a vessel packed (arrow) with neutrophils (H&E, X200); (B) and borders of renal tubules being positive for C. burnetii *(IHC, X100)*

heavily contaminated by birth material (Marrie, 1990). *C. burnetii* is a eukaryotic cell with the characteristic of an intracellular pathogen that lives in an acidic vacuole (Raoult and Marrie, 1995). Reimer (1993) has reported that the pathogenesis of Q-fever, particularly in acute infection, is less understood because of the self-limiting nature of the illness and low mortality rates.

It is important to note that the histology studies of Q-fever have been based on pulmonary, hepatic, spleen, placenta, cardiac, and renal tissues. The immune response during Q-fever is associated with an inflammatory reaction that results in the information of granulomatous lesion which most commonly involves in the lung, liver, and spleen. Meanwhile, lymphocytes and macrophage are usually seen in infiltration and interstitial edema. Histiocytes fill the alveolar space and later form focal necrosis which will take place in intra-alveolar wall that leads to haemorrhages. This will also lead to the development of necrotizing bronchitis and bronchiolitis. In this study, less micro-organism were observed microscopically. Giant cell and plasma cells were also seen, and these could be due to *C.burnetii*. Syncitial-like cells in bronchiole indicated a severe

interstitial pneumonia. During autopsied, most cases resulted in hepatomegaly and speckled with dilated, congested central veins typical of nutmeg liver. This appearance is an indicative of the right heart failure. Meanwhile, hepatic fatty degeneration with the accumulation of inflammatory cells and bizzarecells, granulomatous foci were observed under the microscope. Lesions in the liver are different in acute and chronic Q- fever. In acute cases, granulomatous lesion containing the so-called doughnut granuloma is a pathognomonic finding which is noticeable of dense fibrin rings surrounding a central lipid vacuole (Srigley, 1985). In chronic cases, pathological findings are not restricted to the present of lymphocytic infiltration and foci of spotty necrosis (Janigan and Marrie, 1983). Grossly heart looked normal. The pericardium sac revealed hydropericardium with accumulated light yellow fluid. Under histopathology revealed myocarditis. Grossly spleen was observed congested, fragile, and palpating. Microscopically granuloma, i.e. an early form of doughnut spleenitis was also observed. Renal involvement in acute *C. burnetii* infection has been rarely reported (Tolosa-Vilella, 1995) but from this finding, tubulonephritis which has led to nephrosis is classified as a renal Q-fever. Renal microscopically showed diffuse necrotic interstitial nephritis with intra mononuclear cells attempting to invade the vessel, and containing a lot of neurophils cells which indicate on-going acute inflammation.

Abortion usually occurs during the third trimester of gestation due to *Coxiella burnetii* infection in small ruminant. The aborted foetuses are usually fresh with no lesion. The gross lesions are severe acute necrotizing placentitis with copious amounts of tan-brown exudate and whitish necrotic foci on placentome. Interestingly, numerous intracellular coccobacilli, indicative of *C. burnetii*, were seen as bluish haze within cytoplasm of trophoblasts of *C. burnetii* when stained with Giemsa. These organisms were detected, as clearly revealed by green apple appearance that was seen under the DIC microscopy in IFAT. Placenta vasculitis, characterized by pleomorphic cellular infiltrations of mononuclear cells, neutrophils or eosinophils, was rarely observed under microscopy. Necrotizing placentitis is a common lesion of *C.burnetii* with the appearance of numerous trophoblast containing intracytoplasmic coccobacilli colonies stain pale thin reddish coccobacilli in Modified Ziehl Neelsen. Although Giemsa staining of fresh placental impression smear is a good screening test, the diagnosis should be confirmed through IHC testing.

The statistical data presented in this study revealed that the organs of placenta, lung, and liver are significant samples to look for under histology for quick diagnosis purposes. The findings help pathologist to classify the infection into either acute or chronic Q-fever. This study provides evidence that in spite of placental, lung and liver are also significance for pathological diagnosis of *C.burnetii*. This being proved by Cohen's kappa analysis, i.e. placenta revealed greater than liver greater than lung ($0.95 \geq 0.780 \geq 0.772$) at $p < 0.0001$ indicate higher than ≥ 70.

