Published in final edited form as:

Ticks Tick Borne Dis. 2013 February ; 4(0): 110–119. doi:10.1016/j.ttbdis.2012.09.005.

***Anaplasma odocoilei* sp. nov. (family Anaplasmataceae) from white-tailed deer (*Odocoileus virginianus*)**

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Abstract

Recently, an undescribed *Anaplasma* sp. (also called *Ehrlichia*-like sp. or WTD agent) was isolated in ISE6 tick cells from captive white-tailed deer. The goal of the current study was to characterize this organism using a combination of experimental infection, morphologic, serologic, and molecular studies. Each of 6 experimentally inoculated white-tailed deer fawns (*Odocoileus virginianus*) became chronically infected (100+ days) with the *Anaplasma* sp. by inoculation of either infected whole blood or culture. None of the deer showed evidence of clinical disease, but 3 of the 6 deer evaluated had multiple episodes of transient thrombocytopenia. Light microscopy of Giemsa-stained, thin blood smears revealed tiny, dark, spherical structures in platelets of acutely infected deer. *Anaplasma* sp. was detected in platelets of inoculated deer by polymerase chain reaction, transmission electron microscopy, immunohistochemistry, and in situ hybridization. Five of 6 deer developed antibodies reactive to *Anaplasma* sp. antigen, as detected by indirect fluorescent antibody testing. Phylogenetic analyses of 16S rRNA, groESL, and gltA sequences confirmed the *Anaplasma* sp. is related to *A. platys*. Two attempts to transmit the *Anaplasma* sp. between deer by feeding *Amblyomma americanum*, a suspected tick vector, were unsuccessful. Based on its biologic, antigenic, and genetic characteristics, this organism is considered a novel species of *Anaplasma*, and the name *Anaplasma odocoilei* sp. nov. is proposed with UMUM76^T (=CSUR-A1) as the type strain.

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Keywords

Anaplasma; Cervid; Novel species; White-tailed deer

Introduction

In the United States, white-tailed deer (WTD; *Odocoileus virginianus*) are infected with multiple tick-transmitted rickettsiae in the genera *Ehrlichia* and *Anaplasma* (Lockhart et al., 1997a; Little et al., 1998; Yabsley et al., 2002, 2008a). Three species, *E. chaffeensis*, *E. ewingii*, and *Anaplasma* (A.) *phagocytophilum* are zoonotic pathogens and infect a wide range of mammalian species (Dumler et al., 2001). A fourth organism, detected by PCR in the blood of wild WTD, has been referred to as WTD agent or *Ehrlichia* sp. of WTD (Dawson et al., 1996; Little et al., 1997) and was recently isolated in tick cell culture and identified as a member of the genus *Anaplasma* (Munderloh et al., 2003).

This *Anaplasma* sp. is common among white-tailed deer populations in the southeastern United States and has been reported from Georgia, Oklahoma, Nebraska, Virginia, and Missouri (Little et al., 1997; Lockhart et al., 1997b; Arens et al., 2003; Yabsley, unpublished). A closely-related species has been detected in mule deer (*O. hemionus hemionus*) and black-tailed deer (*O. hemionus columbianus*) from California (Foley et al., 1998; Yabsley et al., 2005) and mule deer and white-tailed deer from Arizona (Yabsley et al., 2005). However, additional data are needed to determine if this *Anaplasma* represents a variant of the *Anaplasma* sp. detected in white-tailed deer in the southeastern United States. In the southeastern United States, it has significant temporal and spatial associations with lone star tick (*Amblyomma americanum*) infestation (Brandsma et al., 1999). Additionally, this *Anaplasma* sp. has been detected by polymerase chain reaction (PCR) in field-collected *Amblyomma americanum* ticks from Missouri (GenBank accession number ELU52514). These previous studies suggest that this *Anaplasma* sp. infects white-tailed deer and could be transmitted by *Amblyomma americanum*, similar to 2 important zoonotic rickettsiae, *E. chaffeensis* and *E. ewingii* (Ewing et al., 1995; Little et al., 1998; Yabsley et al., 2003). The significance of this organism as a pathogen of deer or as a potential zoonotic agent is currently unknown. Previous analysis of partial 16S rRNA and GroESL gene sequences of this *Anaplasma* sp. indicates that it is most closely related to *A. platys*, a canine rickettsia that infects platelets of dogs and potentially humans (Dawson et al., 1996; Arraga-Alvarado et al., 1999; Dumler et al., 2001; Sumner et al., 2003).

In the current study, we experimentally infected deer with this *Anaplasma* sp. in order to (i) investigate infection dynamics and cellular tropism of this organism in vivo, (ii) conduct a small-scale *Amblyomma americanum* transmission study, and (iii) more fully characterize the morphologic, molecular, and antigenic relationships of this so far undescribed *Anaplasma* sp.

Materials and methods

Experimental animals and procedures

Nine orphaned white-tailed deer fawns (WTD76, 77, 81, 86, 128, and 135, and Deer 1, 2, and 3) were hand-raised and housed in a tick-free building at the College of Veterinary Medicine, UGA, Athens, GA. Fawns were acquired within 1–3 days of birth, and prior to inoculation, tested negative for *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum*, and the *Anaplasma* sp. by PCR and negative for antibodies to *E. chaffeensis* and *A. phagocytophilum* by indirect fluorescent antibody (IFA) assays as described (Dawson et al., 1994, 1996; Lockhart et al., 1997a; Little et al., 1998; Yabsley et al., 2002; Munderloh et al., 2003). For inoculations, deer were anesthetized by intramuscular injection of 1.7 mg/kg of xylazine (Mobay Corporation, Shawnee, KS) and 0.1 mg/kg ketamine (Fort Dodge Laboratories Inc., Fort Dodge, IA) and reversed with 1.3 mg/kg of yohimbine (Lloyd Laboratory, Shenandoah, IA).

