NOTE *Bacteriology*

Variation of Genes Encoding GGPLs Syntheses among *Mycoplasma fermentans* Strains

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ABSTRACT. The information of the biosynthesis pathways of *Mycoplasma fermentans* specific major lipid-antigen, named glycoglycerophospholipids (GGPLs), is expected to be some of help to understand the virulence of *M. fermentans*. We examined primary structure of cholinephosphotransferase (*mfl*) and glucosyltransferase (*mf3*) genes, which engage GGPL-I and GGPL-III synthesis, in 20 strains, and found four types of variations in the *mfl* gene but the *mf3* gene in two strains was not detected by PCR. These results may have important implications in virulence factor of *M. fermentans*.

KEY WORDS: cholinephosphotransferase, glucosyltransferase, *Mycoplasma fermentans*.

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Mycoplasma fermentans was first isolated from the urogenital tract of patients with ulcerative balanitis several decades ago [19] and then detected from respiratory tract of children with community-acquired pneumonia [4], adults with acute respiratory distress syndrome [10], the joints of patients with rheumatoid and other inflammatory arthritic disorders [5, 6, 8, 21] and so on. Although it was previously assumed that humans were the only natural hosts, *M. fermentans* has been isolated from genital lesions in sheep [15], suggesting a zoonotic aspect of this particular pathogen.

Interest in this organism has recently increased because of its possible role in the pathogenesis of rheumatoid arthritis and reports indicating that this organism may function as a cofactor accelerating the progression of human immunodeficiency virus infection [17]. Although *M. fermentans* is a typical extracellular microorganism able to adhere to human epithelial cells, ultrastructural studies performed with engulfed *M. fermentans* revealed mycoplasmas within membrane-bound vesicles [24, 25].

In mycoplasmas, adherence is the major virulence factor, and adherence-deficient mutants are avirulent [2, 18]. It seems that *M. fermentans* utilizes at least two surface components for adhesion to HeLa cells, a protease-sensitive surface protein, apparently the lipoprotein recently described [23], and a phosphocholine-containing glycolipid. Phosphocholine-containing lipids were detected in all *M. fermentans* strains tested by Ben-Menachem *et al.* [3].

Matsuda *et al.* have identified several alkali labile glycophospholipids designated as glycoglycerophospholipids (GGPLs) [13]. Of them, GGPL-I and GGPL-III are expressed in *M. fermentans* specifically, and these lipid-antigens are the major lipid-antigens of *M. fermentans* [14]. The structures of GGPL-I and GGPL-III were identified as 6'-O-phosphocholine-α-glucopyranosyl-(1'-3)-1,2-diacylglycerol and 1'-phosphocholine-2'-amino dihydroxypropane-3'-phospho-6'-α-glucopyranosyl-(1'3)-1,2-diacylglycerol, respectively [12, 13], and GGPLs have been chemically synthesized by Nishida *et al.* [16].

Based on unique structures and bioactivities, GGPLs have been considered as a hypothetical factor in the pathogenesis of *M. fermentans* [11]. Because GGPLs have strong immunogenicity, they may play roles as immunodisturbing agents in cell functions such as inflammation and cell differentiation [22]. GGPL-III antigens were detected in synovial tissues from RA patients and significantly induced TNF-α and IL-6 production from peripheral blood mononuclear cells, and also proliferation of synovial fibroblasts [9].

The information of the biosynthesis pathways of relative compounds of GGPLs is expected to contribute to identification of those of GGPLs, and Ishida *et al.* determined one of the putative GGPL-I biosynthetic genes, according to whole genome analysis of *M. fermentans* PG18 [7]. In the present study, we examined the presence of cholinephosphotransferase (*mfl*) gene and glucosyltransferase (*mf3*) gene, which engage GGPL-I and GGPL-III synthesis, in human mycoplasma species as well as in 20 strains of *M. fermentans*.

M. fermentans strains used in this study are listed in Table 1. Other human mycoplasmas examined include *M. genitalium* G37, *M. pneumoniae* Mac, *M. pneumoniae* FH, *M. penetrans* GTU, *M. orale* CH19299, *M. buccale* CH20247, *M. primatum* HRC92, and *M. hominis* PG21. *M. fermentans* strains were obtained from Dr. Tsuguo Sasaki of the National Institute for Infectious Disease, Tokyo, Japan and

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Table 1. Origin of *M. fermentans* strains and *mf1*, *mf3*, *MCGp* sequence type and PCR type based on the major part of the IS*1550* element

Origin	Strain	Year of isolation	*mf1* sequence	*mf3* sequence	IS*1550* PCR type (25)
Genital ulcer	PG18^T	1955	A	+	B
Rheumatoid	KL4	1990	D	+	A
Arthritis	KL8	1990	D	+	B
Joint fluid	GIM	1995	D	+	A
Leukemic bone	E10	1960–1969	D	+	A
marrow	K7	1960–1969	D	+	A
	Z62	1960–1969	D	+	A
Urethral isolate AIDS patients	BRO	1990	D	+	A
Urine	#5	1995	C	+	B
Urine	#29	1995	D	+	B
Blood	AOU	1990	D	+	A
Respiratory	M39	1990–1995	A	+	B
tract	M51	1990–1995	D	+	B
	M52	1990–1995	B	+	B
	M64	1990–1995	D	–	B
	M70	1990–1995	D	+	B
	M73	1990–1995	D	–	B
Cell culture	A6	1982–1992	D	+	B
	C5	1982–1992	D	+	B
	2059	1982–1992	D	+	A
	28AC	1982–1992	D	+	B

they include strains KL4, KL8 (from rheumatoid arthritis), GIM (from human joint), E10, K7, Z62 (from bone marrow of leukaemic patients), BRO (from human urethral), #5, #29 (from urine deposits from AIDS patients), AOU (from blood of an AIDS patient), M39, M51, M52 M64, M70, M73 (from human respiratory tract), 2059, 28AC, A6 and C5 (from cell culture). The *mf1* and *mf3* were PCR-amplified from *M. fermentans* genome. Briefly 1 μl of broth cultures (approximately 10^6 cfu/m*l*) were diluted in 1 m*l* of water and heated at 95°C for 3 min for lysis of mycoplasma cells. Mycoplasma lysate (5 μl) was directly added into 45 μl of the PCR master mixture consisting of 1 unit of KOD plus DNA polymerase (TOYOBO, Osaka, Japan), 5 μl of 10 × PCR buffer, 5 μl of 2 mM deoxynucleoside triphosphates, 75 nmol of $MgSO_4$, and 10 pmol of each primer, combination of mf1F (5'-ATAATAAAAACTATGAATGA-3') and mf1R (5'-CTATTTGTCATTTTTCTT-3'), or mf3F (5'-ATGATATGAAAGTTTTTTGTAAAAAAGAAAGG-3') and mf3R (5'-TTATTTTTTATAATGTTCAATAATTT-TTTTGTATTT-3'). Amplification was done under the following conditions; 30 cycles of 94°C for 40 sec, 50°C for 90 sec, and 68°C for 2 min after 94°C for 2 min. *mf1* and *mf3* amplified products (777 and 1,221 bp respectively) were sent to a reference laboratory (TaKaRa Custom Services, Shiga, Japan) for DNA sequencing.

In our study, *mf1* and *mf3* are thought to be *M. fermentans* specific genes because they were not amplified in case of other human mycoplasmas (*M. genitalium* G37, *M. pneumoniae* Mac, *M. pneumoniae* FH, *M. penetrans*, *M. orale*, *M. buccale* CH20247, *M. primatum* HRC92, *M. hominis* PG21).

