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# Molecular Detection of Previously Unknown *Anaplasma* Genotype in Cattle from Uganda

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**Abstract:** The genus *Anaplasma* includes some of the most economically important tick-borne pathogens of cattle in sub-Saharan African. The main objective of this study was therefore to identify *Anaplasma* species/genotypes circulating in cattle from Kumi and Kiruhuura Districts in eastern and southwestern regions of Uganda respectively where only *A. marginale* has been reported to exist based on serological detection and/or microscopy. Nine farms, 5 from Kumi and 4 from Kiruhuura were randomly selected and blood samples collected from apparently normal calves (<1 year) and adult cattle (>2.5 years). The species- specific oligonucleotide probes based on the differences within the hypervariable V1 region of the 16S rRNA gene were used to detect *Anaplasma* organisms in blood. From this study, previously unknown *Anaplasma* genotype, closely related to *A. marginale* but not hybridizing with *A. marginale*-specific oligonucleotide probe was detected from eastern Uganda. This genotype showed nucleotide differences with the known *Anaplasma* species at the hypervariable V1 region. In addition *A. bovis* and *A. marginale* were detected in cattle from both regions while *A. centrale* was detected only in cattle from southwestern Uganda. The *Anaplasma* infections were detected only in adult cattle. This indicates possible high diversity of *Anaplasma* in these regions of Uganda. The previously unknown genotype may be a different *Anaplasma* species or a variant of *A. marginale*. We suggest the use of the heat shock operon (groESL), citrate synthase (gltA) and surface protein genes to further characterize this *Anaplasma* genotype.

**Key words:** *Anaplasma marginale* variant, cattle, kiruhuura, Kumi (Uganda), 16S rRNA

## INTRODUCTION

The genus *Anaplasma* contains obligate intracellular bacteria of vertebrate hosts. This genus includes tick-borne pathogens of domestic and wild ruminants, dogs and humans. *Anaplasma marginale, A. centrale, A. bovis* and *A. phagocytophilum* are reported pathogenic species of cattle (Inokuma, 2007). Some studies suggest that there may be yet uncharacterized *Anaplasma* or *Ehrlichia* species in cattle from Africa (Awadia *et al*., 2006).

*Anaplasma marginale* and *A. bovis* cause severe bovine Anaplasmosis (Gyles *et al*., 2004). Although severe disease may also occur with *A. centrale* (Ceci *et al*., 2008), it usually causes only a mild anemia in most cases (Ristic and Kreier, 1984). *Anaplasma marginale* is the most widely distributed, infecting red blood cells and causes severe anemia, weakness, fever, anorexia, depression, constipation, decreased milk yield, jaundice, abortion and sometimes death (Alderink and Dietrich, 1981). Calves are less susceptible to infection with *A. marginale* and when infected, are less susceptible to clinical disease and this phenomenon is not well understood (Kocan *et al*., 2003). Animals that recover naturally or after chemotherapy remain carriers of the disease for life and show no signs of the disease. The disease carriers act as sources of infection for susceptible cattle (Zaugg *et al*., 1986). The exotic breeds of cattle or their crosses and indigenous cattle raised from disease-free areas or moved to endemic areas are the most susceptible to these tick-borne diseases (Aguirre *et al*., 1988).

*Anaplasma marginale*, *A. centrale* and *A. bovis* have been detected using the 16S rRNA PCR (Oura *et al.,* 2004) in cattle from central Uganda. In eastern and southwestern regions of Uganda, *Anaplasma* infection in cattle has been reported basing on *A. marginale* serological detection and/or microscopy (Kabi *et al*., 2008; Ocaido *et al*., 2005; Rubaire-Akiiki *et al*., 2004; Ssenyonga *et al*., 2006). This has generated limited information on the diversity of *Anaplasma* from these regions of Uganda. The main objective of this study was therefore to detect *Anaplasma* species/genotypes circulating in cattle from other regions of the country especially eastern and southwestern Uganda. Herein, we report the presence of four *Anaplasma* species/genotypes in cattle from Kumi and Kiruhuura Districts in eastern and southwestern Uganda respectively including a previously unknown *Anaplasma* genotype in Uganda. The species-specific oligonucleotide probes deduced from the hypervariable V1 region of the 16S rRNA gene were used to detect the *Anaplasma*.

