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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

African buffalo is an important reservior of *Anaplasma bovis* in the Ngorongoro Crater, Tanzania

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Abstract

Anaplasma bovis is an intra-cytoplasmic gram negative rickettsia that infects monocytes and causes bovine monocytic anaplasmosis, which can be in peracute, acute, sub-acute or chronic form. A total of 170 tick DNA pools from 11 different tick species from Ngorongoro Crater were amplified for *Anaplasma* species using PCR targeting a gene fragment of 452bp of the 16S rRNA gene. Ten percent of 170 pools tested positive for *Anaplasma* species from seven tick species. On sequence analysis, *Anaplasma bovis* was detected in *Amblyomma gemma*, *A. variegatum*, *Rhipicephalus appendiculatus*, *Rh. evertsi*, *Rh. praetextatus* and *Rh. pulchellus*. The sequenced pools which revealed presence of *A. bovis* were from questing ticks and in moribund grass cover except *Rh. evertsi* which were from buffalo and zebra. This suggests that probably buffalo is an important reservoir of *Anaplasma bovis* and are the favourite hosts for *Rh. evertsi*. Detection of *Anaplasma bovis* suggests that probably the rickettsia contributed significantly in compromising the immunity of nutritionally stressed susceptible animals in the crater in 2000-2001. Therefore, susceptible livestock and wildlife in the crater are at risk of contracting the infections when nutritionally stressed.

Keywords: *Anaplasma bovis*; livestock; Ngorongoro Crater; tick species; wildlife

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Introduction

Originally *Anaplasma bovis* was known as *Ehrlichia bovis* under family *Rickettsiaceae*. However, following phylogenetic sequence similarity analysis of the 16S rRNA gene of families *Anaplasmataceae* and *Rickettsiaceae*, *Ehrlichia bovis* has been renamed as *Anaplasma bovis* (Dumler et al., 2001). The reorganization based on sequence homology of more than 97.3% with sequences of *Anaplasma marginale* and *A. ovis*, and the sequence homology ranging from 96.4 to 97.5% with sequences of *A. phagocytophilum* and *A. platys* (Dumler et al., 2001).

Anaplasma bovis is an intra-cytoplasmic gram negative rickettsia that infects monocytes. In Giemsa stained, blood smears the rickettsia appear as coccoids or cocco-bacilli often polymorphic and situated in vacuoles in the cytoplasm of the circulating mononuclear phagocytes (Sreekumar et al., 1996; Dumler et al., 2001; Goethert and Telford, 2003). The organisms may occur as singly or in compact colonies as a morula in circulating leucocytes which is the characteristic form of the organism (Soulsby, 1982; Sreekumar et al., 1996). Lagomorphs (cotton tail rabbits- *Sylvilagus floridanus*) have been found to harbour the rickettsia in USA and are described as the competent reservoirs of the infection (Goethert and Telford, 2003). *Anaplasma bovis* is a bovine pathogen and has been identified in cattle in many parts of the world and in buffalo and cattle in Africa (Kim et al., 2003; Kwahara et al., 2006). The pathogen causes bovine monocytic anaplasmosis, which can be peracute, acute, sub-acute or chronic form. The clinical symptoms include fever of 39 to 41^{o}C, anorexia, dyspnoea, sometimes a dry cough, tachycardia, diarrhoea, benign nervous disorders (hyper-excitability,

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transient paralysis of larynx, epileptic forms and paralysis), incoordination and enlargement of the lymph nodes (Sreekumar et al., 1996; Kawahara et al., 2006). In peracute form, it has been described to be associated with death within hours to days of the first signs of symptoms and in acute forms death can occur after 5 to 8 days (Sreekumar et al., 1996; Goethert and Telford, 2003). *Anaplasma bovis* is not cultivable *in vitro*; therefore, parasitological diagnosis rests on the examination of the parasites in mononuclear phagocytes in Giemsa stained blood smears (Sreekumar et al., 1996). In Asia, *A. bovis* has been identified in wild deer and in biological vector ticks and co-infection with other *Anaplasma* and *Ehrlichia* species is not uncommon (Kim et al., 2003; Kawahara et al., 2006). Ixodid ticks including *Amblyomma*, *Hyalomma* and *Rhipicephalus* species have been described as important biological vectors of the pathogen (Soulsby, 1982; Goethert and Telford, 2003; Walker et al., 2003). Ticks acquire the infection through transtadial transmission by feeding on infected host and so far transovarian transmission has not been demonstrated.