The sequence data of *mf1* gene were deposited to the international DNA databases under the accession number AB480306 ~ AB480325. Although the *mf1* gene was shown to be conservative in 20 strains of *M. fermentans* by PCR, *mf3* gene was not amplified in two strains, M64 and M73, among these 20 strains examined. Besides, the *mf1* and *mf3* genes were not evident in other human *Mycoplasma* species by specific PCR, suggesting that these two genes are unique to the *M. fermentans* species. Nucleotide sequences of *mf3* gene in the 20 *M. fermentns* strains were identical, but those of *mf1* gene showed a minor variation causing some amino acid substitutions, and categorize A, B, C, and D types for descriptive purposes (Fig. 1). These amino acid changes may be responsible for enzymatic activity of cholinephosphotransferase in *M. fermentans*. In addition, although the *mf3* gene was not amplified from strains M64 and M73, the reason was currently unknown since defection of glucosyltransferase in these strains was not examined in the present study. These diversities in enzymes may engage specific major lipid-antigen syntheses, and also influence immunogenic potential and RA pathogenesis. No significant homology to the *mf3* gene was apparent in other prokayotes in databases, supporting that the GGPLs are unique to *M. fermentans*. Currently recombinant enzymes, based on the nucleotide sequences of the enzyme genes from PG18 strain of *M. fermentans*, have been successfully expressed in *Escherichia coli* [7]. PG18, a type strain of *M. fermentans*, has been shown particularly unique among *M. fermentanss* strains [20]. The difference of *mf1* posttranslational amino-acid sequence may have influenced the activity of choline phosphotransferase, and concern GGPL-I

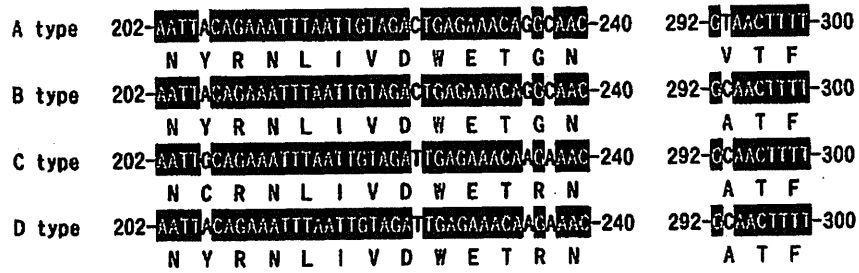

Fig. 1. The part of *mf1* DNA (top) and posttranslational amino-acid sequence data (bottom) of *M. fermentans* PG18 reference strain. The 20 strains tested were differentiate into four types A including PG18 and M39, B including M52, C including #5 and D including the other strains.

antigenicity, so studies on not only PG18 strain but also on other strains will become increasingly important. In addition, all strains presenting A type in IS*1550* PCR pattern were D type in *mf1* and detected *mf3*, however, there seems to be poor correlation among IS*1550*, *mf1* and *mf3*.

In conclusion, our genetic analysis on the 20 strains of *M. fermentans* showed four variations in *mf1* posttranslational amino-acid sequence but undetected *mf3* gene in two strains. Our results may have important implications for the virulence of *M. fermentans* especially in RA pathogenesis.

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FULL PAPER *Bacteriology*

Nitric Oxide Causes Anoikis through Attenuation of E-Cadherin and Activation of Caspase-3 in Human Gastric Carcinoma AZ-521 Cells Infected with *Mycoplasma hyorhinis*

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ABSTRACT. *Mycoplasma hyorhinis* (*M. hyorhinis*) infection leads cultured cells to various biological alterations in cell metabolism including apoptosis. Apoptosis induced by *M. hyorhinis* has mainly been considered to be due to mycoplasmal endonucleases. We previously reported that apoptosis in a human carcinoma cell line AZ-521 infected with *M. hyorhinis* was enhanced by addition of L-ascorbic acid to cell cultures. Since both L-ascorbic acid addition and *M. hyorhinis* infection activated cellular iNOS, we examined the hypothesis that nitric oxide (NO) exerts an apoptotic effect on *M. hyorhinis*-infected cells and down-regulates E-cadherin. In this study, we showed that *M. hyorhinis* infection activates iNOS mRNA synthesis, NO production, and caspase-3 activity and attenuates E-cadherin mRNA synthesis by quantitative real-time PCR, Griess assay and fluorescence caspase-3 detection. L-NAME decreased the numbers of apoptotic cells through inhibition caspase-3 activity. Our results indicate that NO causes anoikis throughout attenuation of E-cadherin and activation of caspase-3 in human gastric carcinoma cell line AZ-521 cells infected with *M. hyorhinis*.

KEY WORDS: anoikis, apoptosis, E-cadherin, *Mycopalsma hyorhinis*, nitric oxide.

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Mycoplasmas are the smallest self-replicating prokaryotes, lack rigid cell walls and belong to the class *Mollicutes* [25]. Most mycoplasma species inhabit plants, insects, animals and humans as normal flora in their hosts. Although some are pathogenic, many possess an opportunistic character [22].

Mycoplsma hyorhinis (*M. hyorhinis*) causes polyserositis [7], otitis media [19], arthritis [12] and pneumonia [1] in piglets. These mycoplasmal infections are responsible for economical losses on swine farms. *M. hyorhinis* is also a major and serious contaminant in cell cultures [2]. In addition, it has been demonstrated in human gastric carcinoma tissues [26], and it has been suggested that lipoprotein P37 of *M. hyorhinis* contributes to invasiveness and metastasis of the tumor cells [5, 14].

M. hyorhinis infection has a significant impact on the physiology of cell cultures, including apoptosis, which has previously been explained by mycoplasmal endonucleases [23], production of nitric oxide (NO) by activating cellular inducible NO synthase (iNOS) and production of reactive oxygen species (ROS) in various cell lines [13]. NO is a multifunctional molecule involved in a variety of physiological and pathological processes [17]. While low concentrations of NO can protect cells from apoptosis, excess NO promotes cell death in various cell types [3].

Apoptosis is characterized morphologically by cell shrinkage and chromatin condensation and biochemically by DNA laddering [30]. Detection of caspase activity is a useful assay for apoptosis. The caspase family participates in a series of reactions that are trigged in proapototic signals and result in the cleavage of substrates, and caspases are synthesized as inactive precursors that undergo proteolytic maturation upon apoptotic stimulation [27].

Adhesion of cells to the extracellular matrix (ECM) is important as detachment from the matrix triggers apoptosis referred to as anoikis [7]. It has recently been reported that anoikis is caspase-3 dependent [6]. Epithelial cadherin (E-cadherin) is the prime mediator of intracellular adhesion [28]. Our preliminary examination suggested marked down-regulation of E-cadherin expression in AZ521 cells infected with *M. hyorhinis* based on a microarray analysis (unpublished data). Therefore, we hypothesized that excessive NO produced by *M. hyorhinis* infection leads to anoikis in AZ-521 cells.

We previously reported that apoptosis in human carcinoma cell line AZ-521 infected with *M. hyorhinis* was enhanced by addition of L-ascorbic acid (AsA) to the cell cultures [21]. Since *M. hyorhinis* infection and/or AsA addition enhance iNOS activity [20], we proposed a hypothesis that *M. hyorhinis* infection results in the presence of another external apoptotic pathway including the NO pathway. The aim of the present study was to obtain insights into the role of NO in cell adhesion-dependent apoptosis in cells infected with *M. hyorhinis*.