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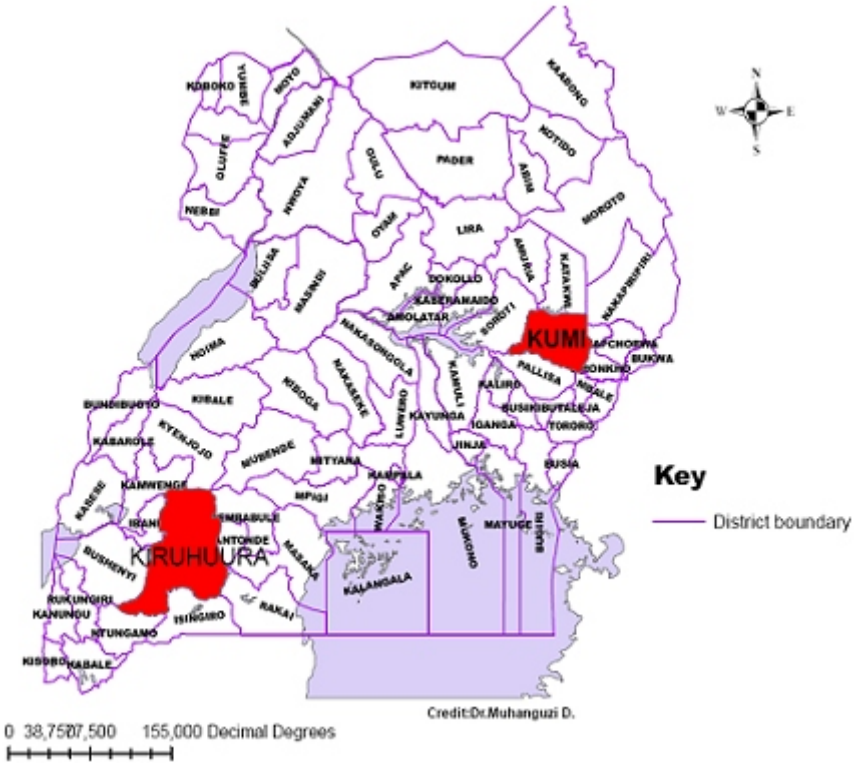


Fig. 1: Map of Uganda showing Kumi and Kiruhuura Districts in eastern and south Western Uganda respectively (marked red) from where blood samples were collected

## MATERIALS AND METHODS

**Study area:** The study was carried out in Ongiino sub-county, Kumi District in eastern Uganda and Sanga sub-county, Kiruhuura District in southwestern Uganda (Fig. 1) between 2006 and 2008. These regions have been known to be endemic to tick-borne diseases. Nine farms were selected using randomly generated computer numbers (5 from Ongiino and 4 from Sanga). Cattle farms from Sanga were at the periphery of Mburo National Park. In total 247 and 109 blood samples were collected and analyzed from Kumi and Kiruhuura Districts respectively. The study was conducted by the Faculty of Veterinary Medicine, Makerere University.

**Collection of blood samples:** Blood was collected from apparently normal Zebu and Ankole longhorn cattle in Kumi and Kiruhuura Districts respectively. A majority of cattle in these areas are from these breeds. Blood was collected from calves and adults <1 year and >2. 5 years of age respectively by jugular venipuncture into EDTA-coated vacutainers and kept on ice packs for transportation to the Laboratory where it was aliquoted into 1.5 mL eppendorf tubes and stored at -20ºC until required for DNA extraction.

**Extraction of DNA from blood:** Blood was processed for DNA extraction as described by d'Oliveira *et al.* (1995). Briefly, 200 µL of thawed blood was washed 3-5 times by mixing with 0.5 mL PBS (137mM NaCl, 2.6 mM KCl, 8.1mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.4), each time followed by centrifugation at maximum speed (13,000 rpm) for 5 min. After the final wash, the cell pellet was resuspended in 100 µL of lysis mixture (10mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5% Tween 20, 100 µg/mL of proteinase K). This mixture was incubated overnight at 56ºC, followed by 10 min of boiling to inactivate the Proteinase K and kept at -20ºC until needed for PCR for amplification of the 16S rRNA gene.