In Ngorongoro Crater, 15 tick species have been identified including some of the described biological vectors of *Anaplasma bovis* (Fyumagwa et al., 2007; 2009). Thirteen of these tick species have been consistently found on buffalo and seven species on cattle (Fyumagwa et al., 2007). Lagomorphs and other small mammals are resident mammals in the Ngorongoro Crater, suggesting that probably the rickettsia can be maintained in the area even in absence of large herbivores. In Tanzania, most often anaplasmosis is provisionally diagnosed based on clinical symptoms of mild fever, dehydration with pelleted dung, anaemia and observation of rickettsia organisms in erythrocytes. This is the classical anaplasmosis caused by *Anaplasma marginale*. However, *Anaplasma bovis* has rarely been reported because it infects monocytes, which in routine field diagnosis is often overlooked. Therefore, despite of high tick density in the crater there is no information regarding the existence of this rickettsia and its clinical manifestations in susceptible animals around Ngorongoro Crater. Detection of *Anaplasma bovis* in ticks in the current study is the first report on the presence of *A. bovis* in ticks from Ngorongoro Crater, Tanzania.

Materials and Methods

Study Area

The Ngorongoro Crater ($03^0$10'S, $35^0$35E) is the world's largest intact, inactive caldera occupying approximately 300km^2 (4%) of the total Ngorongoro Conservation Area (NCA) which has an area of about 8'300km^2 lying at the western edge of the Great Rift Valley in northern Tanzania. The crater is populated by resident wildlife; however, Masai livestock frequently go down the crater for grazing. Although a geographically distinct unit, it is part of the greater Serengeti Ecosystem (25'000 km^2) and regarded 'to some extent' as an ecologically distinct unit (Fyumagwa et al., 2007).

Collection of ticks

Systematic sampling of questing ticks in the crater grassland was performed in 2002 and 2003 by drag and removal of sampling (Fyumagwa et al., 2007). *Amblyomma* tick species (three-host tick species) are nocturnal and do not quest on grass. These tick species were collected under moribund grass cover along the roads and animal treks early in the morning. However, *Amblyomma tholoni* tick species were collected opportunistically on dead elephant in 2004. In 2004 and 2005, other wild herbivore species buffalo (*Syncerus caffer*), wildebeest (*Connochaetus taurinus*) and zebra (*Equus burchelli*) were immobilized to collect ticks that do not quest or hide in moribund grass in adult stage (one-host and two-host *Rhipicephalus* tick species). Immobilizing drugs were Etorphine hydrochloride (M99; Norvatis Pty Ltd, South Africa) in combination with a tranquilizer Azaperone tartrate (Kyron Laboratories Pty Ltd, South Africa). Collected ticks were preserved in liquid nitrogen (-196^{o}C) and transported in dry shipper to the Clinical laboratory of the Vetsuisse Faculty of the University of Zurich, Switzerland.

Identification of tick species

Frozen ticks were decanted in sterile petri dish placed under the dissecting microscope (Wild M38, Heerbrugg, Switzerland). The identification of tick species was performed using the descriptions of Walker et al. (2003). Identified ticks were kept in cold phosphate buffered saline (PBS 1x, Invitrogen, Basle, Switzerland). Ticks of the same species retrieved from the same cryo-vial were pooled in small samples of five ticks, properly labeled and transferred into pre-cooled 1.5ml eppendorfs (Sarstedt, Numbrecht, Germany) and later on preserved at -80^{o}C for subsequent procedures. Forceps and Petri dishes that were used in the identification were cleaned after every batch of ticks using DNA-EX (DNA contaminate removal solution, Inno-Train Diagnostic GmbH, Kronberg, Germany) followed by 70% ethanol (Kantonsapotheke, Zurich, Switzerland), then rinsed with distilled water.

