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A NOVEL CLINICAL SYNDROME AND DETECTION OF *ANAPLASMA OVIS* IN MONGOLIAN REINDEER (*RANGIFER TARANDUS*)

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ABSTRACT: The Tsaatan (or Dhuka) peoples of northern-western Mongolia are one of the few remaining reindeer-herding cultural groups in the world. Recently a disease condition that involves sudden death of reindeer and cases involving fever, lethargy, and pale mucous membranes has been reported. Examination of blood smears collected in the 2005 field season resulted in the identification of intra-erythrocytic inclusions resembling *Anaplasma* spp. in smears from clinically sick animals. Using universal polymerase chain reaction (PCR) primers for the amplification of the 60 kDa chaperonin gene (*cpn*60, also known as *hsp*60 or *gro*EL), we detected sequences corresponding to *Anaplasma ovis* in reindeer blood samples. Species-specific PCR primers for *A. ovis* were designed and validated and used to screen blood samples from Mongolian reindeer. Screening of 66 blood samples collected in the 2006 field season resulted in the detection of *A. ovis* in 80% of the samples. Our results indicate a high prevalence of *A. ovis* in the Tsaatan reindeer herds and an association with clinical disease that is likely to be anaplasmosis. To our knowledge this is the first report of natural *A. ovis* infection in reindeer.

Key words: *Anaplasma ovis*, anaplasmosis, *cpn*60, Mongolia, PCR diagnostic, reindeer, *Rangifer tarandus*.

INTRODUCTION

The Tsaatan (or Dhuka) peoples of northern-western Mongolia are one of the few remaining reindeer-herding cultural groups in the world. The Tsaatan are a migratory people who live and move with their reindeer among herding and camp locations that lie between 1,850 and 2,100 m above sea level in the eastern Sayan mountains that span the Siberian and Mongolian border. The Tsaatans are also the most southerly reindeer herders known, and their animals are adapted to withstand extreme temperatures that may reach as high as 40 C in summer (Vitebsky, 2005; Haigh and Keay, 2006).

A disease condition that involves sudden death of reindeer and cases in which fever, lethargy, and pale mucous membranes has been described (Nansalmaa, pers. comm.). In the summer of 2004, 17 animals died suddenly in one camp. Post mortem examinations on these animals revealed that all tissues in the abdominal cavity were yellow in color, and that the peritoneal fluid had a dark yellow tinge (Borhoo, pers. comm.).

Little information is available regarding bacterial blood pathogens in reindeer, although a recent report suggests that several species of *Mycoplasma* are widespread in North American reindeer (Stoffregen et al., 2006), and *Anaplasma phagocytophilum* has been reported to cause clinical manifestations in reindeer in Europe (Stuen, 2007). Identification and speciation of organisms such as *Mycoplasma* and *Anaplasma* spp. has been problematic because these organisms are not amenable to propagation in the laboratory. Historically, serologic and antibody-based methods have been applied, although sequence-based methods, primarily species- or genus-specific polymerase chain reaction (PCR), are now more widely exploited.

In diagnostic situations in which the target organism is completely unknown and potentially uncharacterized, unbiased methods such as the application of universal PCR primers for phylogenetically

informative targets are particularly useful. The gene encoding the 60 kDa chaperonin (*cpn*60, also known as *hsp*60 or *gro*EL) is found in all eukaryotes, some archaea, and virtually all bacteria. The utility of the *cpn*60 target for bacterial species identification is well established (Goh et al., 1997, 2000; Brousseau et al., 2001; Jian et al., 2001; Lew et al., 2003; Hill et al., 2006). The power of the *cpn*60 target is enhanced by the availability of universal primers for PCR amplification of the "universal target" region (corresponding to nucleotides 274–828 of the *Escherichia coli cpn*60 gene) and a large reference database of *cpn*60 sequences, cpnDB (Hill et al., 2004).

