Tropical Biomedicine 31(4): 769–776 (2014)

Molecular survey and sequence analysis of *Anaplasma* spp. in cattle and ticks in a Malaysian farm

Tay, S.T.[1*], Koh, F.X.[1], Kho, K.L.[1] and Ong, B.L.[2]
[1]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Malaysia
[2]Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[*]Corresponding author email: tayst@um.edu.my
Received 20 February 2014; received in revised form 1May 2014; accepted 9 May 2014

Abstract. This study was conducted to determine the occurrence of *Anaplasma* spp. in the blood samples of cattle, goats, deer and ticks in a Malaysian farm. Using polymerase chain reaction (PCR) and sequencing approach, *Anaplasma* spp. was detected from 81(84.4%) of 96 cattle blood samples. All blood samples from 23 goats and 22 deer tested were negative. Based on the analysis of the *Anaplasma* partial 16S ribosomal RNA gene, four sequence types (genotypes 1 to 4) were identified in this study. Genotypes 1-3 showed high sequence similarity to those of *Anaplasma platys/ Anaplasma phagocytophilum*, whilst genotype 4 was identical to those of *Anaplasma marginale/ Anaplasma centrale/ Anaplasma ovis. Anaplasma* DNA was detected from six (5.5%) of 109 ticks which were identified as *Rhipicephalus* (formely known as *Boophilus*) *microplus* ticks collected from the cattle. This study reported for the first time the detection of four *Anaplasma* sequence types circulating in the cattle population in a farm in Malaysia. The detection of *Anaplasma* DNA in *R. microplus* ticks in this study provides evidence that the ticks are one of the potential vectors for transmission of anaplasmosis in the cattle.

INTRODUCTION

Anaplasmosis is a rickettsial disease of cattle, sheep, horse and other domestic ruminants in the tropical and subtropical regions (Dumler *et al*., 2001). The *Anaplasma* genus is represented by Gram-negative intracellular bacteria which include *Anaplasma marginale*, *Anaplasma centrale*, *Anaplasma ovis*, *Anaplasma phagocytophilum*, *Anaplasma bovis* and *Anaplasma platys*. Bovine anaplasmosis is caused by *A. marginale* and *A. centrale* (Minjauw & McLeod, 2003; Kocan *et al*., 2004; de la Fuente *et al*., 2008). The disease is characterized by progressive anemia and the presence of intra-erythrocytic inclusion bodies (Wanduragala & Ristic, 1993). Sheep and goats in many regions of the world are infected by *A. ovis* (Renneker *et al*., 2013). *Anaplasma phagocytophilum* is the agent of pasture fever or tick-borne fever of ruminants (Stewart, 1992; Woldehiwet, 2006; Stuen, 2007). The organism has been documented as an emerging pathogen of public health importance (causing human granulocytic anaplasmosis) (Bakken & Dumler, 2000; Dumler *et al*., 2001; 2007). *Anaplasma bovis* has been reported in both domestic and wild animals in various parts of the world (Liu *et al*., 2012). *Anaplasma platys* is the causative agent of infectious cyclic thrombocytopenia in dogs (Allsopp *et al*., 1997; Du Plessis *et al*., 1997).

Anaplasmosis causes reduction in the body weight and milk production of cattle (Stuen *et al*., 2003). Animals with acute infections of *Anaplasma* usually suffer from fever and inappetence but seldom die unless there are complications by other infections (Rikihisa, 1991; Greig *et al*., 1996; Egenvall *et al*., 1997; Engvall & Egenvall, 2002). Cattle

can remain as chronically infected carriers for life after recovery from acute phase of infection. Relapse may occur if the cattle are under stress or immunosuppressed (Eriks *et al.*, 1989). Up to 19 different tick species including *Rhipicephalus* (formely known as *Boophilus*) *microplus* have been reported to transmit *A. marginale* (Hoogstraal, 1985; Kocan *et al.*, 2004).