Following inoculation, whole blood was collected periodically for PCR, serologic tests, and blood smears. Complete physical examination of each deer was performed at each blood collection date (Blood et al., 1983). Periodically, whole-blood anticoagulated in ethylenediaminetetraacetic acid (EDTA) from WTD76, WTD81, and WTD77 was submitted to the Clinical Pathology Laboratory, College of Veterinary Medicine, The University of Georgia, for analysis of the following parameters: hematocrit, erythrocyte count, hemoglobin levels, platelet count, total and differential leukocyte counts, and fibrinogen. At the end of the study, all deer were anesthetized as described above, euthanized via intravenous sodium pentobarbital overdose, and a necropsy performed. All procedures were approved by the UGA Institutional Animal Care and Use Committee (A2004-10136).

To detect *Anaplasma* sp. in blood of experimental deer and ticks, RNA was extracted from whole blood with the RNA Blood Minikit (Qiagen, Inc., Valencia, CA) and ticks with the QiAmp® Viral RNA Extraction Kit (Qiagen). For ticks, individuals were frozen in liquid nitrogen and then macerated with glass beads in a Mini Beadbeater-8 (Biospec Products, Inc., Bartlesville, OK). Reverse transcriptase (RT)-nested PCR (nPCR) assays for the 16S rRNA and groESL genes were performed as described (Munderloh et al., 2003; Tate et al., 2005); except that for the groESL assay, secondary amplification was performed using primers EDF10 and EDR11 (Sumner et al., 2003) (Table 1). As a control for the quality of the RNA preparations from frozen ticks, a random subset were tested for the presence of tick RNA using primers 16S+1 and 16S–1 (Table 1) which amplify a fragment of the tick mitochondrial 16S rRNA (Norris et al., 1999). Amplification products were separated by electrophoresis in a 2% agarose gel stained in ethidium bromide and visualized using ultraviolet transillumination.

Experimental inoculation trials

Details of fawn numbers and source of infections are summarized in Table 3. Briefly, two 4-month-old fawns (WTD76 and WTD81) became infected with the *Anaplasma* sp. after inoculation with blood collected from field-collected deer as described (Yabsley et al., 2002; Munderloh et al., 2003). Whole blood (8.0 ml) in EDTA collected from WTD 76, the

original source of UMUM76^T, at 177 DPI was used to inoculate one 10-month-old deer (WTD 77) by each of 4 routes (2 ml each): intradermal, subcutaneous, intravenous, and intraperitoneal (ID, SQ, IV, IP). A 13-month-old deer (WTD 86) and a 6-month-old deer (WTD135) were inoculated with 1×10^6 *Anaplasma* sp. UMUM76^T-infected ISE6 tick cells (passage 7 and passage 14, respectively). A fourth deer, a 5-month-old fawn (WTD 128), subsequently was inoculated with whole blood collected from WTD 86 at 189 DPI in the same manner as WTD 77.

Biomagnetic blood cell fractionation with Dynabeads

Whole blood in sodium citrate Vacutainer tubes (Beckton Dickinson, Rutherford, NJ) obtained from WTD76 and WTD81 was centrifuged at $50 \times g$ for 20 min. The platelet-rich plasma (PRP) was removed, centrifuged at $800 \times g$ for 25 min, and the pellet was resuspended in Dulbecco's phosphate buffered saline (DPBS; Sigma, St. Louis, MO) at a concentration of 1×10^7 cells/ml.

Dynabeads[®] (DynaL Biotech, Brown Deer, WI) were prepared through a series of washes using DPBS (pH 7.4) with 0.1% bovine serum albumin (BSA) solution, using the Magnetic Particle Concentrator (MPC) magnet (DynaL) to sequester beads after each wash. Subsequently, sheep anti-rabbit IgG beads were coated with anti-P-selectin (CD62P) polyclonal rabbit antibody (PharMingen, San Diego, CA) for platelet fractionation. Beads were incubated with the antibody overnight on a rocking plate at 4 °C and then washed 3 times with 0.1% BSA solution, removing the beads after each wash using the MPC magnet.

Platelet preparations were purified by incubating 250 µl of PRP for 24 h at 4 °C with 6 µl of CD62P antibody-coated beads followed by a second 24-h incubation of the non-target cell-depleted sample with 6 µl of the antibody-coated beads. The pellet was washed 3 times with and resuspended in 0.1% BSA solution. To detect the *Anaplasma* sp., DNA was extracted (DNeasy, Qiagen), and PCR targeting the 16S rRNA gene was conducted as described.

Electron microscopy

Transmission electron microscopy (TEM) was completed on PRP collected from WTD76, WTD81, and WTD86. PRP was obtained from citrate anticoagulated blood as described above. The platelets were pelleted and fixed in either 2% glutaraldehyde–2% (para)formaldehyde–0.2% picric acid in 0.1 M cacodylate–HCl buffer (pH 7.0–7.3) or 2% (para)formaldehyde–2% glutaraldehyde in phosphate buffer (pH 7.0). Pellets of fixed platelets were enrobed in agar, routinely embedded in Epon-Araldite (EMS, Hatfield, PA), and ultrathin sections were stained with Reynold's lead citrate and 5% methanolic uranyl acetate and viewed with a JEOL JSM-1210 transmission electron microscope.