Here we describe the application of *cpn*60-based diagnostic methods to the characterization of unidentified blood pathogens in Mongolian reindeer. The amplified sequences, consistent with the identification of *Anaplasma ovis,* were used to design *A. ovis*–specific PCR primers subsequently applied to the screening of reindeer blood samples.

Our results indicate a high prevalence of *A. ovis* in the Tsaatan reindeer herds and an association with clinical disease that is likely to be anaplasmosis. To our knowledge this the first report of natural *A. ovis* infection in reindeer.

MATERIALS AND METHODS

Animals and blood collection

Blood samples were collected from reindeer over two field seasons (2005–06) in the months of August or September. Two samples were collected from each animal by intravenous jugular puncture into Vacutainer® tubes containing EDTA (VNR, Edmonton, Alberta, Canada). Serum was harvested from the clotted samples and used in other studies. Thick and thin smears were made either directly from the intravenous needle, or from the unclotted blood and air dried. They were processed with Dif-Quick® fixative and stain (Fisher Scientific, Ottawa, Ontario, Canada) and stored for later examination.

In 2005, 75 stained blood slides were examined at 100× under oil immersion at the Institute of Veterinary Medicine in Ulaanbaatar, Mongolia. In 2006, 66 blood slides were stained and examined.

DNA extraction

Unclotted blood was stored in a cool pack for up to 2 wk and transported to the laboratories of the Institute of Veterinary Medicine in Ulaanbaatar. Total DNA was extracted from 95 blood samples collected during the 2005 field season, and 66 samples collected during the 2006 field season. The DNA was extracted from blood samples using a rapid extraction method adapted and used by Bova-Can Laboratories, a division of the Saskatchewan Research Council (Saskatoon, SK, Canada). A 150 µl aliquot of whole blood sample was transferred to a labeled 0.5 ml microcentrifuge tube. Then PCR lysis buffer (400 µl; 0.32 M sucrose, 10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1% (v/v) Triton X-100) was added to each, and the samples were vigorously vortexed. Lysates were then centrifuged at 15,000 × G for 1 min in a benchtop microcentrifuge, and the supernatant was gently aspirated, leaving the pellets behind. The sample pellets were washed two more times with lysis buffer to leave a clean pellet at the bottom of the tube. Freshly made 200 mM NaOH (75 µl) was added to each sample, followed by incubation at 97 C for 15 min and vortex mixing. Following incubation, 75 µl of a freshly made solution of 200 mM HCl, 100 mM Tris-HCl was added to each sample, and the samples were mixed by vortexing.

PCR amplification of *cpn*60 universal target and 16S rRNA sequences

For PCR amplification of *cpn*60 universal target sequences from blood DNA extracts, reactions (50 µl total volume) contained 2 µl of template DNA, 2.5 U Taq DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl pH 8.3, 250 mM each of the dNTPs, and 20 pmol each of degenerate primers H729 (5′-CGC CAG GGT TTT CCC AGT CAC GAC GAI III GCI GGI GAY GGI ACI ACI AC-3′) and H730 (5′-AGC GGA TAA CAA TTT CAC ACA GGA YKI YKI TCI CCR AAI CCI GGI GCY TT-3′), which include annealing sites for sequencing primers M13(-40)F and M13(48)R (underlined). Temperature cycling was performed on a Mastercycler EP Gradient thermocycler (Eppendorf Canada Ltd., Mississauga, Ontario, Canada) (3 min at 94 C, followed by 40 cycles 30 sec at 94 C, 1 min at 50 C, 1 min at 72 C, and a final extension at 72 C for 10 min).

Partial 16S rRNA sequences were amplified with primers F1: 5′-GAG TTT GAT CCT

GGC TCA G-3′ and R2: 5′-GWA TTA CCG CGG CKG CTG-3′ (Dorsch and Stackebrandt, 1992). The PCR reactions were carried out as described for the *cpn*60 target with the appropriate primers.