Nucleic acids extractions

The frozen tick pools were thawed and placed on biochemically clean parafilm previously fitted on a sterile glass frame (American National Can$_{TM}$, USA). One tick pool at a time was mechanically disrupted

using sterile scalpel blades followed by decontamination of the glass frame and scalpel with DNA-EX (Inno-Train Diagnostic GmbH), 70% ethanol and rinsed with distilled water prior to processing the next pool. The mechanically disrupted ticks were transferred into 2ml micro centrifuge tubes and to each of the macerated pools 250µl of phosphate buffered saline (PBS 1x, without $MgCl_2$ and $CaCl_2$, Invitrogen) and 375µl of external lysis buffer (Guanidinium thiocynate Triton X-100, MagNA Pure LC Total Nucleic acid isolation kit, Roche Diagnostics, Rotkreuz, Switzerland) were added together with a 5mm silver bead. The tubes were vortexed (Vortex-Genie 2^{TM}, Bender and Hobein, AG, Zurich, Switzerland), fitted into a Mixer mill adapter set (Qiagen, Hombrechtikon, Switzerland) and homogenized at 30'000 hz for 2min in a Mixer Mill MM 300 device (Retsch GmbH, Haan, Germany), cooled in ice for 15min, homogenized again for 2min and cooled for 15min then centrifuged at 8'000 rpm for 1min. From each sample, 500µl of the lysate was used for nucleic acids extraction using the MagNA Pure LC automated system according to manufacturer's instructions (Roche Diagnostics). Sterile aerosol-barrier tips were used during all procedures. At the end of the extraction procedure 90µl of elute was transferred and preserved at -80^{o}C for subsequent molecular analysis. Extraction controls using distilled water were included in each extraction process to monitor absence of cross-contamination.

Determination of 18S rRNA from nucleic acids

The presence of amplifiable nucleic acids in extracted samples was confirmed using a TaqMan real-time PCR assay specific for the 18S rRNA gene (Applied Biosystems, Rotkreuz, Switzerland). Twenty elutes were taken randomly and used in the determination of 18S rRNA. Two negative controls were also analyzed including extraction control and distilled water. The 25µl master mix (1X) for each reaction tube included 12.5µl of 2x qPCR MasterMix (Eurogentec, Seraing, Belgium), 1.25µl of 20X 18S rRNA pre-mixed fluorogenic probe/primers (Applied Biosystems), 6.25µl of distilled water and 5µl of tick DNA template. The assay was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with an initial step of 50°C for 2 min and a denaturation at 95°C for 10min followed by 45 cycles at 95°C for 15 s and 60°C for 1min.

PCR amplification for *Anaplasma* species

A total of 170 DNA pools were amplified for *Anaplasma* species using conventional PCR targeting a gene fragment of 452bp of the 16S rRNA gene as previously described (Goodman et al., 1996). Each of the reaction tube had a total volume of 25µl of the reaction mixture which consisted of 2.5µl of reaction buffer (10x), 2.5µl of $MgCl_2$ (25mM), 0.5µl of dNTPs(10 mM each), 0.625µl each of the forward and reverse primers (20 µM of Ehr1 5'-TTT ATC GCT ATT AGA TGA GCC TAT G-3' and Ehr2 5'-CTC TAC ACT AGG AGG AAT TCC GCT AT-3' respectively), 0.5µl of Taq Polymerase (5U/µl, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), 15.25µl of distilled water and 2.5µl of DNA sample. Negative and positive controls were incorporated in the reaction. The PCR reaction was performed using a Tpersonal 48 Thermocycler (Biometra GmbH, Gottingen, Germany). The thermal profile consisted of an initial denaturation step at 95^{o}C for 5min followed by denaturation at 95^{o}C for 30sec. The temperature was decreased to 45^{o}C to allow for the complimentary DNA primers to anneal for 30sec followed by elongation at 72^{o}C for 45sec. The reaction was repeated for 35 cycles followed by a final elongation at 72^{o}C for 10min and finally cooled down to 4^{o}C. The PCR product was visualized in 1.5% agarose gel stained with ethidium bromide after electrophoresis. The DNA pools which yielded PCR products with fragment of interest were re-amplified and resolved in 1.5% agarose gel. The resolved amplicons were stained with Ethidium bromide for 10min followed by washing in distilled water for 10min and the fragments cut under UV light using a sterile scalpel blade, dissolved in micro centrifuge tubes with buffer QG (Qiagen) and stored at –20^{O}C for subsequent cloning and sequencing.

Purification of DNA fragments

PCR products with fragment of interest were cut under UV light using a sterile scalpel blade and eluted in micro-centrifuge tubes with a Buffer QG (Qiagen). The purification of DNA fragments was performed using the MinElute Gel Extraction Kit following the protocol as described by the manufacturer (Qiagen). The purified DNA fragments were extracted from the column according to the protocol described in the MinElute Gel Extraction Kit (Qiagen). The eluted purified DNA fragments were put in micro-centrifuges and stored at –20^{O}C for subsequent cloning procedures.