Conventional diagnosis of anaplasmosis is mainly based on microscopic examination of blood smears from the infected animals as the organisms are unculturable on agar media (Lew & Jorgensen, 2005). However, detection of *Anaplasma* by this approach may not be suitable if the organism is present in low level in the blood (Shompole *et al.*, 1989; Ndung'u *et al.*, 1995). Diagnosis of anaplasmosis is also hampered by cross-reactivity of serological tests (Torioni de Echaide *et al.*, 1998). With the introduction of molecular techniques, the bacteria in the family *Anaplasmataceae* are reclassified based on 16S rDNA sequences of the bacteria (Dumler *et al.*, 2001). Polymerase chain reaction (PCR) assays based on 16S rDNA and other genes (*Msp1*, *Msp5*, *gltA* and *groEL*) have been used to facilitate the identification and phylogeny studies of *Anaplasma* species (Ndung'u *et al.*, 1995; Allsopp *et al.*, 1997; Parola *et al.*, 2000; Palmer *et al.*, 2001; Liu *et al.*, 2012; Ybañez *et al.*, 2013).

As there is little published data about *Anaplasma* infecting domesticated animals in Malaysia, this study was conducted to determine the presence of *Anaplasma* species in the blood samples of cattle, goats and deer in a local farm. A 16S rDNA PCR assay (Parola *et al.*, 2000) was employed for detection of *Anaplasma* spp. from the samples. Sequencing approach was then used to confirm the amplification results. This study also attempted to identify *Anaplasma* species based on the sequence variation of the *Anaplasma* partial 16S ribosomal RNA gene.

MATERIALS AND METHODS

Blood sampling

Blood samples were collected from 96 cattle, 23 goats and 22 deer from a farm in Selangor, Malaysia in July, 2012. Cattle from two imported breeds, i.e, Brangus/Bradford (n=32) and Friesian Sahiwal (n=32) and a local breed, Kedah Kelantan cross breed (n=32) were included for investigation in this study (Table 1). One ml blood sample was collected from the jugular vein of each animal into EDTA-coated tubes and stored at -20°C prior to processing. Ethical approval for animal care and use was obtained from Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM/FPV/PS/3.2.1.551/AUP-R164).

Tick collection

The animals were examined for tick infestation at the ear, eye, flank, abdomen, tail and perineal regions. Ticks were collected and preserved in 70% ethanol for morphological identification.

Table 1. Distribution of *Anaplasma* genotypes in the cattle blood and ticks samples in this study

Sample	No.(%) positive by PCR assay	No. (%) samples with genotype			
		1	2	3	4
Cattle (n=96)	81 (84.4%)	25 (30.9)	3 (3.7)	15 (18.5)	38 (46.9)
Kedah Kelantan cross breed (n=32)	30 (93.8%)	13 (43.3)	1 (3.3)	8 (26.7)	8 (26.7)
Brangus/Bradford (n=32)	26 (81.3%)	8 (30.8)	1 (3.8)	3 (11.5)	14 (53.8)
Friesian Sahiwal (n=32)	25 (78.1%)	4 (16.0)	1 (4.0)	4 (16.0)	16 (64.0)
Ticks (n=109)	6 (5.5)	5 (83.3)	0 (0.0)	0 (0.0)	1 (16.7)

Note: Genotypes 1-3 showed high sequence similarity to those of *A. platys/A. phagocytophilum*, whilst genotype 4 was identical to those of *A. marginale/A. centrale/A. ovis*

Molecular detection of *Anaplasma* DNA from blood and tick samples

DNA was extracted from blood and tick samples using QIAmp DNA mini extraction kit (Qiagen, Valencia, CA). PCR assay was performed in a thermal cycler (My Cycler^{TM}, Bio-Rad, CA) using primers EHR 16SD (5'- GGT ACC YAC AGA AGA AGT CC- 3') and EHR 16SR (5'- TAG CAC TCA TCG TTT ACA GC- 3') targeting the 16S rRNA gene of *Anaplasma* (Parola *et al.*, 2000). DNA extract (2 µl) was added to 19.4 µl sterile distilled water, 2.5 µl 10X Dream*Taq* buffer, 0.5 µl 10 mM dNTPs, 0.2 µl of each primer (25 µM) and 0.2 µl Dream*Taq*^{TM} DNA Polymerase (Fermentas, Lithuania). Amplified products were then analysed by electrophoresis on a 2% agarose gel at 100V for 45 minutes, stained with ethidium bromide, and the image was captured using InGenius gel documentation system (Syngene, England).