**PCR amplification of the *Anaplasma* 16S rRNA gene:** The DNA samples from cattle blood were used in PCR reactions (Reverse line blot-PCR) to amplify any *Anaplasma* (or even any *Ehrlichia*) 16S rRNA gene present. One primer set was used to amplify a 492-498 bp fragment being part of the V1 region of the16S rRNA gene. The forward primer was the 16S8FE (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') described by Schouls *et al.* (1999) whereas the reverse primer was the B-GAIB-new (biotin-5'-CGG GAT CCC GAG TTT GCC

Table 1: Species-specific probe sequences bound onto the reverse line blot membranes

| Species | Probe sequence from 5' to 3' all with 5'-C6-TFA- aminolinker | Reference |
|---|---|---|
| *Ehrlichia/Anaplasma* catch all | GGG GGA AAG ATT TAT CGC TA | Bekker *et al.*, 2002 |
| *E. ruminantium* | AGT ATC TGT TAG TGG CAG | |
| *A. bovis* | GTA GCT TGC TAT GAG AAC A | |
| *A. marginale* | GAC CGT ATA CGC AGC TTG | |
| *A. centrale* | TCG AAC GGA CCA TAC GC | |
| *A. centrale* | GAC CAT ACG CGC AGC TT | Georges *et al.,* 2001 |
| *A. phagocytophilum species* | TTG CTA TAA AGA ATA ATT AGT GG, | Bekker *et al.*, 2002, |
| | TTG CTA TGA AGA ATA ATT AGT GG, | RLB manual, |
| | TTG CTA TAA AGA ATA GTT AGT GG and | Isogen |
| | TTG CTA TAG AGA ATA GTT AGT GG | |
| *E. chaffeensis* | ACC TTT TGG TTA TAA ATA ATT GTT | RLB manual, Isogen |
| *E.* sp. *omatjenne* | CGG ATT TTT ATC ATA GCT TGC | Bekker *et al.*, 2002 |
| *E. canis* | TCT GGC TAT AGG AAA TTG TTA. | RLB manual, Isogen |

GGG ACT TCT-3') described by Bekker *et al.* (2002), a modification of primer B-GAIB described by Schouls *et al.* (1999). The primer set was procured from Isogen (Maarssen, The Netherlands). The 1xPCR reaction constituents in a final volume of 25 μL were as follows: 1xPCR buffer (Invitrogen), 3.0 mM $MgCl_2$ (Invitrogen), 200 μM each dATP, dCTP, dGTP, 100 μM dTTP (ABgene) and 100 μM dUTP (Amersham), 1.25 U of Taq polymerase (Invitrogen), 0.1U of UDG (Amersham), 25 pmol of each primer and 2.5 μL of template DNA. This was over laid with about 12.5 μL of mineral oil. Positive control DNA (*E. canis*) from Molecular Biology Laboratory, Makerere University and negative control (reaction constituents without DNA) tests were included. The reactions were performed using a three-phase touchdown program as previously described (Bekker *et al.* 2002).

**Hybridization of species-specific DNA probes on to the Biodyne C membrane:** Two RLB membranes were used in this study, the locally prepared and the commercial membrane. The local RLB membrane was prepared as described by Gubbels *et al.* (1999). The species-specific oligonucleotide probes for any *Ehrlichia/Anaplasma* (catch-all probe), *A. marginale*, *A. centrale*, *A. bovis* and *E. ruminantium* procured from Isogen Life Science, The Netherlands (Table 1) were applied to the activated Biodyne C membrane as described by Gubbels *et al.* (1999). The species-specific oligonucleotide probes were synthesized with a 5'-terminal aminogroup (N-terminal N-(trifluoracetamidohexyl-cyanoethyle, N,N-diisopropyl phosphoramidite [TFA]-C6 amino linker (Isogen), which covalently links the oligonucleotide probes to the activated Biodyne C membrane. The commercial RLB membrane was procured from Isogen Life Science already made. Two different *A. centrale* oligonucleotide probes were used in this study. The *A. centrale* probe by Georges *et al.* (2001) was applied onto the locally prepared membrane whereas *A. centrale* probe described by Bekker *et al.* (2002) was on the commercial membrane procured from Isogen.