Anaplasma ovis–specific PCR

The program SignatureOligo (LifeIntel Inc., Port Moody, British Columbia, Canada) and the cpnDB reference database were used to identify PCR primer sequences within the *A. ovis cpn*60 universal target that were unique to this species and would permit species-specific amplification of *A. ovis cpn*60 sequences from complex templates. Based on this analysis, primers JH0011 (5′-TAA AAG CCA AGG AGG CTG TG-3′) and JH0012 (5′-TTG CTC TCC TCG ACC GTT AT-3′) were designed and synthesized. These primers amplify a 181 bp region corresponding to nucleotides 89–269 of the *cpn*60 universal target (or nucleotides 359–539 of the complete *cpn*60 open reading frame). An optimal PCR annealing temperature of 60 C was determined by performing replicate PCR reactions across an annealing temperature gradient of 57 C to 65 C. Specificity of the primers was confirmed by direct sequencing of PCR products obtained from six reindeer blood samples. All amplified sequences were identical to the *A. ovis* reference sequence.

Reindeer blood DNA extracts were screened for the presence of *A. ovis* sequences in 50 µl reactions containing 2 µl of template DNA, 2.5 U Taq DNA polymerase (Invitrogen Canada Inc., Burlington, Ontario, Canada), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl pH 8.3, 250 mM each of the dNTPs, and 20 pmol each of primers JH0011 and JH0012. Temperature cycling was performed on an Eppendorf Mastercycler EP Gradient instrument (3 min at 94 C, followed by 40 cycles 30 sec at 94 C, 30 sec at 60 C, 30 sec at 72 C, and a final extension at 72 C for 10 min). The PCR products were visualized on an ethidium bromide–stained 1.5% (w/v) agarose gel.

DNA sequencing and phylogenetic analysis

The PCR products were purified using the QIAquick Gel Extraction kit (Qiagen Inc., Missisauga, Ontario, Canada) and sequenced directly using primers M13(-40)F and M13(48)R or primers JH0011 and JH0012 where appropriate. Sequence data were assembled and edited using the Staden Package (Staden et al., 2000), and additional sequence manipulation including alignments was done with elements of the EMBOSS software suite (Rice et al., 2000). For identification, sequences of reindeer blood amplicons were compared to cpnDB (http://cpndb.cbr.nrc.ca) using FASTA (Pearson and Lipman, 1988; Hill et al., 2004). Phylogenetic analysis was conducted using the PHYLIP software package (Felsenstein, 1993).

RESULTS

Sick reindeer were examined each year during 2004–06. Seven cases with one or more of the following signs were seen over the three field seasons, two in each of the first 2 yr, three in 2006. Clinical signs included fever (up to 39.7 C), pale mucous membranes, tachycardia, and weight loss.

Of 75 blood smears examined in 2005, intraerythrocytic inclusions resembling *Anaplasma* spp. were seen in 19 (25%) (Fig. 1). Blood smears of clinically sick animals observed in 2004 and 2005 were uniformly positive, but there was no correlation between the number of erythrocytes in a field and the severity of clinical signs or history.

To identify the microorganisms observed in blood smears collected during the 2005 field season, we applied universal PCR primers for amplification of *cpn*60 sequences. Of the 40 samples randomly selected for *cpn*60 PCR, 25 (63%) yielded PCR product that could be visualized on an agarose gel, and 15 of these gave sufficient amounts of product for direct sequencing after purification. Four nearly identical 558 bp sequences were obtained, with a maximum of one nucleotide difference between any pair of sequences. Representatives of the four unique sequences have been deposited to GenBank with the accession numbers EF204475–EF204478. All nucleotides sequences obtained encode identical peptide sequences. A FASTA-based comparison of the reindeer-blood–derived PCR product to cpnDB, a reference database of *cpn*60 sequences, showed that the reindeer derived sequences were 99–100% identical to *Anaplasma ovis* OVI (GenBank accession AF441131), an isolate originally from South Africa (Lew et al., 2003).