Immunohistochemistry

To localize the *Anaplasma* sp., immunohistochemistry (IHC) was conducted on platelets collected from WTD135 on DPI 129. PRP was obtained as described above, pelleted by centrifugation, and the pellet of platelets was fixed in 10% buffered formalin, enrobed in agarose, and routinely embedded in paraffin. Briefly, 3-µm sections of formalin-fixed paraffin embedded tissue (PET) were deparaffinized and rehydrated. Sections were blocked

with Universal Blocking Reagent (BioGenex, San Ramon, CA) for 7 min. Polyclonal primary antibody was prepared using precipitated pre-and post-inoculation (71 DPI) serum from WTD86. Sections were incubated with the primary antibody at a 1:100 dilution in Dako-Cytomation Antibody Diluent (DakoCytomation, Carpinteria, CA) for 60 min and then incubated with 1:20 peroxidase-labeled rabbit anti-deer IgG (KPL, Gaithersburg, MD) for 30 min. Sections were then treated with supersensitive link biotinylated anti-rabbit immunoglobulins (BioGenex) for 20 min, followed by supersensitive label alkaline phosphatase-conjugated streptavidin (BioGenex) and Fast Red (DAKO, Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin.

In situ hybridization assay

The 16S rRNA riboprobe was generated from hemi-nested RT-PCR products amplified from the *Anaplasma* sp. by use of an interior reverse primer with an RNA polymerase promoter sequence. The primers were *A. phagocytophilum*- and *Anaplasma* sp.-specific primers GE9F and GA1UR in the primary reaction (Chen et al., 1994; Little et al., 1997), and DGA (Little et al., 1997) and T7-398R in the secondary reaction (Table 1). The primer T7-398R was complementary to 16S rRNA and had T7 promoter sequence at the 5' end (Table 1) (Dawson et al., 2001). DNA was amplified from *Anaplasma* sp. UMUM76^T-infected tick cells, and the digoxigenin-labeled RNA probe was generated as described (Dawson et al., 2001).

In situ hybridization assays using the digoxigenin-labeled riboprobe and a biotin-streptavidin method were performed. Briefly, 3- μ m sections of formalin-fixed paraffin-embedded pelleted platelets from WTD135 (129 DPI) were deparaffinized and rehydrated. Tissue proteases were digested in PK Buffer (Dawson et al., 2001) for 30 min. Sections were then post-fixed with 4% paraformaldehyde (J.T. Baker Chemical Co., Phillipsburg, NJ) in DPBS, acetylated with fresh 0.25% acetic anhydride (Sigma) in 0.1 M triethanolamine (J.T. Baker), and denatured with 70% deionized formamide (DFA; Sigma) in 2 $\times$ saline sodium citrate (SSC; Roche, Switzerland). Slides were prehybridized with Dig Easy Hyb Buffer (Boehringer Mannheim, Germany) at 42 °C for 30 min. The RNA probe was diluted to 100 ng/ml in Dig Easy Hyb Buffer, denatured, and then applied to sections. Sections were covered with ApopTagTM Plastic Coverslips (ONCOR, Gaithersburg, MD) and hybridized overnight at 42 °C in a humidified chamber. Slides were then washed twice in 50% DFA/2 $\times$ SSC at 52 °C, followed by 2 washes with 2 $\times$ SSC at room temperature. Sections were then blocked with 10% goat serum. Mouse anti-digoxigenin antibodies (Roche) diluted to 0.4 μ g/ml were applied to the sections for 60 min and detected by serial application of goat anti-mouse biotinylated immunoglobulins (Biogenex), streptavidin alkaline phosphatase (Biogenex), and naphthol fast red substrate (DAKO). Sections were counterstained in Mayer's hematoxylin (DAKO) and mounted with Supermount Permanent Aqueous Mounting Adhesive (Biogenex). All steps were carried out at room temperature unless otherwise noted. All solutions were formulated with diethylpyrocarbonate (DEPC; Sigma)-treated deionized water and autoclaved prior to use. All antibodies were diluted with DakoCytomation Antibody Diluent (DAKO).

Indirect fluorescent antibody assays

Actively growing (3–4 days after passage) ISE6 tick cells infected with *Anaplasma* sp. isolate UMUM76^T were gently rinsed with and then resuspended in DPBS to obtain a concentration of approximately 5×10^5 cells/ml. Slides were prepared as described (Yabsley et al., 2008b) except that before use, antigen slides were treated with 0.1 mg/ml Proteinase K (Sigma) for 2 min and then rinsed twice in DPBS for 5 min. IFA assays for antibodies reactive to the *Anaplasma* sp. in the serum of experimentally-inoculated deer were conducted as described (Munderloh et al., 2003; Tate et al., 2005). *A. phagocytophilum* and *E. chaffeensis* IFA assays were conducted using commercially available antigen slides (Focus Technologies, Inc., Cypress, CA) (Munderloh et al., 2003). *A. marginale* IFA assays were conducted with the St. Marie isolate propagated in ISE6 tick cells as antigen prepared as described above except that a blocking step, using 10% non-fat dry milk in DPBS, was added before primary antibody incubation. For IFA cross-reactivity, we used white-tailed deer antisera to *A. phagocytophilum*, *E. chaffeensis*, and *A. marginale* from previous experimental infection studies in our laboratory (Keel et al., 1995; Dugan et al., 2004; Tate et al., 2005).