Sequence determination of partial 16S ribosomal RNA gene

Purification of PCR products was performed using Gene All Expin^{TM} Combo GP kit (GeneAll Biotechnology, Seoul, Korea) prior to sequence determination. Sequencing was performed using both forward and reverse primers on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, USA). The sequences were aligned with BioEdit Sequence Alignment Editor Software (Version 7.0.5.3) and compared for similarity with other sequences in the GeneBank database. A dendrogram was constructed based on the 16S rDNA sequences (228 nucleotides) of representative cattle and tick samples and various published *Anaplasma* species, using neighbor-joining method of the MEGA software and bootstrap analysis with 1,000 resamplings (Tamura *et al.*, 2011). *R. rickettsii* (U11021) was used as an outgroup for comparison.

RESULTS

Of 96 cattle blood samples tested in this study, *Anaplasma* DNA was amplified from 81 (84.4%) samples. The highest detection rate is noted in the Kedah Kelantan cross breed (93.8%). This is followed by Brangus/ Bradford (81.3%) and Shahiwal Friesian (78.1%) breeds. None of the blood samples from goats and deer were positive for *Anaplasma* DNA. Ticks were collected from the cattle, but no ticks were observed on the goats and deer investigated in this study. The ticks were identified tentatively as *R. microplus* based on their morphology chracteristics. Majority of the ticks were adults. Of 109 ticks examined, only 6 (5.5%) were positive by the PCR assays (Table 1).

Based on the *Anaplasma* sequences obtained from the cattle and tick samples in this study, four sequence types (designated as genotypes 1-4) were identified and matched with those *Anaplasma* spp. published in the Genbank database (Figure 1). The sequence of genotype 1 was identical to that of a French isolate of *A. platys* (AF303467). Genotype 2 had one nucleotide difference (99.5% sequence similarity) compared to that of *A. phagocytophilum* type strain (U02521). The sequence of genotype 3 was identical to that of *A. phagocytophilum* (U02521), while genotype 4 had complete matching sequence with those of *A. marginale* (AJ633048), *A. centrale* (AF414868) and *A. ovis* (AJ633049) (Figure 1).

Table 1 shows the distribution of *Anaplasma* genotypes in the cattle blood and ticks samples. Of the 81 *Anaplasma*-positive samples, 25(30.9%), 3(3.7%), 15(18.5%) and 38(46.9%) were identified as genotypes 1, 2, 3 and 4 respectively. Genotype 1 was predominantly detected from the blood samples of the local breed (Kedah Kelantan cross breed), whereas genotype 4 was identified more frequently in the Brangus/ Bradford and Friesian Sahiwal breeds. Genotypes 1 and 4 were identified in 5(4.6%) and 1(0.9%) ticks, respectively in the tick samples (Table 1).

Figure 2 is a dendrogram which was constructed based on the *Anaplasma* sequences of representative cattle and tick samples in this study and those published sequences. Genotypes 1, 2 and 3 were grouped with *A. platys* (AF303467) and *A. phagocytophilum* (U02521) at one branch of the dendrogram (bootstrap values, 33-55%).