**Reverse line blot hybridization of PCR products:** Hybridization of PCR products to the species-specific oligonucleotide probes was performed as previously described (Gubbels *et al.*, 1999) except that 15 μL of the PCR products were mixed with 150 μL 2XSSPE/0.1%SDS as previously done (Oura *et al.*, 2004). Also, the membrane was washed twice in 100ml pre-heated 2XSSPE/0.5%SDS for 10 min at 52ºC to remove any non-specific products that may have hybridized onto the membrane. There after, the membrane was incubated for 1 min at room temperature in 10ml of ECL detection liquids 1 and 2 (Amersham) and placed between two overhead sheets. The covered membrane was then placed on the intensifying screen in an exposure cassette and exposed to an ECL-hyperfilm (Amersham) for 20-30 min in the dark room. After exposure, the hybridization signals were then developed. The membranes were stripped for re-use as previously described (Gubbels *et al.*, 1999).

**Sequencing of the 16S rRNA gene of previously unknown *Anaplasma* genotype:** In one case whereby PCR product strongly hybridized with only the *E/A*-catch-all oligonucleotide probe, the whole 16S rRNA gene was amplified using primers fD1 and Rp2 (Weisburg *et al.*, 1991). To improve on the quality of the sequence result, the amplicon was nested using 2 primer pairs; EHR16SD/EHR16SR (Parola *et al.*, 2000) and RLB-F790/RLB-R1134 (Molad *et al.*, 2006). The first PCR using the universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and Rp2 (5'-ACGGCTACCTTGTTACGACTT-3') was performed and the primer set amplifies the 16S rRNA gene sequence of most eubacterias (Weisburg *et al.*, 1991). The reaction was performed in a final volume of 50 μL containing 5 μL of DNA, 1X PCR buffer, 3.0 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 1.2U of Taq polymerase and 25 pmol of each primer. Cycling conditions were 94ºC for 5 min and then 34 cycles each at 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 1.5 min. Final extension was at 72ºC for 5 min. The middle and 3' portions of the amplified 16S rRNA gene in the first PCR were re-amplified using the primer sets EHR16SD/ EHR16SR (Parola *et al.*, 2000) and RLB-F790/RLB-R1134 (Molad *et al.*, 2006) respectively. The primers EHR16SD and EHR16SR amplify a segment of the 16S

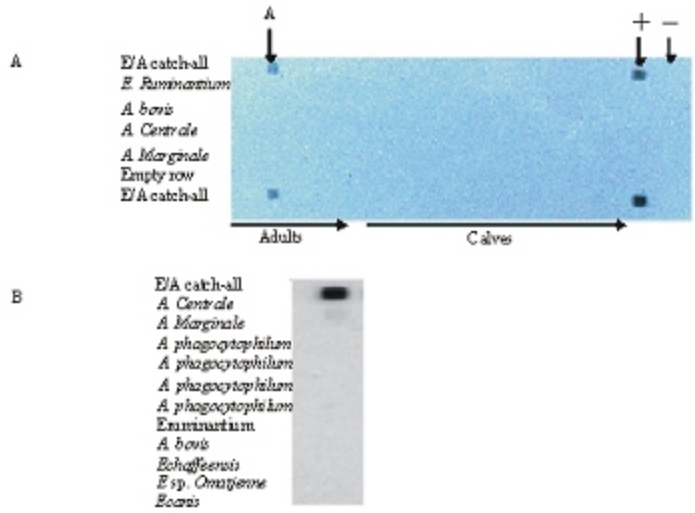


Fig. 2: Reverse line blot analysis of blood samples from Ongiino, Kumi
(A) The species-specific oligonucleotide probes were applied to the horizontal rows and PCR products applied vertically. The positive control (+) was *E. canis* DNA from the Molecular Biology Laboratory, Department of Veterinary Parasitology and Microbioogy, Makerere University. The negative control (-) consisted of PCR mix without DNA. Blot A is from the locally prepared membrane whereas blot B is from the commercial membrane (Isogen). Both membranes were used to detect the genotype in blood sample A. The genotype in blood sample A hybridized strongly with only the E/A catch-all probe on both blots. However non-specific hybridization with *A. central* probe described by Bekker *et al*., (2002) was observed. (B) This suggests that the genotype in blood sample A has a unique nucleotide sequence at the hypervariable VI loop of the 16S rRNA gene