Cloning and sequencing

Purified PCR products were cloned into the vector pCR®II-TOPO® using the TOPO TA Cloning® Kit (Invitrogen). Plasmid DNA was purified by the QIAprep Spin Miniprep kit following the protocol as described by the manufacturer (Qiagen). The clonal DNA was checked for insert by digesting the DNA with the restriction enzyme EcoRI and positive clones were then sequenced from both sides.

Cycle sequencing was performed with approximately 10ng of DNA and 3.3pmol plasmid-

specific primers (M13 forward, M13 reverse) using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Rotkreuz, Switzerland). Cycling conditions were as follows: 1min at 96°C, then 25 cycles at 96°C for 10sec and 50°C for 5sec, followed by 60°C for 4min. Products were purified using the DyeEx Spin column (Qiagen), and analyzed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequences were aligned to one consensus sequence by SeqScape (Version 1.1, Applied Biosystems) and then compared to reference sequences in the Genebank. The reference sequence deposited in the GeneBank is GU361777-GU361780.

Data analysis

For statistical purposes, when a PCR product was obtained from a pool of five ticks, only one tick in the pool was assumed to be infected and the estimation of the rate of exposure was determined using the formula: Maximum Likelihood Estimation (MLE) = $1-(1-Y/X)^{1/m}$, as previously described (Walter et al.,1980). Where Y = number of positive pools; X = number of pools; m=number of organisms per pool.

Results

Tick species

Eleven tick species were identified in 2'000 tick samples which were randomly collected in the Ngorongoro Crater and brought to the Clinical Laboratories at the Vetsuisse Faculty of the University of Zurich. The list of tick species which were identified prior to processing for nucleic acid extraction and used for *Anaplasma* species screening is in Table 1.

Infected tick species

Seven tick species out of 11 identified were detected with *Anaplasma* species. The number of DNA pools tested, tick species infected, positive pools and rate of infection per tick species is also summarized in Table 1.

PCR amplification

Seventeen out of 170 pools tested positive for *Anaplasma* species. Part of the PCR products with fragments of interest resolved in 1.5% agarose gel is shown in Figure 2.

Cloning and sequencing

Cloning and sequencing of some of the amplified fragments revealed presence of *Anaplasma bovis* in *Amblyomma gemma*, *A. variegatum*, *Rhipicephalus appendiculatus*, *Rh. evertsi*, *Rh. praetextatus* and *Rh. pulchellus*.

Table 1: Identified tick species and source, pools tested and estimated prevalence of infection in tick population from Ngorongoro Crater

Tick species	Tick source	Pools tested	Positive pools	Prevalence per tick species
A. gemma	Ground	5	1	4.4%
A. cohaerens	Ground	2	0	0
A. tholoni	Elephant	10	0	0
A. variegatum	Ground	5	3	16.7%
R. appendiculatus	Questing	40	3	1.5%
R. compositus	Questing	23	3	2.8%
R. (*B.*) *decoloratus*	Buffalo	2	0	0
R. evertsi	Buffalo, zebra	16	4	5.6%
R. praetextatus	Questing	15	2	2.8%
R. pulchellus	Questing	13	1	1.6%
R. sanguineus	Lions	6	0	0

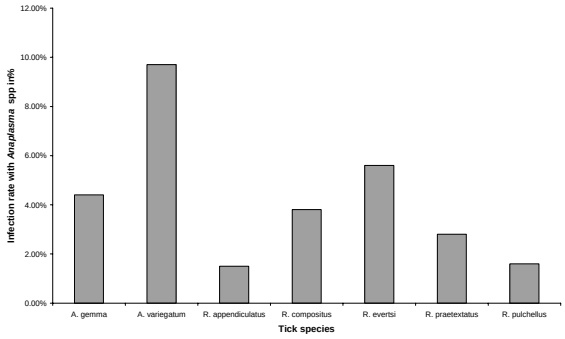

Fig. 1: Prevalence of *Anaplasma* species infection in different tick species collected from Ngorongoro Crater