Tick transmission trials

Laboratory-reared *Amblyomma americanum* nymphs (Oklahoma State University, Stillwater, OK) were placed in tick containment chambers adhered to areas of shaved skin on donor and recipient deer. Pools of nymphs were tested for the *Anaplasma* sp. using RT-PCR and shown to be negative. Briefly, 2 separate transmission trials were conducted in which *Amblyomma americanum* nymphs were first acquisition-fed on *Anaplasma* sp.-positive donor deer (on WTD76 at 119 DPI; on WTD135 at 41 DPI), allowed to molt, and then transmission-fed on 3 naïve recipient deer (Deer 1, 2, and 3). At the time of feeding, WTD135 was RT-nPCR- and culture-positive for the *Anaplasma* sp. For each trial, RT-nPCR was performed on multiple blood samples of recipient deer and on a subset of the adult ticks that had acquisition-fed on positive deer as nymphs.

Phylogenetic analysis

Partial 16S rRNA, groESL, citrate synthase (gltA), and RNA polymerase β -subunit (rpoB) genes were amplified and sequenced from the *Anaplasma* sp. UMUM76^T (Inokuma et al., 2001, 2002; Taillardat-Bisch et al., 2003; Kawahara et al., 2004). Resulting sequences were aligned with those from related organisms in GenBank using ClustalX and the 16S rRNA, groESL, and gltA sequences were concatenated for phylogenetic analyses which were conducted with the MEGA program using the neighbor-joining algorithm with the Kimura 2-parameter model (Kumar et al., 2001) (GenBank numbers provided in Table 2).

Results

Infection dynamics and clinical outcome

All 6 deer experimentally inoculated with the *Anaplasma* sp. became infected and remained infected, as determined by RT-nPCR of blood, at every sampling date for 100–200+ days until euthanasia (Table 3). Light microscopy of Giemsa-stained thin blood smears of acutely

infected deer revealed tiny, dark, spherical structures in platelets consistent with *Anaplasma* inclusions. Clinical signs of illness (i.e., fever, body condition, activity level) were not apparent in any of the infected deer. All 3 deer for which platelet counts were performed experienced 2–4 episodes of transient thrombocytopenia (fewer than 100×10^3 platelets/ μ l blood) during the time period (duration 75–245 days) in which platelets were measured. Thrombocytopenia occurred as early as 13 DPI, as late as 187 DPI, and at intervals of 21–65 days. Mean platelet counts (excluding values $<100 \times 10^3$) were 619, 419, and $363 \times 10^3/\mu$ l blood for WTD76, WTD77, and WTD81, respectively. For comparison, the mean platelet count of 5 age-matched white-tailed deer not infected with the *Anaplasma* sp., but that were housed, anesthetized, and sampled in a similar manner was $685 \times 10^3/\mu$ l blood (Tate et al., 2005). No gross or histopathologic lesions were apparent in any of the deer.

Antibody responses of infected deer

At 72 DPI, serum from WTD86 reacted with individual organisms and clusters of organisms and had a reproducible titer of 1:128 (Fig. 1). The negative control antiserum (WTD128; pre-inoculation) showed no specific fluorescence at dilutions 1:32 on repeated testing. Neither pre-immune sera nor the *Anaplasma* sp. antisera showed reactivity with uninfected ISE6 tick cells. Five of 6 experimentally-inoculated deer developed antibody titers 64 (seropositive cut-off) between 2 and 4 weeks post inoculation, and 2 deer remained seropositive until they were euthanized at 36 weeks (Fig. 2). Peak titer was 512 and gross mean titer was 151.

Immunologic cross-reactivity

Two *Anaplasma* sp. antisera from WTD 86 (titer 512) and 135 (titer 256) were tested for serologic cross-reactivity with related organisms. WTD86 serum only reacted weakly with *A. marginale* antigen (titer 64), and no reaction was noted with *E. chaffeensis* or *A. phagocytophilum* antigens. *A. phagocytophilum* antisera (titers 256 and 2048) reacted weakly with both the *Anaplasma* sp. UMUM76^T antigen and with *A. marginale* antigen (titers of 64 each). Two *E. chaffeensis* antisera (titers of 1024) and 2 *A. marginale* antisera (titers of 128 and 512) did not react with *Anaplasma* sp. UMUM76^T antigen.

Cellular tropism and ultrastructure

Antibody-coated magnetic bead-purified platelets from WTD76 and WTD81 were PCR-positive for the *Anaplasma* sp. in 15 of 16 samples. Immunohistochemistry demonstrated antigen reactive to the *Anaplasma* sp. immune sera in platelets of WTD135 (Fig. 1) that appeared as eccentrically located, densely stained, minute red dots. Positive reactivity of infected platelets was not apparent with non-immune serum. In situ hybridization demonstrated RNA of the *Anaplasma* sp. in platelets of WTD135 (Fig. 1) appearing as eccentrically located, densely stained, minute red dots within platelets. Positive reactivity of infected platelets was absent when DigEasy-Hyb without template RNA was used.

