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Short communication

Sequence analysis of the *msp4* gene of *Anaplasma ovis* strains

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Abstract

Anaplasma ovis (Rickettsiales: Anaplasmataceae) is a tick-borne pathogen of sheep, goats and wild ruminants. The genetic diversity of *A. ovis* strains has not been well characterized due to the lack of sequence information. In this study, we evaluated bighorn sheep (*Ovis canadensis*) and mule deer (*Odocoileus hemionus*) from Montana for infection with *A. ovis* by serology and sequence analysis of the *msp4* gene. Antibodies to *Anaplasma* spp. were detected in 37% and 39% of bighorn sheep and mule deer analyzed, respectively. Four new *msp4* genotypes were identified. The *A. ovis msp4* sequences identified herein were analyzed together with sequences reported previously for the characterization of the genetic diversity of *A. ovis* strains in comparison with other *Anaplasma* spp. The results of these studies demonstrated that although *A. ovis msp4* genotypes may vary among geographic regions and between sheep and deer hosts, the variation observed was less than the variation observed between *A. marginale* and *A. phagocytophilum* strains. The results reported herein further confirm that *A. ovis* infection occurs in natural wild ruminant populations in Western United States and that bighorn sheep and mule deer may serve as wildlife reservoirs of *A. ovis*.
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Keywords: Anaplasmosis; Major surface protein; Reservoir host; Mule deer; Bighorn sheep; Wildlife

1. Introduction

Anaplasma ovis is an intraerythrocytic rickettsial pathogen of sheep, goats and wild ruminants (Krier and Ristic, 1963; Zaugg, 1987, 1988; Kuttler, 1984; Zaugg et al., 1996; Friedhoff, 1997; Yabsley et al.,

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2005; de la Fuente et al., 2006). This pathogen is classified in the genus *Anaplasma* (Rickettsiales: Anaplasmataceae), along with *A. marginale* (the type species), *A. phagocytophilum* and *A. bovis* which also infect ruminants and *A. platys* that infects dogs (Dumler et al., 2001; Kocan et al., 2004). Ticks of the genus *Dermacentor* are biological vectors of *A. ovis* in the western United States. In the Old World, *A. ovis* is transmitted by *R. bursa* and most likely other ticks (Friedhoff, 1997). Mammalian or tick hosts with persistent infection serve as reservoirs of the pathogen in nature (Kocan et al., 2004).

Anaplasma major surface proteins (MSPs) are involved in interactions with both vertebrate and invertebrate hosts (de la Fuente et al., 2005a; Kocan et al., 2004; Brayton et al., 2006; Dunning Hotopp et al., 2006), and therefore are likely to evolve more rapidly than other genes because they are subjected to selective pressures exerted by host immune systems. The *msp4* gene, the function of which is currently unknown, is part of the MSP2 protein superfamily (de la Fuente et al., 2005a; Brayton et al., 2006). The *msp4* gene and protein sequences have proven useful for phylogenetic studies of *A. marginale* and *A. phagocytophilum* (de la Fuente et al., 2002a, 2005a,b) and for the genetic characterization of *Anaplasma* spp. (reviewed by de la Fuente et al., 2005a).

Many geographic strains of *Anaplasma* have been identified which differ in biology, genetic characteristics and/or pathogenicity (reviewed by de la Fuente et al., 2005a). While the genetic diversity of *A. marginale* and *A. phagocytophilum* has been studied extensively, the genetic diversity for *A. ovis* has not been well characterized due to the lack of sequence information (reviewed by de la Fuente et al., 2005a). Few studies have analyzed *A. ovis msp4* sequences from both domesticated sheep and wild sheep and deer populations with the identification of three different genotypes only (de la Fuente et al., 2002b, 2005c, 2006; Yabsley et al., 2005).

In this study, we evaluated bighorn sheep (*Ovis canadensis*) and mule deer (*Odocoileus hemionus*) from Montana for infection with *A. ovis* by serology and sequence analysis of the *msp4* gene. The four new *msp4* genotypes identified herein were analyzed together with the three sequences reported previously to characterize the genetic diversity of *A. ovis* strains in comparison with other *Anaplasma* spp.

2. Materials and methods

2.1. Animals and sample preparation

During November 2002–October 2005, 83 bighorn sheep were darted and captured in Glacier National Park, MT, USA, using combinations of carfentanil and xylazine, primarily during the October–November and April–May periods. During March 2006, 13 additional bighorn sheep were captured via helicopter net-gunning in adjacent Waterton Lakes National Park, AB, Canada. Samples from 57 of these animals were submitted for serologic testing. Mule deer ($N = 70$) were physically restrained using a net gun fired from a helicopter for the purpose of radio-collaring. These animals were captured between 10 and 14 January 2006 on privately owned land in southeastern Montana. Blood was collected into separate sterile tubes with and without anticoagulant (EDTA) and maintained at 4 °C until processed. Plasma and serum were then separated after centrifugation and stored at −20 °C. DNA was extracted from blood samples of seropositive animals using Tri Reagent (Sigma, St. Louis, MO, USA) and following manufacturer's recommendations. Experiments were performed according to the U.S. laws and approved by the Montana State University ethical committee.