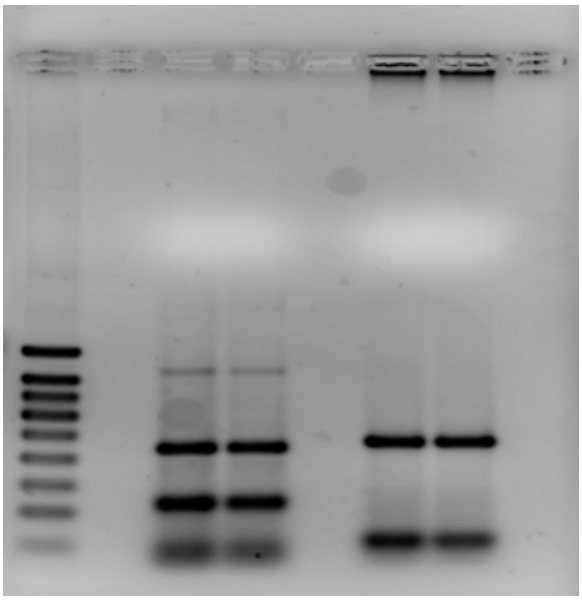

Fig. 2: PCR products which were resolved in 1.5% agarose gel prior to cloning indicating *Anaplasma* species positive pools in lane 3, 4, 6 and 7 (from left) lying between ladder of 400bp and 500bp.

Discussion

The PCR amplification for *Anaplasma* species has shown that seven tick species were infected, which is a wide range out of 11 identified tick species under this study. In the PCR amplification using real-time PCR, *Amblyomma variegatum* tested negative for *Anaplasma marginale* and *A. Phagocytophilum* (Fyumagwa et al., 2009). The tick species is known to be a biological vector of *Anaplasma bovis* (Goethert and Telford, 2003; Walker et al 2003). In the PCR analysis for *Anaplasma* species, *A. variegatum* had the highest rate of infection among seven tick species which tested positive for *Anaplasma* species (Fig.1). *Rhipicephalus evertsi* was the second in having a high rate of infection with *Anaplasma* species and is described to be a biological vector of *Anaplasma marginale* (Walker et al., 2003). However, in the PCR amplification for *A. marginale* using real- time PCR with primers specific for *A. marginale* none of the 17 *Rh. evertsi* pools tested positive for *A. marginale* or *A. phagocytophilum* (Fyumagwa et al., 2009). Therefore, the detected *Anaplasma* species in the PCR amplification were neither *A. marginale* nor *A. phagocytophilum*. In the sequence analysis, *Rh. evertsi* selected pools tested positive for *Anaplasma bovis*. The observation suggests that probably in Ngorongoro Crater, *Rh. evertsi* is a biological vector of *A. bovis*.

Amblyomma gemma is not described to be important to the health of domestic animals (Walker et al., 2003), however, in the PCR amplification it has shown to be infected by *Anaplasma* species and was the third in prevalence after *A. variegatum* and *Rh. evertsi*. *Rhipicephalus appendiculatus* is described to be a biological vector of *Anaplasma bovis* but not *A. marginale*. However, in a different study using real-time PCR, *Rh. appendiculatus* was found to be infected with *A. marginale* (Fyumagwa et al., 2009). This observation suggests that this tick species is probably infected by many more of the known tick-borne pathogens than it was thought before and is the most important tick species in transmitting a wide range of tick-borne diseases in eastern, central and southern Africa where the tick species is endemic (Soulsby, 1982; Walker et al., 2003; Lynen et al., 2007). *Amblyomma variegatum* and *Rh. appendiculatus* cause tick toxicosis which is immunosuppressive to infested animals (Walker et al., 2003). Presence of *Anaplasma bovis* can have a synergistic effect in compromising the immunity of infected animals because the rickettsia causes leucopenia apart from other pathological effects to susceptible animals.

Rhipicephalus compositus, Rh. praetextatus and *Rh. pulchellus* are not described to transmit *Anaplasma* species (Walker et al., 2003). However, in this amplification the three tick species tested positive for *Anaplasma* species and in sequence analysis the later two revealed presence of *A. bovis* suggesting that probably are important vectors. In real-time PCR *A. phagocytophilum* was not detected in the three tick species, therefore, the positive pools for *Anaplasma* species could be *A. bovis* and probably other *Anaplasma* species (Fyumagwa et al., 2009).

Some of the sequenced pools which revealed presence of *A. bovis* from *Rh. evertsi* were collected on buffalo and zebra. This suggests that probably buffalo is an important reservoir of *Anaplasma bovis* in the Ngorongoro Crater (Kwahara et al., 2003). In the crater, buffalo and zebra have been observed to be the favourite host for *Rh. evertsi* (Fyumagwa et al., 2007).