In the present study, we examined the role of NO in apoptosis induced by *M. hyorhinis* infection. We used a general competitive inhibitor of NOS, N^{ω}-nitro-L-arginine methyl ester (L-NAME), to measure NO based on the Griess assay

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and analyzed iNOS and E-cadherin expression by real-time PCR (RT-PCR) and caspase-3 detection. This study may also promote understanding of the mechanism of piglet diseases caused by *M. hyorhinis*.

MATERIALS AND METHODS

Cell cultures: Human gastric carcinoma cell line AZ-521 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and was maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, U.S.A.), 1% penicillin and streptomycin (100 U/ml) at 37°C in a humidified 5% CO_2 atmosphere. To minimize the pH change by L-NAME (Nacalai Tesque Inc., Kyoto, Japan) addition to the cell culture, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5) was added to the DMEM at a final concentration of 50 mM. The cell culture was tested for the absence of mycoplasma contamination by using a PCR Mycoplasma Detection Set (TaKaRa-Bio, Shiga, Japan) according to the manufacturer's instructions. The cells were seeded into chamber flasks at 8.5×10^5 cells per chamber (Asahi Techno Glass, Tokyo, Japan).

Five hundred μM of L-NAME were added to the final concentration as indicated. The vehicle for L-NAME was distilled water, and distilled water was used as a control. Addition of distilled water or 500 μM of L-NAME to AZ-521 cells did not produce any differences in this study (data not shown) using trypan blue staining after 30 hr of incubation in order to monitor the cytotoxic effect of distilled water [16]. The reagents were freshly prepared and dissolved in distilled water before pretreatment.

M. hyorhinis strain and its growth conditions: The BTS-7 strain of *M. hyorhinis* was used in present study. *M. hyorhinis* was grown at 37°C in PPLO broth (Difco, Detroit, MI, U.S.A.) supplemented with 20% horse serum (Gibco BRL, Grand Island, NY, U.S.A.), 5% fresh yeast extract (ICN Biomedicals, Inc., OH, U.S.A.) and 0.5% glucose instead of bacteriological mucin [15]. The propagation of *M. hyorhinis* was expressed as Colony-forming units (CFU). *M. hyorhinis* was inoculated into the AZ-521 cell culture at a multiplicity of infection (MOI) of 10^3 after seeding the cells into Chamber Slide II wells (Asahi Techno Glass, Tokyo, Japan). *M. hyorhinis* grown in PPLO broth containing 500 μM of L-NAME and HEPES (pH 7.5) was calculated after 30 hr of incubation at 37°C. Viability of the AZ-521 cells and *M. hyorhinis* strain were not affected by incubation with distilled water or 500 μM of L-NAME after 30 hr post-inoculation (data not shown).

Quantitative RT-PCR for iNOS and E-cadherin mRNA transcription: Total RNA was extracted from AZ-521 cells inoculated with *M. hyorhinis*, after 20, 24 and 28 hr using RNA-Bee™ (Tel-Test, Inc., Pearland, TX, U.S.A.), precipitated by isopropanol and dissolved in diethyl pyrocarbonate-treated water.

Similarly, total RNA was extracted from AZ-521 cells inoculated with *M. hyorhinis* and treated or not treated with 100, 250 and 500 μM of L-NAME after 24 and 28 hr. The RNA was transcribed with *Moloney murine leukemia* virus reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. First-strand complementary DNA was quantitatively analyzed for the expression of iNOS and E-cadherin gene in a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with TaKaRa Ex *Taq* R-PCR (TakaRa Bio, Shiga, Japan). The reaction mixture contained 1 μl of each primer (10 pmol/μl), 2.5 μl of 10 × reaction buffer, 20 nmol of each deoxynucleotide, 0.25 μl of 250 mM $MgCl_2$, 0.2 μl (5 U/μl) TaKaRa Ex *Taq* HS DNA polymerase (TaKaRa Bio, Shiga, Japan), 2.5 μl of 1:3,000 SYBR Green I (TaKaRa Bio, Shiga, Japan) and water to a volume of 23 μl. Finally, 2 μl of RNA as a template was added to this mixture. The conditions of amplification were as follows: after initial melting at 94°C for 2 min, amplification was performed with 40 cycled of 94°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec. The primer sequences used were as follows: 5'- CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3', forward, and 5'-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3', reverse, for iNOS (394 bp) [13]; 5'-AGA ATG ACA ACA AGC CCG AAT-3', forward, and 5'-CGG CAT TGT AGG TGT TCA CA-3', reverse for E-cadherin (132 bp) [4]; and 5'- GTC TTC ACC ACC ATG GAG AAG GCT-3', forward, and 5'-CAT GCC AGT GAG CTT CCC GTT CA-3', reverse, for glyceraldehydes-3-phosphate dehydrogenase (GAPDH; 420 bp) [13]. The amount of target gene transcription was calculated from the standard curves and was normalized using the transcription of the housekeeping GAPDH gene as an internal control. The value for the mock-infected control was scored as one.

Measurement of NO oxidation products, nitrite and nitrate: NO is chemically unstable and undergoes rapid oxidation to nitrite, and cellular components catalyze its further oxidation to nitrate. Therefore, production of NO was determined by measuring the formation of the stable oxidation products of NO, nitrite and nitrate as described previously [10]. After the cells had been infected with *M. hyorhinis* for 20, 24 and 28 hr, total nitrite and nitrate concentrations in the cell culture were determined based on the Griess reaction by using a Total Nitric Oxide Assay Kit (Assay Designs Inc., Ann Arbor, MI, U.S.A.) according to manufacturer's instructions. The cell culture was collected after centrifugation at 1,000 × g for 5 min. The collected supernatant fluid was stored at −80°C until measurement of nitrate. The absorbance was measured at a wavelength of 570 nm in an NJ-2000 multiwell plate reader (InterMed, Tokyo, Japan).

Fluorescent microscopic analysis for apoptotic cell count and caspase-3 activity detection: The AZ-521 cells were examined for apoptotic changes by using a fluorescent dye, Hoechst 33258 (Dojin Chemicals, Kumamoto, Japan). We observed fluorescent nuclei showing apoptotic changes, mainly due to chromatin condensation, at 400 × magnification by using 352 and 461 nm band pass filters for excitation and emission, respectively, on an Eclipse E400 fluorescent

microscope (Nikon, Tokyo, Japan). At 24 and 28 hr post-inoculation, we counted the number fluorescent cells infected with BTS-7 showing apoptotic changes at 400 × magnification in 200 AZ-521 cells each treated with or without 100, 250 or 500 μM of L-NAME. Apoptosis was expressed as the percentage of cells with an apoptotic nuclear morphology in relation to the total cell number.

Furthermore, to detecte caspase-3 activity in AZ-521 cells, we used an APO LOGIX™ FAM-DEVD-FMK Carboxyfluorescein Caspase Detection Kit (Cell Technology Inc., Mountain View, CA, U.S.A.). AZ-521 cells grown in the DMEM containing 500 μM of L-NAME were examined more than 24 and 28 hr after inoculation with *M. hyorhinis*, according to manufacturer's instructions. Caspase-3 activity of AZ-521 cells was examined by counting the number fluorescent cells that were positive for caspase-3 in 200 AZ-521 cells at 400 × magnification.