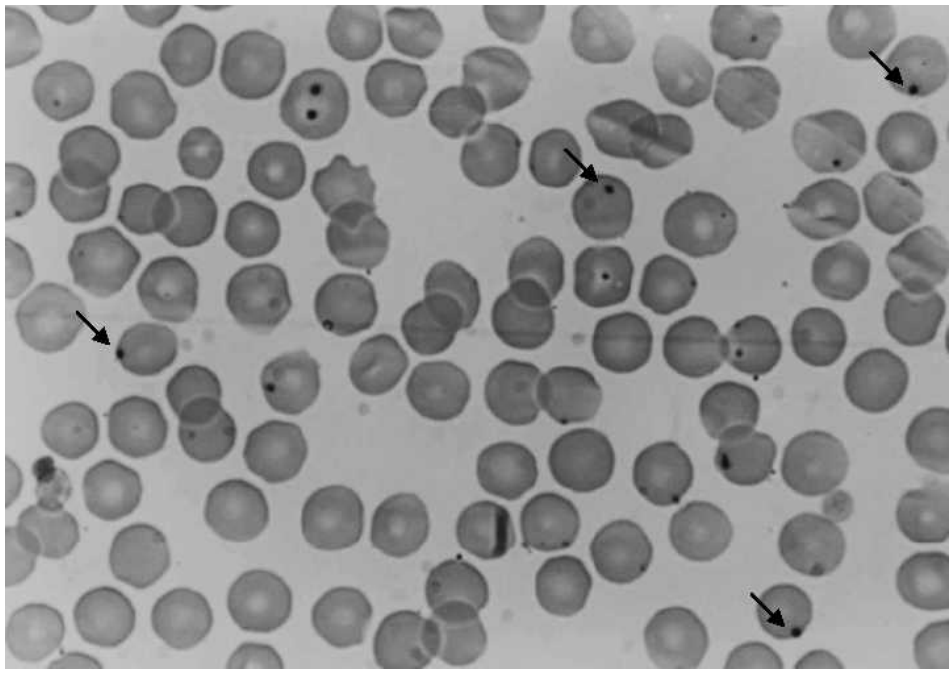

FIGURE 1. Intra-erythrocytic inclusions (arrows) in reindeer blood smear.

Phylogenetic relationships between the reindeer-derived sequences and other members of the Anaplasmataceae family are shown in Figure 2. The tree shows robust clustering of isolates within species and obvious discrimination between species.

Support for the identification of *A. ovis* was obtained by the independent amplification of partial *16S rRNA* gene sequences from three samples that were positive by *cpn*60 PCR but gave insufficient product for sequencing. The 485 bp sequences obtained were 99% identical to *A. ovis* strains in GenBank, and to *Anaplasma marginale*, *Anaplasma centrale*, and *A. phagocytophilum* (data not shown).

The *A. ovis*–specific PCR primers were applied to the analysis of 66 DNA extracts prepared from reindeer blood samples collected during the 2006 field season. Negative controls included a reaction containing no template DNA and a reaction containing DNA from the blood of a healthy, Canadian reindeer. Positive results (a 181 PCR product) were obtained from 53 of 66 samples (80%). Typical PCR results are illustrated in Figure 3.

DISCUSSION

Anaplasma spp. are widespread in domestic and wild animals (Kuttler, 1984). Among cervids, clinical signs of disease associated with *A. marginale* have been detected in the black-tailed deer (*Odocoileus hemionus columbianus*), for which Kuttler (1981) reported they were "more responsive to infection than intact WTD" (white-tailed deer, *Odocoileus virginianus*). In the latter, he stated that "Intact WTD rarely showed outward signs following induced *A. marginale* infection." Evidence of *A. phagocytophilum* infection has also been found in moose (*Alces alces*), red deer (*Cervus elaphus*), and roe deer (*Capreolus capreolus*) in Europe (Jenkins et al., 2001; Stuen et al., 2002). Although *A. ovis* is traditionally associated with domestic ruminants, particularly sheep and goats, there is sequence-based evidence for *A.*