rRNA gene from bacteria within the family *Anaplasmataceae* while primers RLB-F790/RLB-R1134 are specific for *Anaplasma* species. The following were used when amplifying the middle portion: 25 pmol of each primer [forward primer EHR16SD (5'-GGTACCTACAGAAGAAGTCC-3') and reverse primer EHR16SR (5'-TAGCACTCATCGTTTACAGC-3')], 1xPCR buffer, 3.0 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP and dTTP, 1.2 U of Taq polymerase and 1 µL of purified product from the first PCR in a final volume of 50 µL. The cycling conditions were 94ºC for 5 min and then 30 cycles each at 94ºC for 30 sec, 52ºC for 1 min and 72ºC for 1.5 min. Final extension was at 72ºC for 5 min. Twenty five Picomoles of each primer [forward primer RLB-F790 (5'-GGCTTTTGCCT CTGTGTTGT-3') and reverse primer RLB-R1134 (5'-CTTGACATCA TCCCCACCTT-3')], 1xPCR buffer, 3.0 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP and dTTP, 1.2 U of Taq polymerase and 1 µL of purified product from the first PCR in a final volume of 50 µL were used to amplify the 3' portion. The cycling conditions were 94ºC for 5 min and then 30 cycles each for 30 sec at 94º C, 30 sec at 55º C and 1.5 min s at 72º C. Final extension was at 72ºC for 7 min. Thereafter, the products from RLB-PCR and nested PCR were purified using QIAquick PCR purification kit (QIAGEN) and sent for sequencing at MWG Biotech, Germany or ILRI, Kenya.

## RESULTS

**Detection of previously unknown *Anaplasma* genotype in cattle from Uganda:** The *Anaplasma* genotype in blood sample A from Ongiino, Kumi was detected by blotting (Fig. 2) and sequence analysis of its 16S rRNA gene (Fig. 3 and 4). The genotype in sample A was first detected using the locally prepared RLB membrane (Fig. 2A) and repeated using the commercial RLB membrane (Fig. 2B).

**Analysis of the 16S rRNA gene of the genotype in sample A:** The 16S rRNA gene of the genotype in sample A was PCR amplified and sequenced. The BLAST and phylogenetic analyses (Fig. 4) showed that this is *Anaplasma* genotype.