Statistical analysis: The data was analyzed using analysis of variance (ANOVA) for comparison between groups using StatView ver. 5 (Hulinks Inc., Tokyo, Japan). Differences were accepted as significant values at $P<0.05$. Each test was repeated three times. Data were expressed as mean ± SEM values.

RESULTS

RT-PCR analysis of iNOS and E-cadherin transcription: The transcription levels of iNOS and E-cadherin were examined by using RT-PCR (Fig. 1). At 20, 24 and 28 hr post-inoculation, a significant amount of iNOS mRNA was detected in the *M. hyorhinis*-infected AZ-521 cells. On the other hand, no significant difference in iNOS mRNA transcription was observed in the mock-infected controls at any hour post-inoculation. These results suggested that *M. hyorhinis* induced iNOS mRNA expression in the AZ-521 cells.

Similarly, E-cadherin mRNA transcription was examined in AZ-521 cells treated with or without 100, 250 or 500 μl of L-NAME at 24 and 28 hr post-inoculation (Fig. 2). *M. hyorhinis* infection caused decreases of E-cadherin mRNA transcription at 24 and 28 hr post-inoculation as compared with the mock-infected controls. Also, the amount of E-cadherin mRNA was significantly decreased in the *M. hyorhinis*-infected AZ-521 cells treated with 500 μl of L-NAME at 24 hr and in those treated with 250 and 500 μl of L-NAME at 24 hr and 28 hr post-inoculation. *M. hyorhinis* infection did not alter GAPDH mRNA transcription. These results suggested that *M. hyorhinis* infection attenuates E-cadherin mRNA transcription by NO-dependency in AZ-521 cells.

Measurement of NO oxidation products, nitrite and nitrate: To determine whether or not the expressed iNOS in AZ-521 cells produces NO, NO synthesis was assayed by measuring the accumulation of the NO end products, nitrite and nitrate, in the medium (Fig. 3). At 20 hr post-inocula-

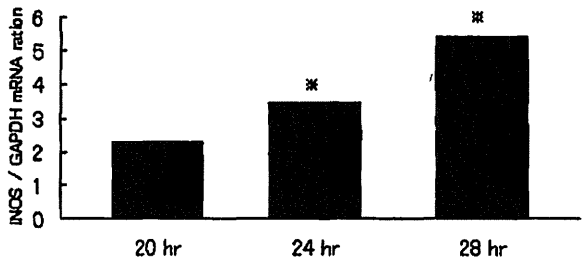

Fig. 1. Expression of iNOS in AZ-521 cells was analyzed by quantitative real-time PCR. The cells were treated with *M. hyorhinis* for 24 and 28 hr. The intensity of iNOS mRNA expression was normalized by that of GAPDH, and the value for mock was estimated as 1. Means ± SEM for 2 separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

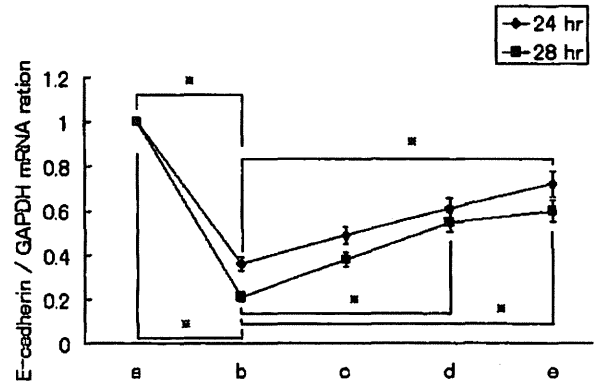

Fig. 2. E-cadherin expression in AZ-521 cells was analyzed by quantitative real-time PCR. The cells were infected with *M. hyorhinis* and treated with (b) no L-NAME, (c) 100, (d) 250 or (e) 500 μM of L-MAME or (a) mock for 24 and 28 hr. The intensity of E-cadherin mRNA expression was normalized by that of GAPDH. The value for mock was set at 1. Means ± SEM for 2 separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

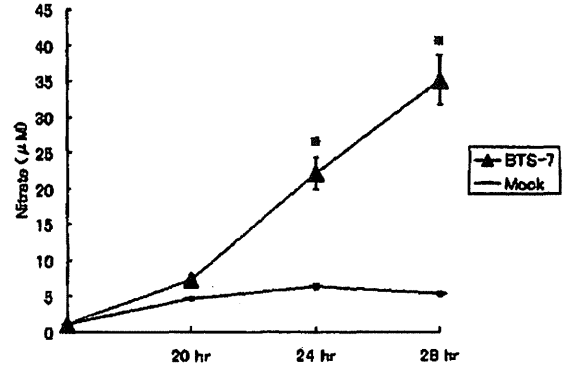

Fig. 3. Nitrate production in AZ-521 cells infected with *M. hyorhinis* at 20, 24 and 28 hr post inoculation. Means ± SEM for two separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

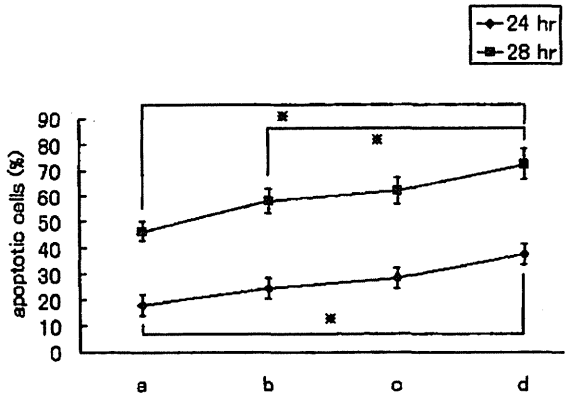

Fig. 4. The effect of L-NAME on *M. hyorhinis*-induced apoptosis in AZ-521 cells. The cells were infected with *M. hyorhinis* treated with (a) 500, (b) 250 or (c) 100 μM of L-MAME or with (d) no L-NAME for 24 and 28 hr. Means ± SEM for 2 separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

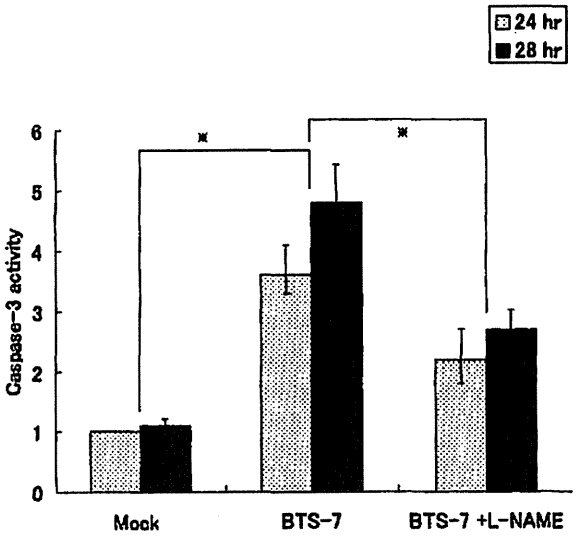

Fig. 5. Caspase-3 activity of AZ-521 cells infected with *M. hyorhinis* at 24 and 28 hr post inoculation. AZ-521 cells were treated with 500 μM of L-NAME. AZ-521 cells infected with *M. hyorhinis* at 24 and 28 hr post inoculation; in the non-treated AZ-521cells, the number of caspase-3 positive cells was scored as 1, and the numbers of caspase-3 positive AZ-521 cells were counted in *M. hyorhinis*-infected cells treated with 500 μM L-NAME or without. Means ± SEM for 2 separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

tion, no significant NO production was evident among the different *M. hyorhinis*-infected AZ-521 cells or in the mock-infected controls. Together with the augmentation of iNOS mRNA expression, nitrite and nitrate increased in the *M. hyorhinis*-infected AZ-521 cells at 24 and 28 hr post-inoculation compared with the mock-infected controls AZ-521 cells. These results corresponded to the results of the RT-PCR analyses for iNOS expression.