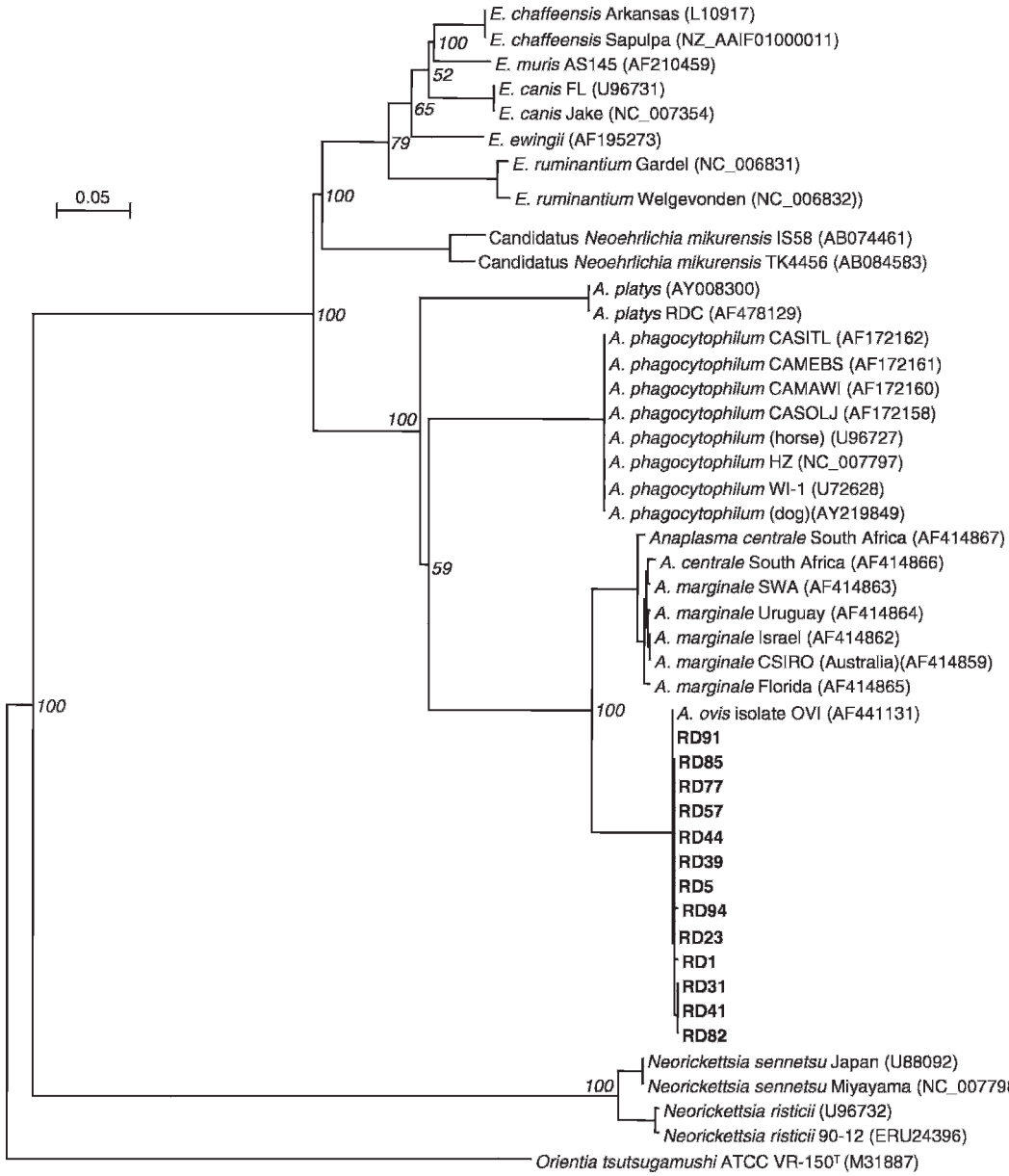

FIGURE 2. Phylogenetic relationships of 558 nucleotide *cpn*60 sequences amplified from 13 reindeer blood samples to reference sequences from the Anaplasmataceae. Reindeer blood sample names are shown (RD=reindeer). The tree is the consensus of 100 neighbor-joined trees, and bootstrap values (out of 100) are shown at the species nodes. The tree is rooted with *Orientia tsutsugamushi* (Rickettsieae). GenBank accession numbers for the reference sequences are shown in parentheses.

ovis in wild ruminant populations in North America (mule deer [*Odocoileus hemionus*] and bighorn sheep [*Ovis canadensis*]; Yabsley et al., 2005; de la Fuente et al., 2006).

Anaplasma organisms can be transmitted with varying efficiency by a range of methods including tick-borne transmission and mechanical transfer by biting flies, needles and other instruments and even blood (Wiesenhütter, 1975; Scoles et al., 2005). We have no information on tick