**Sequence alignment of the hypevaribale V1 region of the 16S rRNA gene:** The 16S rRNA gene sequence of the *Anaplasma* genotype in blood sample A (GenBank Accession number: HM061603) was aligned with 16S rRNA gene sequences of closely related *Anaplasma* species (*A. marginale* and *A. centrale*) to demonstrate the differences within the hypervariable V1 region from where species-specific oligonucleotide probes are deduced (Fig. 3).

```
                          70          80          90
                          +           +           +
A. marginale-Florida      GTATACGCAGCTTGCTGCGTGTATGGTTAGTGGC
A. marginale-Zimbabwe     GTATACGCAGCTTGCTGCGTGTATGGTTAGTGGC
Anaplasma-Uganda          ATACACGCAGCTTGCTGCGTGTATGGTTAGTGGC
A.centrale-S.A            ATACGCGCAGCTTGCTGCGTGTATGGTTAGTGGC
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Fig. 3: Alignment showing nucleotide differences within the hypervariable VI region of the 16S rRNA gene
The hypervariable VI region was identified from previous alignment (Bekker *et al*., 2002). The underlined part is only where variability is exhibited. The *Anaplasma* genotype in Sample A (*Anaplasma*-Uganda), differs from *A. centrale* at position 70 only (Red nucleotide) whereas it differs from *A. marginale* at positions 66 and 69 (bold nucleotides). This therefore confirms that the *A. marginale*- or *A. centrale*- specific oligonucleotide probes could not strongly hybridize with *Anaplasma* genotype in blood sample A because of these differences. The nucleotide positions are referred to the sequence of *A. marginale* –Florida (Accession number AF309867). Accession number for *A. marginale*- Zimbabwe and *A. centrale*- South Africa are AF414878 and AF414869, respectively.

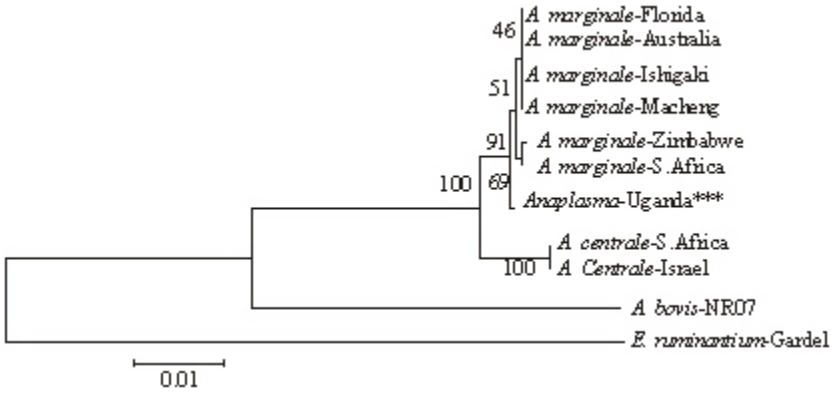


Fig. 4: Phylogenetic relationship of *Anaplasma* in sample A with other rickettsiae
Using MEGA 4 program (Tamura *et al*., 2007) the evolutionary history was inferred using the Neighbor-Joining method. The *Anaplasma* genotype in sample A (matched with asterisks) is closely related to *A. marginale*

**Phylogenetic analysis of *Anaplasma* genotype in blood Sample A:** A partial 16S rRNA gene sequence (1088 bases, GenBank Accession number: HM061603) of the *Anaplasma* genotype in blood sample A was used for phylogenetic analysis (Fig. 4). The GenBank accession numbers of the 16S rRNA gene sequences used to construct the phylogenetic tree are: *A. marginale*-Florida, AF309867, *A. marginale*-Australia, AF414874, *A. marginale*-Ishigaki, FJ226454, *A. marginale*-Macheng Buffalo, DQ341370, *A. marginale*-Zimbabwe, AF414878, *A. marginale*-S. Africa, AF414873, *A. centrale*-S. Africa, AF414869, *A. centrale*-Israel, AF309869, *A. bovis* NR07, AB196475 and *E. ruminantium*-Gardel, CR925677. The evolutionary history was inferred using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in Molecular Evolutionary Genetics Analysis 4, MEGA4 (Tamura *et al*., 2007).

**Other *Anaplasma* detected in cattle:** *Anaplasma bovis* and *A. marginale* were detected in cattle blood from both Districts. *Anaplasma centrale* was detected only in cattle blood from Kiruhuura. All the *Anaplasma*-positive samples were from adult cattle >2.5 years old while calves <I year of age tested negative.

## DISCUSSION

In this study, DNA-DNA hybridization based assay was used to detect *Anaplasma* organisms in cattle from two districts of Uganda. Blotting, sequencing and phylogenetic analysis of the 16S rRNA gene (partial sequence) from a previously unknown *Anaplasma* genotype showed that this organism is closely related to *A. marginale*. However, this *Anaplasma* genotype could not hybridize with *A. marginale*-specific oligonucleotide probe, suggesting that the two have genetic differences at the hypervariable V1 region of the 16S rRNA gene from where the species-specific oligonucleotide probes are deduced. This was confirmed by sequencing. This observation suggests presence of *Anaplasma* genotype in cattle from Uganda not yet characterized. Previously, Inokuma *et al.* (2005) reported presence of previously unknown *Anaplasma* sp. related to *A. phagocytophilum* in a dog from South Africa. By using RLB assay, Awadia *et al.* (2006) also reported that there may be a

new species of *Ehrlichia/Anaplasma* infecting cattle in the Sudan, North of Uganda. This indicates that there are *Anaplasma* species in domestic animals not yet identified. The genotype in sample A from Kumi in eastern Uganda therefore may be a different *Anaplasma* species or a variant of *A. marginale*. This should be confirmed by gene sequencing and analysis of the entire 16S rRNA, heat shock operon (groESL), citrate synthase (gltA) and surface protein genes. The animal infected with this *Anaplasma* genotype, like all other animals infected with other *Anaplasma* organisms did not show any observable signs of disease, suggesting that the infected animal was a recovered carrier or this *Anaplasma* genotype causes less severe disease. Furthermore, other *Anaplasma* organisms i.e., *A. marginale*, *A. centrale* and *A. bovis* were detected indicating possible high diversity of the *Anaplasma* in these regions of Uganda.

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