Fluorescent microscopic analysis for apoptotic cell counts and caspase-3 activity detection: The AZ-521 cells were examined for apoptotic changes by using a fluorescent microscope. Addition of 100, 250 and 500 μM L-NAME to AZ-521 cells infected with *M. hyorhinis* at 24 and 28 hr post-inoculation showed that L-NAME significantly inhibited apoptosis depending on the concentrations, except for 100 μM of L-NAME at 28 hr (Fig. 4).

To examine whether caspase-3 was involved in apoptosis induced by BTS-7 infection, we examined caspase-3 activity in AZ-521 cells by using an FAM-DEVD-FMK kit (Fig. 5). *M. hyorhinis*-infected AZ-521 cells showed significant caspase-3 activity at 24 and 28 hr post-inoculation compared with the mock-infected controls. This indicated that *M. hyorhinis* has another apoptotic pathway other than its endonucleases as previously reported [23]. Furthermore, at both 24 and 28 hr after inoculation with 500 μM of L-NAME, *M. hyorhinis*-infected AZ-521 cells were significantly less caspase-3 positive than non-treated *M. hyorhinis*-infected AZ-521 cells. These results suggested that NO produced by AZ-521 cells infected with *M. hyorhinis* plays an important role in activating caspase-3 and finally promoting apoptosis.

DISCUSSION

The present study is the first to provide data showing that the BTS-7 strain of produces excessive amounts of NO and attenuates E-cadherin expression, leading to anoikis.

Two regular apoptotic pathways have previously been identified. The extrinsic pathway is triggered by receptor/ligand interaction such as TNF receptor-1/TNF-alpha [31] and is mediated by "initiator" caspase-8 to the death receptor complex [24]. This leads to activation of "executioner" caspase-3, which can further activate downstream substrates involved in apoptotic changes [27]. In the present study, we showed that caspase-3 activity increased in AZ-521 cells infected by *M. hyorhinis*, and L-NAME-treated AZ-521 cells infected by *M. hyorhinis* had decreased caspase-3 activity as compared with the mock-infected AZ-521 cells (Fig. 5). Activation of caspase-3 is a downstream event in apoptosis and may occur before apoptosis. Thus, the "true" caspase-3 activity may be higher than in this study (at 24 and 28 hr). The more NO increased, the more cellular apoptosis and caspase-3 activity increased.

Different stress situations may alter matrix proteins, and such alteration may affect cell-matrix adhesion [28]. During cell-matrix adhesion, adhesion molecules on cell membrane surfaces recognize their matrix protein receptors as an initial step of adhesion. The present study showed that *M. hyorhinis* infection decreased E-cadherin mRNA expression in AZ-521 cells (Fig. 2). Since E-cadherin is the prime mediator of intracellular adhesion [28], E-cadherin down-regulation means the collapse of cell-matrix adhesion,

occurs anoikis in dependent on caspase-3.

NO can promote or inhibit cell death [3] in various cells depending on the concentration of intracellular NO in combination with the intracellular environment and its interactions with other biological molecules such as oxygen or superoxide. Excess NO induces apoptosis in various cells [3]. Also, NO rapidly reacts with superoxide anion radicals to form peroxynitrite, which is an oxidant substance producing cytotoxic effects in many cells [29]. It is known that peroxynitrite induces apoptosis [29]. Overproduction of NO resulting from *M. hyorhinis* infection may induce a caspase cascade. The present paper presents evidence showing that the unselective NOS inhibitor L-NAME prevented apoptosis induced by *M. hyorhinis* infection (Fig. 4). This suggests that excess amounts of NO or the NO reactant peroxynitrite resulting from *M. hyorhinis* decrease E-cadherin expression (Fig. 2) and lead to anoikis in AZ-521 cells.

M. hyorhinis infection in cell cultures causes diverse biological effects. For example, it has been reported that *M. hyorhinis* infection activates nNOS expression in human neuroblastoma cell line YT-nu [9]. Therefore, it is necessary to ascertain the absence of mycoplasma in cell cultures prior to study [11].

In conclusion, we first showed that *M. hyorhinis* induces anoikis in AZ-521 cells through NO production and E-cadherin down-regulation. Although we have obtained similar results in MDBK cells infected with *M. hyorhinis* (unpublished data), our results await application to other host cell types. Since *M. hyorhinis* is very common in nasal and tracheobronchial secretions of young swine [22], a few of strains of *M. hyorhinis* may induce apoptosis *in vitro*. In fact, we have shown that there is a difference in the amount of induced iNOS mRNA expression, NO production and apoptosis in AZ-521 cells between *M. hyorhinis* strains (data not shown). The difference of virulence to piglets among *M. hyorhinis* strains may depend on the ability to produce NO and induce apoptosis *in vivo*. However, we did not elucidate the relation between pathogenecity to piglets and the difference in ability to produce NO by cells infected with *M. hyorhinis* strains. It might be necessary to confirm whether serious lesion of piglets infected with *M. hyorhinis* presents mainly apoptotic cells. The present study will lend us further understanding of the mechanism of the piglet diseases caused by *M. hyorhinis*. Our results also underline the potential application of NOS inhibitor in the treatment of *M. hyorhinis*-induced swine diseases. However, the molecular mechanism of effect of NO on swine diseases needs further exploration.

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NOTE *Bacteriology*

Novel Hemoplasma Species Detected in Free-Ranging Sika Deer (*Cervus nippon*)

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ABSTRACT. Hemoplasma infections in wild ungulates have not been reported yet in Japan. We examined presence of hemoplasmas in blood samples collected from 147 sika deer (*Cervus nippon*) in the Iwate prefecture by real-time PCR, and found 13 (9%) were positive. Almost entire region of the 16S rRNA gene of the representative strains from positive samples was amplified by conventional PCR. The nucleotide sequences of the 16S rRNA gene were further determined and compared with those of other hemoplasmas. Our examinations 1st revealed the presence of 2 distinct hemoplasma species in sika deer, which are previously not described. One of them was closely related to *M. ovis* by the 16S rRNA sequence analysis, but was found distinct by comparison of the RNase P RNA gene sequences. Pathogenicity of these two hemoplasma species in sika deer is currently unknown.

KEY WORDS: hemoplasma, mycoplasma, sika deer.

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Hemoplasmas are tiny epierythrocytic bacterial parasites that lack a cell wall like other mycoplasmas, but have never been cultured *in vitro* [6]. Hemoplasmas infections, may lead to hemolytic anemia in animals, have been reported in wild ungulates such as reindeer (*Rangifer tarandus*) raised on a farm in Michigan [12] and splenectomized deer captured from a wild population in Texas [5] in the United States. However prevalence of hemoplasma infections in free-living ungulates have not well been understood. Sika deer (*Cervus nippon*), middle-sized ungulates, are found in the temperate forests of eastern Asia, and Japan has large populations of native sika deer. Therefore, we have examined hemoplasma infections among free-ranging sika deer in Japan.