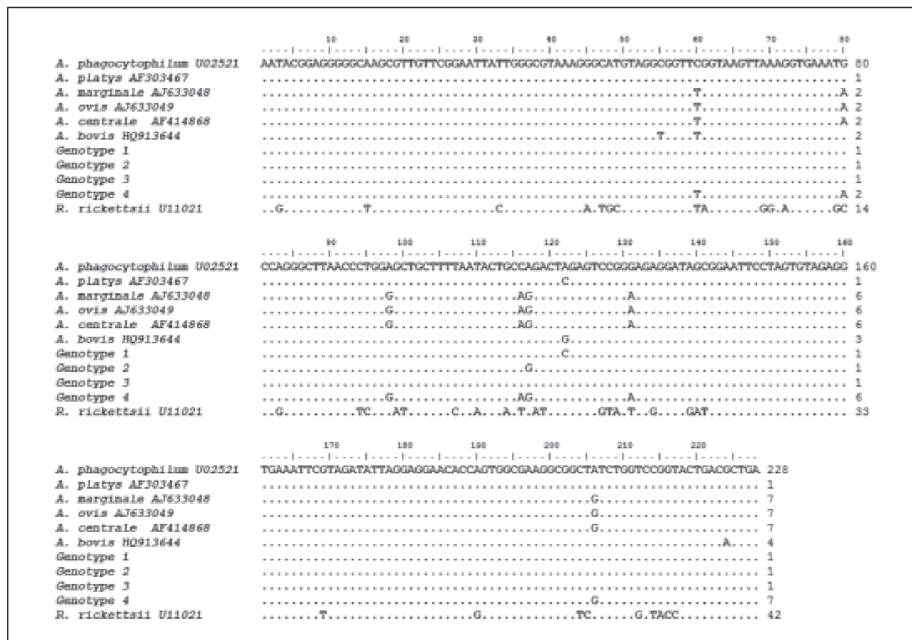

Figure 1. Comparison of the sequences derived from the partial fragments of 16S ribosomal RNA gene of *Anaplasma* genotypes (1-4) with published sequences of *Anaplasma* species. *R. rickettsii* (U11021) was included for comparison purpose

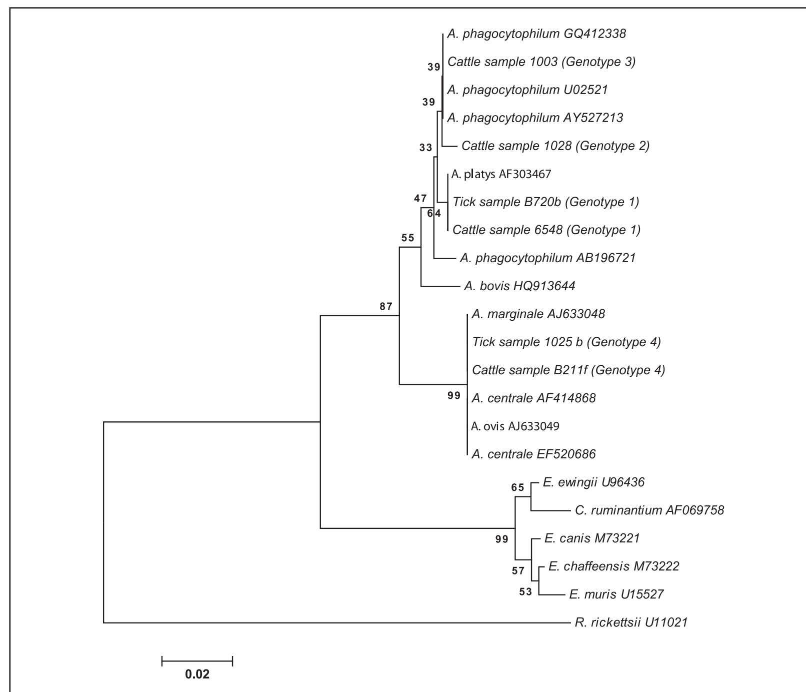

Figure 2. Dendrogram depicting phylogenetic placement of *Anaplasma* genotypes based on sequences of the partial 16S ribosomal RNA gene. Sequences obtained from cattle blood samples (1003, 1028, 6548, B211f) and ticks (B720b, 1025b) representing different *Anaplasma* genotypes 1-4 from this study, and published sequences from the Genbank database were included in the analysis. *R. rickettsii* (U11021) was used as an outgroup for comparison

Meanwhile, genotype 4 was grouped with *A. marginale/A. centrale/A. ovis* at another branch with a high bootstrap value (99%).

DISCUSSION

This is the first study reporting the detection of *Anaplasma* DNA in cattle and *R. microplus* ticks by molecular approach in Malaysia. *Anaplasma* DNA was detected from 84.4% of cattle blood samples in this study. None of the blood samples from goats and deer from the same farm were positive. The goats were kept under *stall feeding* (*zero grazing*) system in a pen 250 meters away from the cattle paddock, while the deer were allowed to graze in a paddock 100 meter away from the cattle paddock. Despite of the detection of *Anaplasma* DNA in the blood, the cattle were clinically healthy. Nevertheless, these infected cattle were probably chronic carriers of *Anaplasma* and might serve as a source of infection for other susceptible cattle in the herd, as reported by previous investigators (Lykkesfeldt & Svendsen, 2007; De *et al.*, 2012).