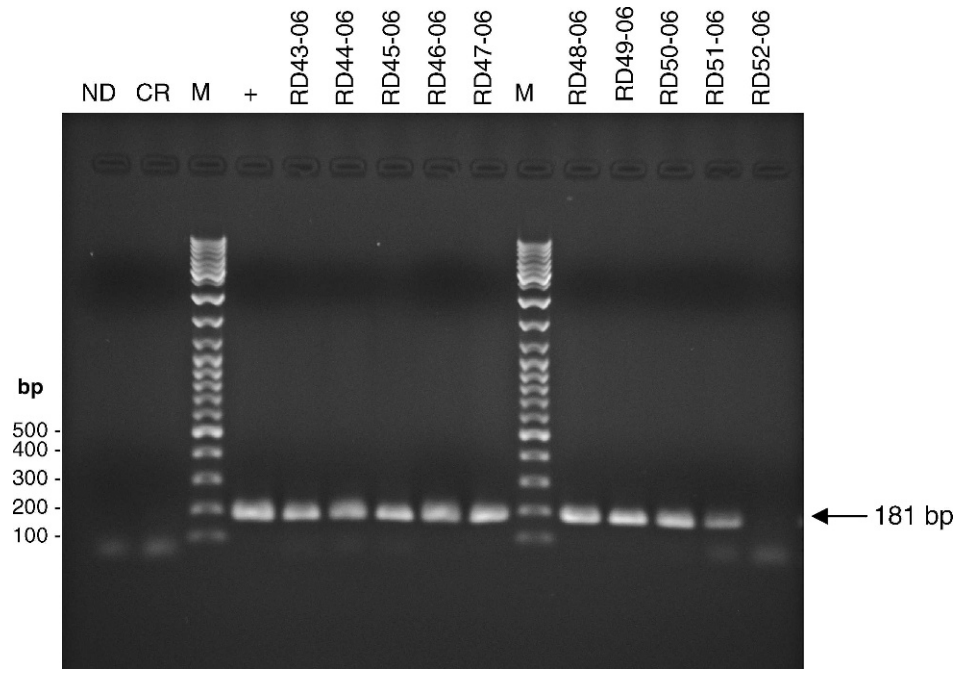

FIGURE 3. Typical PCR results using *A. ovis*–specific primers JH0011 and JH0012. Five microliters of each 50 μL PCR reaction is loaded on a 1.5% agarose gel. ND=No DNA template, CR=blood DNA from healthy Canadian reindeer, +=positive control template (sequenced *A. ovis cpn*60 universal target PCR product), M=DNA size ladder. The remaining lanes are Mongolian reindeer samples RD43-06 to RD52-06. DNA fragment sizes are indicated on the left-hand side, and the position of the 181 bp PCR product is indicated on the right-hand side of the figure.

species associated with Mongolian reindeer, but herders report that they are present. Another possible transmission factor contributing to the high infection rate observed in 2005 is the practice of removal of reindeer antlers in velvet. In the years up to and including 2004, antlers were removed with unsterilized saws and with no use of tourniquets to prevent bleeding.