Blood samples were collected from 147 wild sika deer hunted for the population control measures in Iwate prefecture, Japan from 2004 to 2006, and stored at –20°C prior to examination. Total DNA was extracted from 200 μl blood samples collected from sika deer using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Negative controls consisting of 200 μl phosphate-buffered saline were prepared with each batch. Extracted DNA samples were stored at –80 °C prior to use.

For the preliminary screening of hemoplasma infections, specific PCR primers (forward primer: 5'-ATATTCCTACGGGAAGCAGC-3' and reverse primer: 5'-ACCGCAGCTGCTGGCACATA-3') for the 16S rRNA gene of hemoplasmas were used as described previously [9]. Real-time PCR was performed in a SmartCycler instrument (Cepheid, Sunnyvale, CA) with SYBR Premix Ex *Taq* (Code #RR041A, TaKaRa Bio., Shiga). The reaction mixture contained 1 μl of each primer (10 pmol/μl), 12.5 μl of 2X premix reaction buffer and water to volume of 23 μl. Finally, 2 μl of DNA samples as templates were added to this mixture. Amplification was achieved with 40 cycles of denaturation at 95°C for 5 sec, renaturation at 57°C for 20 sec, and elongation at 72°C for 15 sec, after the initial denaturation at 94°C for 30 sec. Fluorescence readings in a channel for SYBR Green I were taken throughout the experiments.

After real-time PCR, melting experiment was performed from 60 to 95°C at 0.2°C/sec with smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature (*Tm*) as described previously [2]. The *Tm* was defined as a peak of the curve, and if the highest point was a plateau, then the mid-point was identified as the *Tm*. The input amount of DNA, the copy number of the target as well as presence of co-infections with several targets did not influence the *Tm*. Out of the 147 blood samples tested by real-time PCR, 13 (9%) were found to be positive for hemplasma infection. The *Tm* of the positive samples was 82.79 ± 0.18°C.

The positive samples were further subjected to conventional PCR to amplify entire region of the 16S rRNA gene. The conventional PCR was carried out with 50-μl reaction mixtures containing 1 μl of DNA solution, 0.5 m*l* of TaKaRa LA *Taq* ™ (5 units/μl), 5 μl of 10X LA PCR™ Buffer II, 8 μl of 25 mM $MgCl_2$ (final 4.0 mM), 8 μl of dNTP mixture (2.5 mM each), 0.2 μl of forward primer (5'-AGAGTTTGATCCTGGCTCAG-3', equivalent to nucleotide numbers 11 to 30 of *M. wenyonii* (AY946266), or 5'-ATATTCCTACGGGAAGCAGC-3', equivalent to nucleotide numbers 328 to 347 of *M. wenyonii*), reverse primer (5'-ACCGCAGCTGCTGGCACATA-3', equivalent to

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nucleotide numbers 503 to 522 of *M. wenyonii*, or 5'-TAC-CTTGTTACGACTTAACT-3', equivalent to nucleotide numbers 1446 to 1465 of *M. wenyonii*) (50 pmol/μl each) and water to a final volume of 50 μl. After the mixture was overlaid with 20 μl of mineral oil, the reaction cycle was carried out 35 times with denaturation at 94°C for 30 sec, annealing at 58°C for 120 sec and extension at 72°C for 60 sec in a thermal cycler. Conventional PCR products that showed a clearly visible band were subjected to direct sequencing and the nucleotide sequences of the almost entire region of the 16S rRNA gene have been deposited to the DNA database under the accession numbers AB558897 to AB558899. The remaining 10 samples are still examined for determination of the nucleotide sequences.

The 16S rRNA gene sequences from the sika deer isolates were aligned with other hemoplasma sequences from DNA database using Clustal W [12]. An unrooted phylogenetic tree was generated with the neighbor-joining method [10] from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model [4]. The dataset was resampled 1,000 times to generate bootstrap values (Fig. 1). 16S rRNA gene sequences of the two strains, Sika99 and Sika122, from the blood samples of sika deer showed 100% homology each other, but were distinct from the sequence of Sika152. The 16S rRNA gene sequences are widely used in microbiology for assigning uncultivable microorganisms as new species, and reclassification of haemotropic *Mycoplasma* species has been based on the16S rRNA gene sequences [7, 8]. In our examinations, hemoplasmas were divided into two genetic groups, haemofelis and wenyonii clusters, and the hemoplasmas detected in sika deer were both assigned into the wenyonii cluster.

This is the 1st demonstration of the existence of 2 genetically distinct hemoplasma species in sika deer. Although Sika99 and Sika122 showed close relatedness to '*Candidatus* M. haemotaradirangiferis' detected from reindeer [12], similarity of the 16S rRNA gene sequences between them was 95.7%, suggesting different species [1]. Thus, we ten-