Different breeds of cattle may demonstrate varying levels of resistance to *Anaplasma* infection, for instance, *Bos indicus* has been reported to show greater resistance to *A. marginale* infection than *Bos taurus* (Wilson, 1979; Otim *et al.*, 1980). Although the local breed of cattle (Kedah Kelantan cross breed) is known to be more resistant to tick infestation, the detection rate of *Anaplasma* DNA (93.8%) is higher as compared to two imported cattle breeds (Table 1). The reason for this high detection rate is unknown.

Ticks were found on the cattle despite of regular tick control programme in the farm in this study. However, no ticks were collected from the goats and deer understudied. Beside ticks, *Anaplasma* may be transmitted through other biting arthropods such as horseflies, mosquitoes and use of unsterilized surgical instruments and needles (Kocan *et al.*, 2004). Due to the high vectorial capacity of *R. microplus* ticks, the prevalence of anaplasmosis has been reported to be highest in region where the ticks are endemic (Lincoln *et al.*, 1987; Palmer *et al.*, 2001; Futse *et al.*, 2003). *Rhipicephalus microplus* has been identified as a common cattle tick species in Malaysia (Nadchatram, 2006). The detection of *Anaplasma* DNA in 5.5% of ticks investigated in this study provides molecular evidence that the ticks are one of the potential vectors for transmission of anaplasmosis in our cattle. Our finding is consistent with that of a recent study conducted in Philippines where *Anaplasma* DNA was identified in 66.7% cattle and 13.3% *R. microplus* ticks using 16S rRNA-based screening-PCR and DNA sequencing approach (Ybañez *et al*, 2013).

The results of *Anaplasma* PCR assays were confirmed by sequence determination of the amplified products in this study. The sequences were further analysed for genotypic identification of *Anaplasma spp*. However, only little sequence variation was noted in the partial 16S ribosomal RNA genes of the *Anaplasma* spp. in the cattle blood samples (Figure 1). There is only one nucleotide difference between *A. platys* and *A. phagocytophilum* over the short nucleotide region (Figure 1). It is not possible to differentiate *A. marginale/A. centrale/A. ovis* as their sequences are identical. The sequence analysis is also reflected by the low bootstrap values (33-55%) exhibited by *A. platys/A. phagocytophilum* in the dendrogram. Hence, definite discrimination of *Anaplasma* species based on the short sequence (228 nt) is not possible. The use of full 16S rRNA gene sequences or other genes having more variations between species may provide more informative data on the *Anaplasma* species identified in this study.

The preliminary finding from this study suggests that at least four *Anaplasma* genotypes are circulating in our cattle population. These genotypes shared high sequence similarity to those of *A. platys*, *A. phagocytophilum*, *A. marginale*, *A. centrale* and *A. ovis*. The high prevalence of *A. marginale* has been reported in many other countries (De Waal, 2000; Palmer *et al.*, 2001; Futse *et al.*, 2003; Ybañez *et al.*, 2013). *Anaplasma phagocytophilum* has been recognized as an animal and human pathogen

which may spread through tick bites or contact with infected animal blood (Bakken & Dumler, 2000; Woldehiwet, 2006; Dumler *et al.*, 2007; Stuen, 2007). The presence of *A. platys* has been demonstrated using molecular approach in Malaysian dogs in a recent study (Mokhtar *et al.*, 2013). Ruminant can also be a potential reservoir for *A. platys* as the organism has been reported to cause infection in sheep and impalas (Allsopp *et al.*, 1997; Du Plessis *et al.*, 1997).

In view of the high prevalence of anaplasmosis in the cattle population investigated in this study, implementation of effective tick control programme is essential as anaplasmosis may impact animal health and production. Further study to identify and characterize *Anaplasma* spp. is necessary to provide further information on the biology, virulence and epidemiology of the organisms.

Acknowledgements. We thank Ms AS Mokhtar for her technical assistance in this study. The assistance of Dr Baljit Singh and his staff from Universiti Putra Malaysia in sample collection is greatly acknowledged. This study was supported by University of Malaya [grant numbers: HIR/E000013-20001 (subprogramme 4) and RP013/2012A].

REFERENCES

Allsopp, M.T.E.P., Visser, E.S., du Plessis, J.L., Vogel, S.W. & Allsopp, B.A. (1997). Different organisms associated with heart water as shown by analysis of 16S ribosomal RNA gene sequences. *Veterinary Parasitology* **71**: 283-300.