Compared to the high percentage of DNA positive samples, the relatively low number of blood smears with anaplasma-like inclusions (25% positive smears vs. 63% positive by PCR in 2005) is likely the result of low parasitemia at the time of collection. In a study of *Anaplasma marginale* in cattle, Eriks et al. (1989) used nucleic acid probes to determine that animals in the "carrier state" following recovery from a period of acute anaplasmosis had parasitemia levels as low as 0.000025% or 500–1,000 organisms per 0.5 mL of blood. Organisms at this low density could not reliably be detected in blood smears.

An inability to discriminate *Anaplasma* spp. microscopically combined with the lack of a laboratory culture system has led to a lack of information regarding the epidemiology and clinical significance of these organisms. These difficulties, and the increasing emphasis on sequence-based identification, are also reflected in the volatile taxonomy of this group and changes to the nomenclature describing it (Dumler et al., 2001).

The *cpn*60 (*gro*EL or *hsp*60) sequences have been used in the speciation of many groups of human and animal pathogens including *Enterococcus* spp. (Goh et al., 2000), *Staphylococcus* spp. (Goh et al., 1997), *Streptococcus* spp. (Goh et al., 1998; Brousseau et al., 2001), and *Cam-*

pylobacter, Helicobacter, and *Arcobacter* spp. (Hill et al., 2006). It has also been demonstrated that in most cases *cpn*60 sequences are more informative than *16S rRNA* sequences for discriminating between species, including *Anaplasma* spp. (Lew et al., 2003). In the current study the robust speciation of *Anaplasma* based on *cpn*60 universal target sequences provides clear identification of the reindeer blood–derived sequences as *A. ovis* and further evidence that the combination of direct sequencing of *cpn*60 universal target sequences and the reference database, cpnDB, is a powerful tool for bacterial identification. Other genetic targets, particularly the outer membrane protein gene *msp*4, have been used to identify and detect *Anaplasma* spp. (de la Fuente et al., 2005, 2006, 2007). However, this approach is useful only for focused studies of *Anaplasma* and is not applicable in challenging diagnostic situations where the identity of the pathogen is not known.

For the reasons described above, information about blood-borne bacterial pathogens of reindeer is limited. In a recent study of anemic, farmed North American reindeer, Stoffregen et al. (2006) found evidence for at least two *Mycoplasma* spp. in these populations. The authors suggest that hemomycoplasmas may be widespread in reindeer since they identified sequences in animals from two distinct geographic locations. In our study we detected only *Anaplasma* and found no evidence for other bacterial species. There is a possibility that additional pathogens were present and went undetected, especially if they were eukaryotes such as *Babesia* spp. (Holman et al., 2002) or other bacterial species present at such a low level that although they would be amplified in the universal primer PCR reactions, the signal from these templates would be masked by the more abundant *A. ovis* sequence.

With the positive blood smear and DNA sequence results from 2005, the use of tetracyclines was advised for treatment of animals exhibiting clinical signs that might be related to anaplasmosis. Tetracyclines are the drug of choice for both cattle and cervids, although the administration protocols differ (Wilson, 1983; Haigh et al., 1997; Smith, 2002).

Ideally, if budget and logistical constraints were not a factor, as they were in this study, serum harvested in the field would be brought out after storage in liquid nitrogen. If this becomes possible, it would be desirable to conduct enzyme-linked immunosorbent assay tests for further confirmation of our findings. However, the *A. ovis*–specific PCR primer set used in this study will be useful in future surveys of *A. ovis* in other host species. The test could be easily adapted for quantitative real-time PCR for investigation of bacteria load and clinical outcomes.

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