Ultrastructurally, platelets of deer infected via both blood (WTD76 and WTD81) and UMUM76^T culture (WTD86) contained *Anaplasma*-like organisms (Figs. 3 and 4). Infected platelets contained one to several organisms in individual membrane-bound vacuoles or 2–5 organisms in a single large membrane-bound vacuole (Fig. 3). The single organisms were

spherical, ranged from 0.250 to 0.786 μm ($n = 8$) in diameter, and typically tightly filled the vacuole (Fig. 4A and B). Organisms were surrounded by 2 smooth-contoured trilaminar membranes, one protoplasmic and one cell wall, with a very narrow intervening periplasmic space. Peripherally, organisms had a rim of electron-dense granular material and centrally were either relatively electron-lucent with dispersed fibrillar material or moderately electron-dense with uniformly dispersed fibrillar and granular material. Vacuoles containing multiple organisms (morulae) were irregularly spherical, approximately 1.0–1.15 μm in greatest diameter ($n = 5$) and contained loosely packed organisms that varied in size, shape, and protoplasmic characteristics (Fig. 4C and D). These organisms were either spherical, 0.250–0.675 μm in diameter ($n = 8$), or ovoid, 0.250–0.450 μm in width and 0.450–0.675 μm in length ($n = 4$), and were surrounded by 2 relatively smooth-contoured trilaminar membranes with a small periplasmic space. Larger organisms had central moderately electron-dense protoplasm with evenly distributed fibrils and clumps of electron-dense densely granular material and a peripheral rim of more electron-dense granular material. As organisms decreased in size there was condensation of the fibrils and granular material making the protoplasm diffusely more electron-dense with the eventual formation of an eccentric electron-dense condensation. Overlying these condensations, the periplasmic space was enlarged. The vacuolar matrix was generally electron-lucent, but had a few small bits of moderately electron-dense fibrillar material.

Phylogenetic analysis

The UMUM76^T partial 16S rDNA sequence (1403 bp) was identical to sequences previously amplified from this *Anaplasma* sp. from deer in Georgia and Oklahoma (100% similarity of overlapping 1163 bp; U27101–U27104) and an *Anaplasma* sp. from a pool of *Amblyomma americanum* ticks from Missouri (99.8% similarity, 1403 bp, ELU52514) followed by *A. platys* (98.2%, EF139459). Sequences of groESL (1603 bp) and gltA (912 bp, DQ020101) were most similar to *A. platys* with 69.6–70% and 83.2–83.3% similarity to numerous strains of *A. platys*. Phylogenetic analysis of the 16S rRNA, groESL, and gltA concatenated sequences (3502 bp) confirmed the close relationship of the *Anaplasma* sp. with *A. platys* (Fig. 5). Phylogenies of each individual gene confirmed the result of the concatenated sequence analysis (data not shown). A sequence of the rpoB gene was not available for *A. platys* for comparison, but the *Anaplasma* sp. UMUM76^T sequence (1722 bp) was 80.1% similar to *A. phagocytophilum* (AF237414), 76% similar to *A. centrale* (CP001759), 75.2% similar to *A. marginale* (CP001079), 70% similar to *E. chaffeensis* (CP000236), and 60.4% similar to *Neorickettsia risticii* (CP001431).

Tick transmission trials

Attempts to transmit the *Anaplasma* sp. to naïve deer using *Amblyomma americanum* nymphs acquisition-fed on infected deer were unsuccessful in 2 separate trials, as determined by RT-nPCR of blood samples on multiple days post tick infestation (Table 4). In addition, 10 and 20 adult ticks from WTD76 and WTD135, respectively, were RT-nPCR-negative.

Discussion

Numerous studies have detected a previously undescribed *Anaplasma* sp. in white-tailed deer throughout the southeastern United States (Little et al., 1997; Lockhart et al., 1997b). An identical, or closely related, organism has been detected in *Odocoileus* spp. from the western United States (Foley et al., 1998; Yabsley et al., 2005). Recently, the *Anaplasma* sp. was isolated in tick cells (Munderloh et al., 2003). In the current study, we determined that white-tailed deer were experimentally susceptible to a recently obtained isolate of the *Anaplasma* sp. (strain UMUM76^T), that they developed a detectable antibody response following infection, and that the *Anaplasma* sp. infected platelets. In addition, phylogenetic analyses of multiple gene targets indicated that the *Anaplasma* sp. was most similar to *A. platys*, another platelet-dwelling rickettsia. Two attempts to transmit the organism between deer with feeding *Amblyomma americanum* failed. Because of the unique cellular tropism and host and molecular analyses, we propose the name *Anaplasma odocoilei* sp. nov.

All 6 deer exposed to the *Anaplasma* sp. UMUM76^T, whether by infected whole blood or culture, became persistently infected for more than 100 days, and 3 maintained infections for over 200 days. It has been suggested that low-level persistent infection is a common feature of *Anaplasma* species (Palmer et al., 1998), documented in cattle infected with *A. marginale* (Eriks et al., 1989), goats infected with *A. ovis* (Ndung'u et al., 1995; Palmer et al., 1998), rodents, dogs, and sheep infected with *A. phagocytophilum* (Engvall et al., 2000; Castro et al., 2001; Stuenkel and Bergström, 2001), and dogs infected with *A. platys* (Harvey et al., 1978; Engvall et al., 2000). Similar to dogs infected with *A. platys* (Harvey et al., 1978), infected deer revealed no apparent clinical signs of illness despite 2–4 episodes of thrombocytopenia.