2.2. Anaplasmosis serologic test

Animals were tested for antibodies to *Anaplasma* spp. by the State of Montana Department of Livestock Diagnostic Laboratory (Bozeman, MT, USA) using the cELISA anaplasmosis test (VMRD Inc., Pullman, WA, USA) (Knowles et al., 1996), which detects antibodies to the MSP5 antigen conserved between *A. marginale*, *A. phagocytophilum* and *A. ovis* (Dreher et al., 2005). Percent inhibition values greater than 30% were considered positive as recommended by the manufacturer.

2.3. msp4 PCR and sequence analysis

The *Anaplasma* spp. *msp4* gene was amplified by PCR and sequenced as reported previously (de la Fuente et al., 2002a, 2005b). Briefly, 1 μl (1–10 ng) DNA was used with 10 pmol of each primer (*A. marginale*/*A. ovis*: MSP45: 5′-GGGAGCTCCTATGAATTACAGAGAATTGTTTAC-3′ and MSP43:

5′-CCGGATCCTTAGCTGAACAGGAATCTTGC-3′; *A. phagocytophilum*: MAP4AP5:5′-ATGAATTA-CAGAGAATTGCTTGTAGG-3′ and MSP4AP3: 5′-TT-AATTGAAAGCAAATCTTGCTCCTATG-3′) in a 50-μl volume PCR (1.5 mM $MgSO_4$, 0.2 mM dNTP, 1× AMV/*Tfl* reaction buffer, 5 u *Tfl* DNA polymerase) employing the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler (Eppendorf Mastercycler® personal, Westbury, NY, USA) for 35 cycles. After an initial denaturation step of 30 s at 94 °C, each cycle consisted of a denaturing step of 30 s at 94 °C, an annealing for 30 s at 60 °C and an extension step of 1 min at 68 °C for *A. marginale*/*A. ovis* and an annealing-extension step of 1 min at 68 °C for *A. phagocytophilum*. Negative control reactions were performed with the same procedures, but adding water instead of DNA to monitor contamination of the PCR. The program ended by storing the reactions at 10 °C. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 kb DNA Ladder, Promega). Amplified fragments were resin purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both strands by double-stranded dye-termination cycle sequencing (Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University). At least two independent clones were sequenced for each PCR.

The *msp4* coding region was used for sequence alignment. Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) with an engine based on the Clustal W algorithm (Thompson et al., 1994). Nucleotides were coded as unordered, discrete characters with five possible character-states: A, C, G, T, or N and gaps were coded as missing data. Maximum parsimony (MP) analyses were conducted with equal weights for all characters and substitutions and heuristic searches with 10 random additions of input taxa. To examine the effect of method of analysis on the resulting phylogeny, phylogenetic trees were constructed based on the sequence distance method using the Neighbor-Joining (NJ) algorithm of Saitou and Nei (1987) with Kimura 2 parameters correction. Phylogenetic analyses were conducted using Mega 2 (Kumar et al., 1994). Stability or accuracy of inferred topology(ies) were assessed via bootstrap analysis (Felsenstein, 1985) of 1000 iterations. *A. ovis msp4* sequences discovered in this study and those reported previously were included in the analysis (Table 1). Character-state changes for *A. ovis msp4* were polarized by designating *A. marginale* strains Oklahoma (AY010247) and Mexico (AF345868) as outgroups.

2.4. Statistical analysis

Anaplasma spp. seroprevalence was compared between various subpopulations of bighorn sheep by comparing approximate confidence intervals, calculated using the Clopper–Pearson method (Johnson et al., 1993).

Table 1
Nucleotide sequence differences among *msp4* from different strains of *Anaplasma ovis*

Sequence[a] (reference)	Host	Location	Genotype	*msp4* nucleotide positions[b]						
				360	366	400	470	522	630	774
AF393742 (de la Fuente et al., 2002b)	Sheep	ID, USA	I	A	C	G (A)	T (V)	T	A	C
AY702924 (de la Fuente et al., 2005c)	Sheep	Sicily, Italy	II	*	T	*	C (A)	*	*	*
AY702923 (de la Fuente et al., 2005c)	Sheep	Sicily, Italy	III	*	T	*	*	*	*	*
DQ674246 (this work)	Bighorn sheep	MT, USA	IV	G	*	*	*	*	*	*
DQ674247 (this work)	Mule deer	MT, USA	V	*	*	*	*	C	G	T
DQ674248 (this work)	Mule deer	MT, USA	VI	*	*	A (T)	*	*	*	T
DQ674249 (this work)	Mule deer	MT, USA	VII	*	*	*	*	*	*	T

[a] Genbank accession number.
[b] The numbers represent the nucleotide position starting at translation initiation codon adenine. Conserved nucleotide positions with respect to the Idaho strain are represented with asterisks. Amino acid changes are represented in parentheses using the single letter code.