Conclusion

Detection of *Anaplasma bovis* suggests that the rickettsia probably contributed significantly in compromising the immunity of nutritionally stressed animals in Ngorongoro Crater (Grootenhuis, 2000; Fyumagwa et al., 2004). Therefore, susceptible livestock and wildlife in the crater are at high risk of contracting multiple infections with clinical symptoms during severe and prolonged drought.

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References

Dumler, J.S., Barbet, A.F., Bekker, C.P.J., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y. and Rurangirwa, F.R. 2001. Reorganization of genera in the families *Rickettsiaceae*: 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 phagocytophila*. *International Journal of Systematic Evolutionary Microbiology*, 51: 2145-2165.

Fyumagwa, R.D., Mkumbo, S.S. and Morkel, P.B. 2004. Remote treatment of black rhinos against babesiosis in Ngorongoro Crater, Tanzania. *Pachyderm*, 37: 80-83.

Fyumagwa, R.D., Runyoro, V., Horak, I.G. and Hoare, R. 2007. Ecology and control of ticks as disease vectors in wildlife of the Ngorongoro Crater, Tanzania. *S. African Journal of Wildlife Research*, 37: 79-90.

Fyumagwa, R.D., Simmler, P., Meli, M.L., Hoare, R., Hofmann-Lehmann, R. and Lutz, H. 2009. Prevalence of *Anaplasma marginale* in different tick species from Ngorongoro Crater, Tanzania. *Veterinary Parasitology*, 161: 154-157.

Goethert, H.K., and Telford, S.R. 2003. Enzootic transmission of *Anaplasma bovis* in Nantucket cotton tail rabbits. *Journal of Clinical Microbiology*, 41: 3744-3747.

Goodman, J.L., Nelson, C., Vitale, B., Madigan, J.E., Dumler, J.S., Kurtti, T.J. and Munderloh, U.G. 1996. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. *New England Journal of Medicine*, 334: 209-215.

Grootenhuis, J.G. 2000. Wildlife, Livestock and Animal Disease Reservouirs, In: Prins, H.H.T., Grootenhuis, J.G., and Dolan, T.T. (Eds). Wildlife Research for Sustainable Use. Kluwer Academic Publishers, Dordrecht, the Netherlands. Pp: 81-113.

Kawahara, M., Riskihisa, Y., Lin, O., Isogai, E., Tahara, K., Itaqaki, A., Hiramitsu, Y. and Tajima, T. 2006. Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale* and *Ehrlichia* species in wild deer and ticks on two major islands in Japan. *Applied Environmental Microbiology*, 72: 1102-1109.

Kim, C.M., Kim, M.S., Parks, M.S., Park, J.H. and Chae, J.S. 2003. Identification of *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum* and *Anaplasma bovis* in *Haemophysalis longicornis* and *Ixodes persulcatus* ticks from Korea. *Vector Borne Zoonotic Diseases*, 3: 17-26.

Lynen, G., Zeman, P., Bakuname, C., Di Giulio, G., Mtui, P., Sanka, P. and Jongejan, F. 2007. Cattle ticks of the genera *Rhipicephalus* and *Amblyomma* of economic importance in Tanzania: distribution assessed with GIS based on an extensive field survey. *Experimental and Applied Acarology*, 43: 303-319.

Soulsby, E.J.L. 1982. *Helminths, Arthropods, and Protozoa of Domesticated animals*. 7th edition. Bailliere Tindall, London. Pp: 752-759.

Sreekumar, C., Anandan, R., Balasundaram, S., & Rajavelu, G. 1996. Morphology and staining characteristics of *Ehrlichia bovis*. *Comparative Immunology and Microbiology*, 19: 79-83.

Walker, A.R., Bouattour, A., Camicas, J.L., Estrada-Pena, A., Horak, I.G., Latif, A.A., Pegram, R.G. and Preston, P.M. 2003. *Ticks of Domestic Animals in Africa: a Guide to Identification of Species*. Bioscience Reports, 42 Comiston Drive, Edinburgh EH10 5QR, Scotland, U.K. Pp: 1- 221.

Walter, S.D., Hildreth, S.W. and Beaty, B.J. 1980. Estimation of infection rates in populations of organisms using pools of variable size. *American Journal of Epidemiology*, 112: 124-128.