CONCLUSIONS

Coxiella burnetii can be detected in various organ samples by using impression smear, histopathology and immunohistochemistry testing. In other words, immunohistochemical techniques are sufficiently sensitive to detect the antigen in various organ samples. The fact that *C.burnetii* causes Q-fever in man, safeguards against inhalation of infective material should be done when laboratory personnel work with infective material from abortion cases.

REFERENCES

Anonymous. (2008). Q fever (Chapter 2.1.12). *OIE Terresterial Manual*, 292-303.

Baca, O.G. and Paretsky, D. (1983). Q fever and *Coxiella burnetii*: A model for host-parasite interactions. *Microbiology Reviews*, *47*, 127-149.

Bildfell, R.J., Thomson, G.W., Haines, D.M., McEwen, B.J. and Smart, N. (2000). *Coxiella burnetii* infection is associated with placentitis in cases of bovine abortion. *Journal of Veterinary diagnostic investigation: Official publications of the American Association of Veterinary Laboratory Diagnosticians, Inc.*, *12*, 419-425.

Henning, K. and Sting, R. (2002). Definitive ability of Stamp-staining, antigen-ELISA, PCR and cell culture for the detection of *Coxiella burnetii. Berliner and Munchener tierarztliche Wochenschrift*, *115*, 381-384 (in German).

Janigan, D.T. and Marrie, T.J. (1983). An inflammatory pseudotumor of the lung in Q fever pneumonia. *The New England Journal of Medicine*, *308*, 86-87.

Marrie, T.J. **(**1990). Q-fever - A Review. *Canadian Veterinary Journal*, *31*, 555-563.

Norina, L., Shamsad, B., Pritipal, S. and Noordin, M.M. (2008). First report of *Coxiella burnetii* infection, a new emerging disease in goats in Penang, Peninsular Malaysia. *Proceedings of the 20th Veterinary Association Malaysia, 29.*

Raoult, D. and Marrie, T. (1995). Q fever. *Clinical infectious diseases: An official publication of the Infectious Diseases Society of America*, *20*, 489-96.

Reimer, L.G. (1993). Q fever. *Clinical Microbiology Reviews, 6,* 193-198.

Rodolakis, A. (2005). Q fever, state of art: Epidemiology, diagnosis and prophylaxis. *Small Ruminant Research*, *62*(200), 121-12.

Russo, P. (1997). Infection à *Coxiella burnetii* ou Fièvre Q. In A. Rodolakis and P. Nettleton (Eds.), *Manuel pratique de diagnostic de laboratoire des avortements infectieux des petits ruminants* (pp. 103-114). L'espace Vètèrinaire, Casablanca, Morocco.

Russo, P. and Malo, N. (1981). Q fever in the Vienne departments of France: Antibody kinetics and abortion in goats. *Recueil de Medecine Veterinaire*, 157, 585.

Seshadri, R., Paulsen, I.T., Eisen, J.A., Read, T.D., Nelson, K.E., Nelson, W.C., Ward, N.L., Tettelin, H., Davidsen, T. M., Beanan, M. J., Deboy, R.T., Daugherty, S. C., Brinkac, L.M., Madupu, R., Dodson, R.J., Khouri, H.M., Lee, K. H., Carty, H.A., Scanlan, D., Heinzen, R.A., Thompson, H.A., Samuel, J.E., Fraser, C. M. and Heidelberg, J.F. (2003). Complete genome sequence of the Q fever pathogen *Coxiella burnetii*. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 5455-5460.

Srigley, J.R., Vellend, H. and Palmer, N. (1985). Q fever. The liver and bone marrow pathology. *The American Journal of Surgical Pathology*, *9*, 752-8.

Tolosa-Vilella, C., RodrıB guez-Jornet, A., Font-Rocabanyera, J.and Andreu- Navarro, X. (1995). Mesangioproliferative glomerulonephritis and antibodies to phospholipids in a patient with acute Q fever: Case report. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *21,* 196-8.