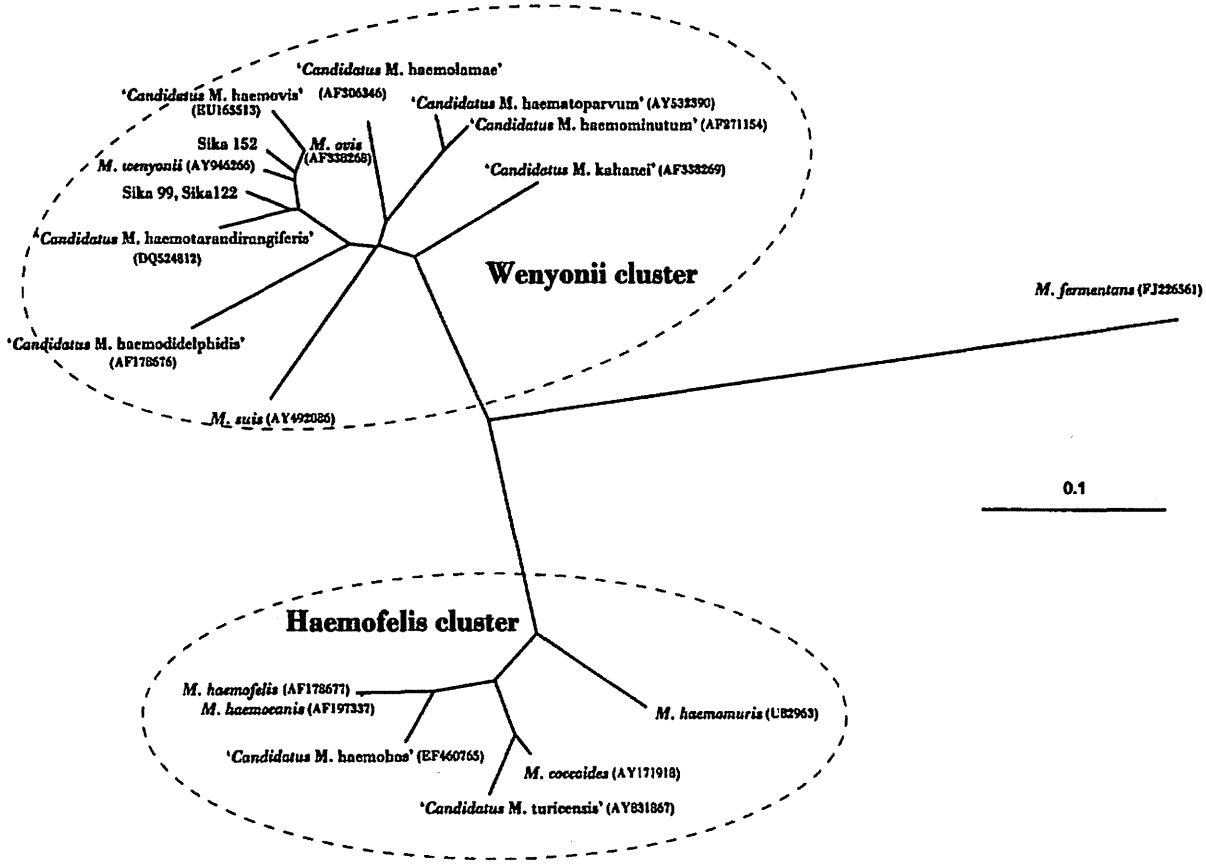

Fig. 1. An unrooted phylogenetic tree based on the 16S rRNA gene sequence comparison among mycoplasmas including 16 hemoplasna species (accession numbers are given in a parenthesis) and putative taxa created by Sika99, Sika122 and Sika152. Genetic distances were computed with CLUSTAL W [14]. Hemoplasmas were divided into two genetic clusters, haemofelis and wenyonii. A nucleotide sequence of the 16S rRNA gene of *M. fermentans* PG18 strain with accession number FJ226561 was included as an out-group. Sika99, Sika122 and Sika152 represent hemoplasma strains detected from the sika deer in the Iwate prefecture. Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications. Scale bar indicates the estimated evolutionary distance.

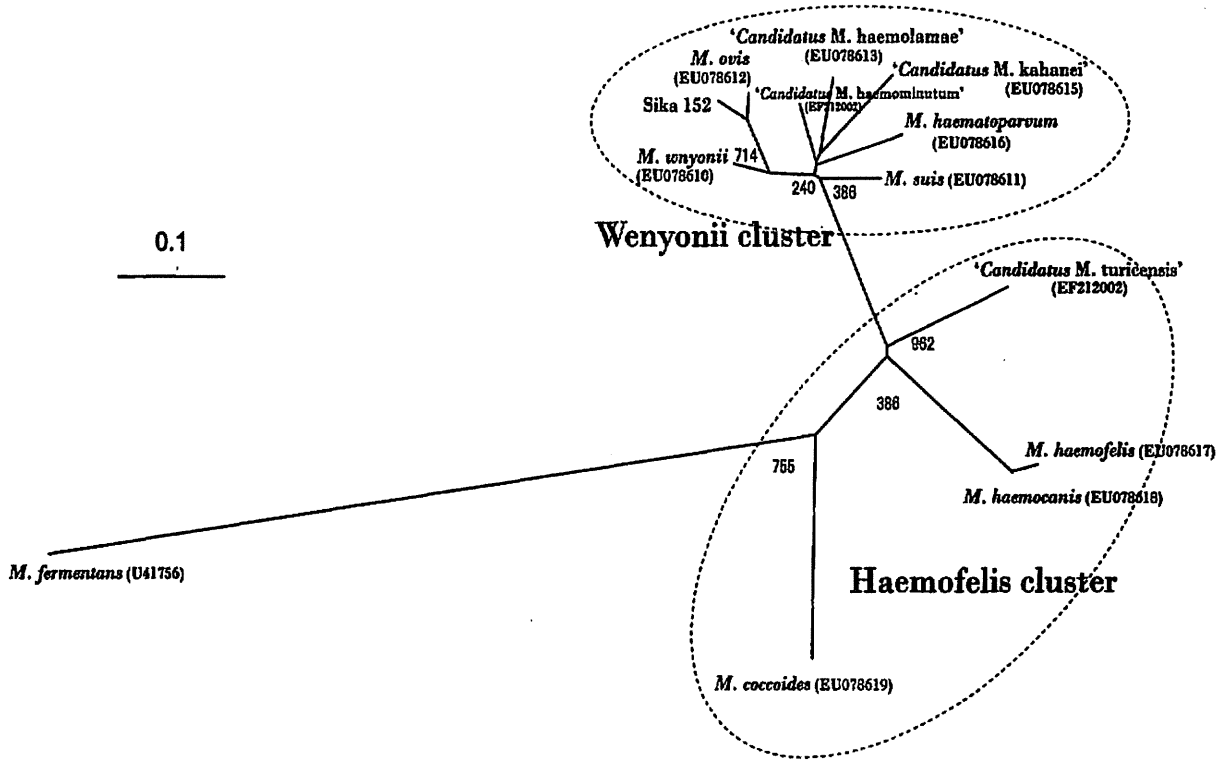

Fig. 2. An unrooted phylogenetic tree generated by comparison of the RNase P RNA (*rnpB*) gene sequences among mycoplasmas including 11 established species (accession numbers are given in a parenthesis) and a putative taxon created by Sika152. Genetic distances were computed with CLUSTAL W [14]. Two genetic clusters, haemofelis and wenyonii, were also revealed in this tree. A nucleotide sequence of the *rnpB* gene of *M. fermentans* with accession number U41756 was included as an out-group.Sika152 represents a hemoplasma sequence detected from the sika deer in the Iwate prefecture. Numbers in the relevant branches refer to the values of bootstrap probability of 1,000 replications. Scale bar indicates the estimated evolutionary distance.

tatively designated '*Candidatus* M. erythrocervae' for Sika99 and Sika122. On the other hand, Sika152 showed close relatedness to *M. ovis*, and sequence similarity between them was 98.4%, which may not be enough for establishment of a new species according to the cutoff value of the 16S rRNA gene sequence identity for species definition [1]. *M. ovis* is also close to '*Candidatus* M. haemovis', and their similarity is 97.9% [3]. Thus, we further examined the nucleotide sequence of the RNase P RNA (*rnpB*) gene of Sika152 to compare with other hemoplasma species [10]. The *rnpB* gene has been shown to be suitable for phylogenetic discrimination of closely related taxonomic groups when examined by 16S rRNA sequence comparison [13]. Partial nucleotide sequence of the *rnpB* gene of Sika152 was amplified by conventional PCR with forward primer rnpB-F (5'-AGTCTGAGATGACTRTAGTG-3' equivalent to nucleotide numbers 1 to 20 of *M. wenyonii* (EU078610)) and reverse primer rnpB-R (5'-TRCTTGMGGGGTTTGC-CTCG-3' equivalent to nucleotide numbers 170 to 189 of *M. wenyonii* (EU078610)). Reaction was the same as used for the amplification of the 16S rRNA gene except for the annealing temperature at 60°C instead of 58°C. Amplified PCR product was subjected to direct sequencing and the nucleotide sequence of the *rnpB* gene of Sika152 has been deposited to the DNA database under the accession number AB561882. The *rnpB* gene sequence of Sika152 was aligned with other hemoplasma sequences from DNA databases using Clustal W [14]. A phylogenetic tree was constructed with the neighbor-joining method [11] from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model [4]. The dataset was resampled 1,000 times to generate bootstrap values (Fig. 2). The *rnpB* gene sequence of Sika152 showed close relationship to *M. ovis* (90% similarity) as well as *M. wenyonii* (84%similarity), which was compatible with the results from the 16S rRNA gene comparison, and supported the notion that the Sika152 was distinct from other hemoplasma species. Similarly, *M. haemocanis* and *M. haemofelis* have been recognized as a distinct species due to difference of the *rnpB* gene, though they showed 99.2% homology in the 16S rRNA gene sequences. Therefore, we provisionally designated '*Candidatus* M. haemocervae' for Sika152.