Phylogenetic analyses of multiple genes confirmed that the *Anaplasma* sp. UMUM76^T is most closely related to, but genetically distinct, from *A. platys*, a platelet-infecting organism of dogs (Dumler et al., 2001). Based on light and electron microscopy, PCR testing of purified platelets, immunohistochemistry, and in situ hybridization, we have confirmed that the *Anaplasma* sp. infects platelets. By both immunocytochemistry and in situ hybridization, *A. odocoilei* appeared as small, eccentrically located inclusions in platelets. This appearance corresponds to the small, eccentrically located, darkly stained structures visualized within platelets in Giemsa-stained blood smears. By PCR, both purified platelets and monocytes were positive for the *Anaplasma* sp. UMUM76^T which was somewhat surprising because *A. platys* has no known stage in monocytes (Dumler et al., 2001). Although the *Anaplasma* sp. may infect both cell types, other possible explanations for PCR-positive monocytes include monocyte preparations that were contaminated with platelets, monocytes that had phagocytosed infected platelets, or organisms could have been released from platelets during sample processing. The latter possibility has been suggested in a previous study in which the *Anaplasma* sp. was detected by PCR in serum samples of wild WTD (Brandsma et al., 1999).

Five of 6 experimentally-infected deer developed low titers of antibodies that remained detectable (1:64) for several weeks. The single deer that failed to develop a detectable titer was only intermittently PCR-positive. Although this assay has not been validated, we believe that these low-titer antibody levels are due to reaction with the *Anaplasma* sp.

UMUM76^T because the deer were free of known co-infecting *Anaplasma* and *Ehrlichia* spp. prior to inoculation, and the serum from one deer reacted specifically with the *Anaplasma* sp. UMUM76^T inclusions in infected platelets and the *Anaplasma* sp. UMUM76^T-infected tick cells as detected by IHC. Because antigenic cross-reactivity among *Anaplasma* and *Ehrlichia* spp. has been reported when testing polyclonal antibodies by immunofluorescence (Nicholson et al., 1997; Rikihisa et al., 1997; Wong et al., 1997; Walls et al., 1998; Comer et al., 1999), we tested cross-reactivity of the *Anaplasma* sp. UMUM76^T with related organisms. Antigenically, the *Anaplasma* sp. UMUM76^T antigens reacted weakly with *A. phagocytophilum* antisera, but not with *A. marginale* and *E. chaffeensis* antisera. Antisera against the *Anaplasma* sp. UMUM76^T were slightly reactive when tested with *A. marginale* antigen, but unreactive with *A. phagocytophilum* and *E. chaffeensis* antigens. Because *A. phagocytophilum* antisera reacted weakly with the *Anaplasma* sp. UMUM76^T antigens in an IFA assay, field serologic studies on the *Anaplasma* sp. would be complicated due to the common infection of white-tailed deer with *A. phagocytophilum* (Dugan et al., 2006).

Ultrastructurally, the organisms and morulae in platelets are similar to other Anaplasmataceae including other platelet-dwelling species such as *A. platys* in dogs (Arraga-Alvarado et al., 2003), a rickettsia-like organism in humans (Arraga-Alvarado et al., 1999), and a rickettsia-like organism seen in impala (*Aepyceros melampus*) (DuPlessis et al., 1997). The singlet organisms and those with similar morphology in morulae resemble the reticulated forms whilst the cells with electron-dense condensations resemble the dense forms of other *Anaplasma* and *Ehrlichia* spp. (Popov et al., 1995, 1998; Munderloh et al., 2004). However, the *Anaplasma* sp. UMUM76^T in platelets differed morphologically from the *Anaplasma* sp. UMUM76^T that was previously isolated and grown in tick cell culture (Munderloh et al., 2003). In tick culture, the *Anaplasma* sp. UMUM76^T forms much larger morulae with numerous pleomorphic and elongated organisms. This may be due to the cell type infected, with the small size of the platelets perhaps conferring a limit of the size of a developing morula. Differences in morphology between in vivo-infected cells and various cell culture-infected cells have been documented with related organisms (e.g. *A. phagocytophilum*) (Sells et al., 1976; Chen et al., 1994; Munderloh et al., 1996, 2004; Rikihisa et al., 1997; Woldehiwet et al., 2002).

The majority of *Anaplasma* and *Ehrlichia* spp. are transmitted by ticks and, in the southeastern and midwestern United States, deer are infested with multiple species of ticks, most commonly with *Amblyomma americanum* (Paddock and Yabsley, 2007). A previous study found that the *Anaplasma* sp. had a spatial and temporal association with *Amblyomma americanum* in the southeastern United States (Brandsma et al., 1999), but the only evidence of infected *Amblyomma americanum* was a single PCR-positive pool of ticks which were collected from deer and likely contained the *Anaplasma* sp.-infected blood meals (Lockhart et al., 1997b) and a sequence submitted to GenBank indicating the organism was detected in a pool of *Amblyomma americanum* from Missouri (GenBank ELU53514). In the current study, our attempts to experimentally transmit the *Anaplasma* sp. UMUM76^T by *Amblyomma americanum* failed; however, few potentially infected ticks were ultimately fed on transmission deer. Further, transmission studies with related bacteria have failed despite the tick being an appropriate vector (Mathew et al., 1996). Additional studies are needed to

determine the route of transmission of the *Anaplasma* sp., including potentially transmission-feeding wild-caught questing *Amblyomma americanum* from an endemic area on naïve fawns as has been done for related tick-borne organisms (Varela-Stokes, 2007).