2.5. Sequence accession numbers

The GenBank accession numbers for *msp4* sequences of *A. ovis* strains are DQ674246–DQ67 4249 (Table 1).

3. Results and discussion

Antibodies to *Anaplasma* spp. were detected in 21 (37%) and 27 (39%) of bighorn sheep and mule deer tested, respectively (Table 2). The observed seroprevalence of *Anaplasma* spp. was similar to that reported in previous studies and suggested a high prevalence of infection in these populations (Table 2). However, seroprevalence varied among various subpopulations of bighorn sheep. In the Waterton–Glacier area, radiotelemetry and genetic data indicate that bighorn sheep in the southern half of Glacier Park comprise a distinct subpopulation from those in northern Glacier and Waterton Lakes National Parks (K.A. Keating, unpublished data). The observed seroprevalence of *Anaplasma* spp. in the southern herd was 0/17 = 0% (95% CI = 0–16%), while the estimated incidence for the adjacent northern herd was 21/40 = 53% (95% CI = 36–68%), a difference that is both statistically ($\alpha < 0.05$) and biologically significant. Within the northern herd, seroprevalence varied with gender. The observed seroprevalence of *Anaplasma* spp. was lower ($\alpha = 0.07$) for females (5/18 = 28%; 95% CI = 10–53%) than for males (16/22 = 73%; 95% CI = 50–89%).

The *Anaplasma* spp. *msp4* sequences were amplified and sequenced from 11 and 12 of the bighorn sheep and mule deer seropositive samples, respectively (Table 2). The difference between the results of serologic and PCR analysis most likely resulted from the sensitivity of the PCR (5 copies *msp4*/ng DNA) which may not detect the low infections of carrier animals. Sequence analysis of *msp4* amplicons demonstrated that all sequences corresponded to *A. ovis*. *A. phagocytophilum* and *A. marginale* were not detected in any of the samples by use of the *msp4* PCR.

It has been demonstrated that mule deer are susceptible to experimental infections with *A. ovis* and *A. marginale* (Zaugg, 1988). The absence of *A. marginale* and *A. phagocytophilum* infections in this herd of mule deer may be due to the absence of biological and mechanical vectors for these pathogens and/or the possibility that some *Anaplasma* genotypes may exclude the multiplication of other genotypes as has been described for *A. marginale*, *A. ovis* and *A. phagocytophilum* strains (de la Fuente et al., 2002b; Stuen et al., 2005). However, the absence of *A. marginale* and *A. phagocytophilum* infections in bighorn sheep may be due primarily to the pathogens host-range and to a lesser extent to the factors discussed above for mule deer.

Five *A. ovis msp4* genotypes were identified infecting bighorn sheep and mule deer in this study (Table 1). However, despite the high prevalence of *A. ovis* infections, the genetic diversity of strains characterized was low (Table 2). In the Western United States, the genotype I was the most prevalent genetic variant, which corresponded to the *msp4* sequence of the Idaho strain (AF393742) (Tables 1 and 2). The *A. ovis msp4* genotypes identified in Italy were unique to this region, which suggested that *msp4*

Table 2
Prevalence of *Anaplasma ovis* by serology and corroborated by *msp4* PCR and sequence analysis

Host	Location	Seroprevalence[a] infected/tested (%)	*msp4* PCR positive/ tested (%)	Genetic diversity[b] genotype (%)	Reference
Sheep	Sicily, Italy	6/8 (75)	7/8 (87)	II (14) III (86)	de la Fuente et al. (2005c)
Bighorn sheep	MT, USA	25/180 (14)	9/23 (39)[c]	I (100)	de la Fuente et al. (2006)
Bighorn sheep	MT, USA	21/57 (37)	11/15 (73)[c]	I (91) IV (9)	This work
Mule deer	CA, USA	4/6 (67)	3/11 (27)	I (100)	Yabsley et al. (2005)
Mule deer	MT, USA	27/70 (39)	12/27 (44)[c]	I (50) V (16.66) VI (16.66) VII (16.66)	This work

[a] Determined using the cELISA for *Anaplasma* MSP5 (VMRD Inc., Pullman, WA, USA).
[b] Percent of each genotype in PCR positive samples. Genotypes were described in Table 1.
[c] The *msp4* PCR and sequence analysis was done on seropositive animals, except for six seropositive bighorn sheep from Waterton Lakes National Park, whose samples were not yet available at the time of processing.

sequences may provide some phylogeographic information.