Bakken, J.S. & Dumler, J.S. (2000). Human granulocytic ehrlichiosis. *Clinical Infectious Diseases* **31**: 554-560.

de la Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M. & Sonenshine, D.E. (2008). Overview: ticks as vectors of pathogens that cause disease in humans and animals. *Frontiers in Bioscience* **13**: 6938-6946.

De, U.K., Dey, S., Banerjee, P.S. & Sahoo, M. (2012). Correlations among *Anaplasma marginale* parasitemia and markers of oxidative stress in crossbred calves. *Tropical Animal Health and Production* **44**: 385-388.

De Waal, D.T. (2000). Anaplasmosis control and diagnosis in South Africa. *Annals of the New Year Academy of Science* **916**: 474-483.

Du Plessis, L., Reyers, F. & Stevens, K. (1997). Morphological evidence for infection of impala, *Aepyceros melampus*, platelets by a rickettsia-like organism. *Onderstepoort Journal of Veterinary Research* **64**: 317-318.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y. & Rurangirwa, F.R. (2001). Re-organization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophilia*. *International Journal of Systematic and Evolutionary Microbiol*ogy **51**: 2145-2165.

Dumler, J.S., Madigan, J.E., Pusterla, N. & Bakken J.S. (2007). Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. *Clinical Infectious Diseases* **45**: 45-51.

Egenvall, A.E., Hedhammar, A.A. & Bjoersdorff, A.I. (1997). Clinical features and serology of 14 dogs affected by granulocytic ehrlichiosis in Sweden. *Veterinary Record* **140**: 222-226.

Engvall, E.O. & Egenvall, A. (2002). Granulocytic ehrlichiosis in Swedish dogs and horses. *International Journal of Medical Microbiology* **291**: 100-103.

Eriks, I.S., Palmer, G.H., McGuire, T.C., Allred, D.R. & Barbet, A.F. (1989). Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *Journal of Clinical Microbiology* **27**: 279-284.

Futse, J.E., Ueti, M.W., Knowles, D.P. Jr. & Palmer, G.H. (2003). Transmission of *Anaplasma marginale* by *Boophilus microplus*: retention of vector competence in the absence of vector-pathogen interaction. *Journal of Clinical Microbiology* **41**: 3829-3834.

Greig, B., Asanovich, K.M., Armstrong, P.J. & Dumler, J.S. (1996). Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. *Journal of Clinical Microbiology* **34**: 44-48.

Hoogstraal, H. (1985). Ticks. In *Parasites, Pests and Predators*, Gaafer, S.M., Howard, W.E. & Marsh, R.E. (eds.). Elsevier, Amsterdam.

Kocan, K.M., de la Fuente, J., Blouin, E.F. & Garcia-García, J.C. (2004). *Anaplasma marginale* (*Rickettsiales: Anaplasmataceae*): recent advances in dening host-pathogen adaptations of a tick-borne rickettsia. *Parasitology* **129**: 285-300.

Lew, A. & Jorgensen, W. (2005). Molecular approaches to detect and study the organisms causing bovine tick borne diseases: babesiosis and anaplasmosis. *African Journal of Biotechnology* **4**: 292-302.

Lincoln, S.D., Zaugg, J.L. & Maas, J. (1987). Bovine anaplasmosis: susceptibility of seronegative cows from an infected herd to experimental infection with *Anaplasma marginale*. *Journal of American Veterinary Medical Association* **190**: 171-173.

Liu, Z., Ma, M., Wang, Z., Wang, J., Peng, Y., Li, Y., Guan, G., Luo, J. & Yin, H. (2012) Molecular survey and genetic identification of *Anaplasm*a species in goats from central and southern China. *Applied and Environmental Microbiology* **78**: 464-470.

Lykkesfeldt, J. & Svendsen, O. (2007). Oxidants and antioxidants in disease: oxidative stress in farm animals. *Veterinary Journal* **173**: 502-511.

Minjauw, B. & McLeod, A. (2003). Tick-borne diseases and poverty: the impact of ticks and tick-borne diseases on the livelihoods of small-scale and marginal livestock owners in India and Eastern and Southern Africa. In *Research Report, DFID-AHP, Centre for Tropical Veterinary Medicine (CTVM)*. University of Edinburgh, United Kingdom, pg. 116.