Description of *Anaplasma odocoilei* sp. nov

Anaplasma odocoilei (odo coil'ei L. gen. n. odocoileus, of deer in the genus *Odocoileus*. The reference strain is UMUM76^T (=CSUR A1). Originally isolated from a white-tailed deer fawn that was experimentally infected with *A. odocoilei* by inoculation with blood from naturally infected adult deer from central Georgia, USA (Munderloh et al., 2003). Has also been detected in white-tailed deer in numerous locations in the eastern United States (Little et al., 1997; Lockhart et al., 1997b). Experimental infections of white-tailed deer can be achieved by inoculation with either whole blood of infected donor deer or infected cell culture preparations. Clinical signs of illness were not apparent in experimentally infected white-tailed deer. Natural infections, as determined by 16S rRNA PCR, have also been reported in related *Odocoileus* spp. (mule deer and black-tailed deer) from California and Arizona (Foley et al., 1998; Yabsley et al., 2005). Inclusions of *A. odocoilei* UMUM76^T were observed in platelets of acutely infected deer by electron microscopy, light microscopy of Giemsa-stained, thin blood smears, immunohistochemistry, and in situ hybridization.

Morphology of organisms in naturally infected platelets and ISE6 tick cells was similar to other members of the family Anaplasmataceae. The bacterium is classified as a member of the family Anaplasmataceae and genus *Anaplasma* based on 16S rRNA, groESL, gltA, and rpoB gene sequences and growth within membrane-bound cytoplasmic vacuoles. In ISE6 tick cells, the organism tends to form larger morulae with numerous organisms and is very pleomorphic and elongate, rather than spherical. Organisms are Gram-negative. Ultrastructurally, organisms are small, pleomorphic cocci within membrane-lined vacuoles, and each organism is surrounded by 2 trilaminar membranes, one protoplasmic and one cell wall, with a narrow periplasmic space. Within platelets, organisms may occur singly or in morulae. Singlet organisms are spherical (mean diameter $0.521 \pm 0.200 \mu\text{m}$) while clustered organisms vary in shape and size (mean diameter $1.070 \pm 0.057 \mu\text{m}$ with spherical organisms $0.423 \pm 0.11 \mu\text{m}$ in diameter and ovoid organisms $0.350 \pm 0.913 \mu\text{m}$ wide and $0.503 \pm 0.823 \mu\text{m}$ long).

A. phagocytophilum antisera reacted weakly with *A. odocoilei* UMUM76^T whole-cell organisms. *A. odocoilei* UMUM76^T antisera from infected white-tailed deer did not cross-react with *A. phagocytophilum* or *E. chaffeensis* antigens in IFA assays, but in some cases did weakly cross-react with *A. marginale* antigen.

Analysis of gltA sequence fragment revealed 3 novel insertions compared with *A. platys*. A single 3-bp deletion was present in groESL sequence compared with *A. platys*. This strain can also be differentiated from other *Anaplasma* species based on its unique 16S rDNA, groESL, gltA, and rpoB sequences. The G + C content of the 4 sequenced genes of *A. odocoilei* UMUM76^T is 49.2 mol%.

Acknowledgments

We are grateful to David Stallknecht for insightful guidance, Jane Huffman for facilitating acquisition of fawns, Andrea Varela-Stokes and Molly Murphy for significant technical help, Mary Ard for electron microscopy expertise, and Jeff Tucker and Frank Waters for animal handling and husbandry assistance. Work was supported primarily by the National Institutes of Allergy and Infectious Diseases (5 R01 AI044235-02). Further support was provided by the Federal Aid to Wildlife Restoration Act (50 Stat. 917) and through sponsorship from fish and wildlife agencies in Alabama, Arkansas, Florida, Georgia, Kansas, Kentucky, Louisiana, Maryland, Mississippi, Missouri, North Carolina, Oklahoma, Puerto Rico, South Carolina, Tennessee, Virginia, and West Virginia.

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Fig. 1.

In situ hybridization and immunohistochemical localization of *Anaplasma* sp. UMUM76^T in infected tick cell culture and platelets of experimentally infected deer. Signal appears as minute red dots within cells. Results of in situ hybridization (ISH) assays (top), on infected tick cell culture control (A) and platelets of WTD135 (129 DPI) (D). Results of immunohistochemistry (IHC) assays (bottom), on infected tick cell culture control (C) and platelets of WTD135 (129 DPI) (E). Result of indirect fluorescent antibody assay (B) performed on tick cell culture antigen slides using WTD86 (71 DPI) serum at a dilution of 1:64.

Fig. 2.

Antibody response of deer experimentally infected with the *Anaplasma* sp. UMUM76^T, as determined by IFA. (A) Animals inoculated with blood from infected deer. (B) Animals inoculated with tick cell isolate UMUM76^T or with blood from animals inoculated with UMUM76^T.

Fig. 3.

Transmission electron micrograph of white-tailed deer platelets from WTD81 at 20 DPI. One platelet contains a single organism (arrow), and one has a membrane-bound morula loosely filled with 3 organisms (1, 2, 3). Bar = 0.2 μ m. Lead citrate/uranyl acetate.

Fig. 4.

Transmission electron micrograph of white-tailed deer platelets. (A) Platelet from WTD86 at 33 DPI containing single reticulated form of the organism (R) tightly filling a membrane-bound vacuole. Note electron-lucent center primarily containing a few DNA fibrils and peripheral granular layer of ribosomes. Inset is enlargement of square showing membrane of vacuole (long black arrow) and the 2 trilaminar membranes surrounding the organism (2 short white arrows) separated by narrow periplasmic space (long white arrow). The platelet contains normal platelet alpha granules (A). Bar = 0.1 μ m. (B) Platelet from WTD81 at 20 DPI containing single reticulated form of the organism (R) tightly filling a membrane-bound vacuole. The center of this organism is denser due to the fairly evenly distributed DNA fibrils and clumps of ribosomes. The platelet contains normal platelet alpha granules (A). Bar = 0.1 μ m. (C) Platelet from WTD76 at 20 DPI as seen on edge. A membrane-bound morula loosely filled with 3 organisms forms a bulge in the platelet (arrow). The organisms are surrounded by 2 membranes and have fairly evenly dispersed DNA fibrils and ribosomal aggregates. Bar = 0.2 μ m. (D) Platelet from WTD81 at 20 DPI containing a morula loosely filled with 5 organisms. The matrix of the membrane-bound vacuole is electron-lucent and contains a small amount of fibrillar material. Organisms are bound by 2 trilaminar membranes, and there is an eccentric electron-dense condensation in 3 of the organisms. The periplasmic space is enlarged over the condensation (arrow). Bar = 0.1 μ m. Lead citrate/uranyl acetate.