Phylogenetic analysis of *msp4* sequences by MP and NJ resulted in trees with similar topology (Fig. 1 and data not shown). A weak (>60%) support was found for the clade containing the genotypes II and III of Italian *A. ovis* strains (Fig. 1). The phylogenetic analysis also differentiated between sheep and deer *A. ovis* strains with genetic variants different from genotype I (Fig. 1).

Genetic diversity of *A. marginale* strains has been described within cattle herds and geographic locations, suggesting that multiple introductions of genetically diverse strains of the pathogen occurred in most geographic areas with further diversification by cattle movement (de la Fuente et al., 2005a). Maintenance of different genotypes by independent transmission events was shown to occur by infection exclusion of *A. marginale* in cattle and ticks which results in the establishment of a single genotype in most animals (de la Fuente et al., 2002b, 2005a). Genetic diversity of *A. phagocytophilum* strains also occurs in geographic regions, but ruminant and non-ruminant strains of the pathogen can be differentiated by use of the *msp4* sequence (de la Fuente et al., 2005b).

The results of the studies reported herein demonstrated that *A. ovis msp4* genotypes may vary among geographic regions. These results also suggested that *A. ovis msp4* genotypes may vary between sheep and deer hosts, although such differences could not be demonstrated with certainty because the sheep and deer populations studied were widely separated geographically. However, as in previous studies (Yabsley et al., 2005; de la Fuente et al., 2006), the variation observed thus far is less than that observed in *A. marginale* and *A. phagocytophilum*. This finding may have resulted from restricted movement of infected hosts. Additionally, the limited host range of *A. ovis* as compared with *A. phagocytophilum* may have also contributed to the lack of genetic diversity of this rickettsia.

The results reported herein together with recent reports (Yabsley et al., 2005; de la Fuente et al., 2006), documented using the *msp4* PCR and sequence analysis the presence of *A. ovis* infection in natural wild ruminant populations in Western United States. These results support the potential for bighorn sheep and mule deer to serve as wildlife reservoirs of *A. ovis*. However, the role of these species as reservoirs of *A. ovis* on the epidemiology

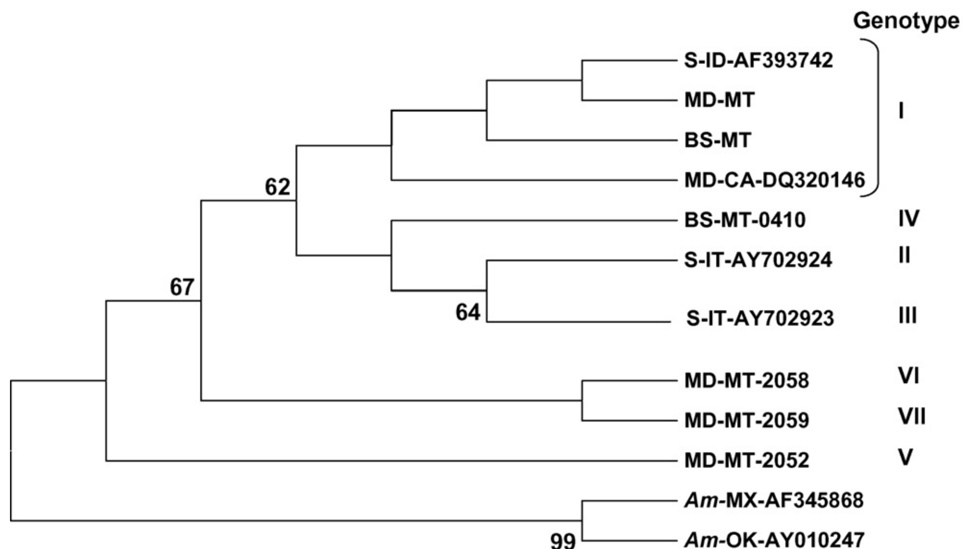

Fig. 1. Phylogenetic analysis of *Anaplasma ovis* strains based on MP analysis of *msp4* sequence data and bootstrap analysis with 1000 iterations. Numbers on branches indicate >60% support for each clade. Strains of *A. ovis* are described in Table 1 and are represented as host-location-sequence accession number. Abbreviations: S, sheep; MD, mule deer; BS, bighorn sheep; ID, Idaho; MT, Montana; IT, Italy; MX, Mexico; OK, Oklahoma; *Am*, *A. marginale*.

of the disease is unknown and warrants further investigation.

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