Mokhtar, A.S., Lim, S.F. & Tay, S.T. (2013). Molecular detection of *Anaplasma platys* and *Babesia gibsoni* in dogs in Malaysia. *Tropical Biomedicine* **30**: 345-348.

Nadchatram, M. (2006). A review of endoparasitic acarines of Malaysia with special reference to novel endoparasitism of mites in amphibious sea snakes and supplementary notes on ecology of chiggers. *Tropical Biomedicine* **23**: 1-22.

Ndung'u, L.W., Aguirre, C., Rurangirwa, F.R., McElwain, T.F., McGuire, T.C., Knowles, D.P. & Palmer, G.H. (1995). Detection of *Anaplasma ovis* infection in goats by major surface protein 5 competitive inhibition enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* **33**: 675-679.

Otim, C., Wilson, A.J. & Campbell, R.S.F. (1980). A comparative study of experimental anaplasmosis in *Bos indicus* and *Bos taurus* cattle. *Australian Veterinary Journal* **56**: 262-266.

Palmer, G.H., Rurangirwa, F.R. & McElwain, T.F. (2001). Strain composition of the ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. *Journal of Clinical Microbiology* **39**: 631-635.

Parola, F., Roux, V., Camicas, J.L., Baradji, I., Brouqui, P. & Raoult, D. (2000). Detection of *Ehrlichiae* in African ticks by polymerase chain reaction. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**: 707-708.

Renneker, S., Abdo, J., Salih, D.E., Karagenç, T., Bilgiç, H., Torina, A., Oliva, A.G., Campos, J., Kullmann, B., Ahmed, J. & Seitzer, U. (2013). Can *Anaplasma bovis* in small ruminnats be neglected any longer? *Transboundary and Emerging Diseases* **60**: 105-112.

Rikihisa, Y. (1991). The tribe *Ehrlichieae* and ehrlichial diseases. *Clinical Microbiology Reviews* **4**: 286-308.

Shompole, S., Waghela, S.D., Rurangirwa, F.R. & McGuire, T.C. (1989). Cloned DNA probes identify *Anaplasma ovis* in goats and reveal a high prevalence of infection. *Journal of Clinical Microbiology* **27**: 2730-2735.

Stewart, C.G. (1992). Bovine ehrlichiosis, in *Tick vector biology: medical and veterinary aspects*, Fifaz, B., Petney, T. & Horzk, I., (eds). Springer-Verlag, Berlin, Germany, pg. 101-107.

Stuen, S., Bergström, K., Petrovec, M., Van de Pol I & Schouls, L.M. (2003). Differences in clinical manifestations and hematological and serological responses after experimental infection with genetic variants of *Anaplasma phagocytophilum* in sheep. *Clinical and Diagnostic Laboratory Immunology* **10**: 692-695.

Stuen, S. (2007). Anaplasma *phagocytophilum* – the most widespread tick-borne infection in animals in Europe. *Veterinary Research Communications* **31**: 79-84.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Torioni de Echaide, S., Knowles, D.P., McGuire, T.C., Palmer, G.H., Suarez, C.E. & McElwain, T.F. (1998). Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *Journal of Clinical Microbiology* **36**: 777-782.

Wanduragala, L. & Ristic, M. (1993). Anaplasmosis. In *Rickettsial and Chlamydial Diseases of Domestic Animals*. Woldehiwt, Z. and Ristic, M. (eds). Pergmon Press, Oxford, pg. 65-83.

Wilson, A.J. (1979). Observations on the pathogenesis of anaplasmosis in cattle with particular reference to nutrition, breed and age. *Journal of the South African Veterinary Association* **50**: 293-295.

Woldehiwet, Z. (2006). *Anaplasma phagocytophilum* in ruminants in Europe. *Annals of the New York Academy Sciences* **1078**: 446-460.

Ybañez, A.P., Sivakumar, T., Ybañez, R.H., Ratilla, J.C., Perez, Z.O., Gabotero, S.R., Hakimi, H., Kawazu, S., Matsumoto, K., Yokoyama, N. & Inokuma, H. (2013). First molecular characterization of *Anaplasma marginale* in cattle and *Rhipicephalus (Boophilus) microplus* ticks in Cebu, Philippines. *Journal of Veterinary Medical Science* **75**: 27-36.