Fig. 5.

Phylogenetic analysis of the 16S rRNA, groESL, and gltA concatenated sequences (3502 bp) of the *Anaplasma* sp. UMUM76^T and related organisms. Numbers at nodes indicate levels of bootstrap support based on 500 replicates.

Table 1

Oligonucleotide sequences of primers used in this study.

Primer pair	Gene target	Target organism(s)	Nucleotide sequence (5' → 3')	Product size (bp)	References
ECC ECB	16S rRNA	<i>Anaplasma</i> and <i>Ehrlichia</i> spp.	CGTATTACCGCGCTGCTGGCA AGAACGAAAGCTGGCGCAAGCC	480	Dawson et al. (1994)
GE9F GAIUR	16S rRNA	<i>A. phagocytophilum</i> and <i>Anaplasma</i> sp. UMUM ^r	AACGGATTATTCTTTATAGCTTGCT GAGTTTGCCGGGACTTCTTCT	411	Chen et al. (1994) and Little et al. (1997)
DGA GAIUR	16S rRNA	<i>Anaplasma</i> sp. UMUM ^r	TTATCTCTGTAGCTTGCTACG GAGTTTGCCGGGACTTCTTCT	411	Little et al. (1997)
DGA T7-398R	16S rRNA	<i>Anaplasma</i> sp. UMUM ^r	TTATCTCTGTAGCTTGCTACG T7-GCATAGCTGGATCAGGCTTTC	325	Dawson et al. (1994) and Little et al. (1997)
APF1 APR1	<i>groESL</i>	<i>A. phagocytophilum</i> and <i>Anaplasma</i> sp. UMUM ^r	TAGTGATGAAGGAGAGTGAC CCAGGIGCCTTACAGCWGCAAC	1603	Sumner et al. (2003)
EDF10 EDR11	<i>groESL</i>	<i>Anaplasma</i> sp. UMUM ^r	GATTCTCCGGTTTGTTCTGT GGAGAAAGATAAACCCTG	650	Sumner et al. (2003)
F4b HG1085R	<i>glfA</i>	<i>Anaplasma</i> and <i>Ehrlichia</i> spp.	CCGGGTTTATGTCTACTGC ACTATACCKGAGTAAAGTC	935	Inokuma et al. (2001, 2002)
16S+1 16S-1	16S rRNA	Tick	CCGGTCTGAACCTCAGATCAAGT CTGCTCAATGATTTTAAATTGCTGTGG	460	Norris et al. (1999)

Table 2

GenBank accession numbers used in phylogenetic analyses.

Organism	16S rRNA	<i>groESL</i>	<i>gltA</i>
<i>Anaplasma</i> sp. UMUM ^T	JX876644	JX876642	DQ020101
<i>Anaplasma platys</i>	AF536828	AY044161	AB058782
<i>Anaplasma phagocytophilum</i>	AY055469	AF172161	AF304136
<i>Anaplasma marginale</i>	AF309867	AF414865	AF304140
<i>Ehrlichia ruminantium</i>	X62432	U13638	AF304146
<i>Ehrlichia ewingii</i>	EEU96436	AF195273	DQ365879
<i>Ehrlichia chaffeensis</i>	AF147752	L10917	AF304142
<i>Ehrlichia canis</i>	AF373613	U96731	AF304143
<i>Neorickettsia sennetsu</i>	M73225	ESU88092	AF304148

Table 3
RT-nPCR detection of *Anaplasma* sp. UMUM76^T in blood of experimentally inoculated deer.

WTD ^a	Source of infection	DPI ^b first tested	DPI first PCR positive	No. of times positive/no. of times sampled	DPI last tested
76	Wild deer blood ^c	2	5	35/36	250
81	Wild deer blood ^c	2	2	22/22	117
77	Blood from WTD76	10	10	13/13	252
86	Tick cell isolate (UMUM76 ^T)	12	12	8/8	214
128	Blood from WTD86	4	4	5/9	153
135	Tick cell isolate (UMUM76 ^T)	15	15	5/9	129

^aWTD, white-tailed deer.
^bDPI, days post inoculation.
^cDetails of inoculation are given in Munderloh et al. (2003).

Table 4

Attempted transmission trials of *Anaplasma* sp. UMUM76^T between white-tailed deer (*Odocoileus virginianus*) with *Amblyomma americanum*.

Acquisition deer	No. of nymphs fed/no. molted to adults	Transmission deer	No. of adult ticks fed per deer/no. that fed to repletion	No. of times transmission deer were tested by PCR (during days post tick infestation)
WTD76	103/55	Deer 1	45/21	6 (13–79)
WTD135	390/222	Deer 2	102/10	8 (4–60)
		Deer 3	100/17	8 (4–74)