In conclusion, we found 13 (9%) out of 147 blood samples collected from free-ranging sika deer in the Iwate prefecture were positive for hemoplasma infection by real-time PCR. We identified two hemoplasma species, '*Candidatus* Mycoplasma erythrocervae' and '*Candidatus* Mycoplasma haemocervae', which have not been described previously in sika deer population. Pathogenicity of these two putative species, both assigned in the wenyonii cluster, is currently unknown, since no clinical significance was apparent in the affected animals.

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CHARACTERIZATION OF *MYCOPLASMA* ISOLATED FROM AN IBEX (*CAPRA IBEX*) SUFFERING FROM KERATOCONJUNCTIVITIS IN NORTHERN ITALY

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ABSTRACT: In 2005 a *Mycoplasma* species was isolated from ocular-conjunctival swabs from an adult male Alpine ibex (*Capra ibex*) from the Valle d'Aosta Region, Northern Italy. The animal suffered from bilateral ocular discharge with diffuse inflammation, severe corneal involvement of the left eye and mild corneal opacity of the right eye. Histologic examination revealed a keratoconjunctivitis characterized by lymphocytic and plasmacellular infiltration. Laboratory investigations of the isolate included culture, transmission electron microscopy, PCR, and denaturing gradient gel electrophoresis, as well as DNA sequencing of the 16S rDNA gene. These tests identified the isolate as *Mycoplasma mycoides* subsp. *capri* large-colony serovar, an organism that has occasionally been associated with keratoconjunctivitis in goats. For a correct diagnosis, it is necessary to carry out laboratory investigations, as clinical cases of keratoconjunctivitis in wild ruminants are not always ascribable to *Mycoplasma conjunctivae*.

Key words: Characterization, denaturing gradient gel electrophoresis (DGGE), ibex (*Capra ibex ibex*), *Mycoplasma*, northern Italy, phylogenetic analysis.

INTRODUCTION

Many pathogens have been reported to cause eye disease in wild ruminants. Among the bacterial agents, *Mycoplasma conjunctivae* is probably the most known and studied. *Mycoplasma conjunctivae* causes infectious keratoconjunctivitis (IKC), a highly contagious ocular disease. This etiologic agent was first reported by Surman (1968) from an Australian sheep suffering from phlogosis of the ocular mucosa and was further identified and classified by Barile et al. (1972). Phylogenetically, *M. conjunctivae* belongs to the *Mycoplasma neurolyticum* cluster of the hominis group, and is closely related to *Mycoplasma bovoculi* and *Mycoplasma ovipneumoniae* (Pettersson et al., 1996). The disease, recently described in Europe (in the Alps and Pyrenees) and in New Zealand, affects domestic small ruminants and various wild ruminants, such as chamois (*Rupicapra rupicapra rupicapra*), ibex (*Capra ibex ibex*), and mouflon (*Ovis orientalis musimon*; Sarrazin et al., 1990; Mayer et al., 1996; Giacometti et al., 1998; Degiorgis et al., 2000). In sheep and goats the infection can cause blindness, but it is more often associated with mild symptoms or it can occur asymptomatically, possibly because of more favorable conditions such as good shelter and medication. In wild animals the clinical course is generally more serious and can include perforation of the cornea and blindness, with subsequent death of the animal from starvation or falling from cliffs. Diagnosis is based on the isolation of the causative agent in culture with subsequent immunologic identification, or by using molecular techniques such as PCR. Isolation in culture is difficult and requires enriched and selective media, whereas PCR is rapid, sensitive, and specific. The PCR/denaturing gradient gel electrophoresis (DGGE)

method offers significant advantages over standard PCR methods because it detects most animal *Mycoplasma* species including mixed species in a single reaction (McAuliffe et al., 2005).

In northern Italy, infections caused by *Mycoplasma* species have not been investigated in wild fauna. However, *M. conjunctivae* has been reported previously in wild animals in the Alpine regions of Italy (Grattarola et al, 1999). Genomic characterization has been performed on only six isolates from chamois from the provinces of Bergamo and Sondrio, Lombardia Region (Belloy et al., 2003). No eradication measures have been taken and no further investigations have been reported.

Other organisms can cause similar clinical disease, such as ocular *Chlamydophila* infection, *Staphylococcus* species, *Moraxella* species or members of the *Mycoplasma mycoides* cluster. The *M. mycoides* cluster includes *M. mycoides* subsp. *mycoides* small-colony (SC) type (*MmmSC*), *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*) and *Mycoplasma mycoides* subsp. *capri*, a single subspecies comprising two serovars which can only be distinguished by serological methods: *M. mycoides* subsp. *capri* (*Mmc*), and *M. mycoides* subsp. *capri* large-colony (LC) type (*MmcLC*), previously defined as *M. mycoides* subsp. *mycoides* large-colony (LC) type (Manso-Silvàn et al., 2009). *Mycoplasma mycoides* subsp. *capri* subspecies members (*Mmc* and *MmcLC*) and *M. capricolum* subsp. *capricolum* are associated with mastitis, arthritis, pleurisy, pneumonia, keratoconjunctivitis, and septicemia in small ruminants. *Mycoplasma mycoides* subsp. *capri* LC type is mostly confined to goats but has been isolated occasionally from sheep with balanoposthitis and vulvovaginitis (Trichard et al., 1993), and from cattle (Perreau and Bind, 1981), Vaal rhebok (*Pelea capreolus*; Nicolas et al., 2005), Spanish ibex (*Capra pyrenaica hispanica*; Gonzàlez-Candela et al. 2006), and wild goats (*Capra aegagrus cretica*; Perrin et al., 1994).

Several outbreaks of IKC have been reported in wild fauna, in particular chamois and ibex, in the Alpine regions (Mayer et al., 1996; Grattarola et al., 1999; Degiorgis et al., 2000). A regular and wide monitoring program was therefore activated in the northern Italian region of Valle d'Aosta under the guidance and supervision of the National Reference Center for Wild Animal Diseases (CeRMAS). In the framework of this survey, 86 carcasses of wild ruminants with ocular lesions were examined at CeRMAS from 2003 until 2006. Samples were collected from ibex, roe deer (*Capreolus capreolus*), and chamois and submitted to gross pathologic, histologic, and bacteriologic examinations.

We report the diagnostic examinations performed on an adult male ibex (number 130104/2005), which was euthanized in 2005 by forest rangers in Val Veny (Valle d'Aosta Region) due to severe blindness. We also report the subsequent isolation and characterization of *Mycoplasma* species.

MATERIALS AND METHODS

Anatomopathology

Gross pathology lesions were determined at postmortem examination and histologic procedures completed the anatomopathologic investigations. Ocular bulbs were fixed in 10% neutral buffered formalin for 48 hr. Samples from the cornea and conjunctiva were cut to 4 mm thickness, washed, dehydrated, cleared, and placed in paraffin using an automated system (Leica Microsystems Inc., Bannockburn, Illinois, USA). Samples were embedded in paraffin wax for 14 hr. After cooling at 4 C, the paraffin blocks were cut into 5-μm sections using a microtome. The sections were placed on slides, deparaffinized in Bioclear (Gene Linx International, Inc., Dublin, Ohio, USA), rehydrated in decreasing gradations of alcohol, washed, and stained with hematoxylin and eosin followed by dehydration in increasing concentrations of alcohol, cleared in Bioclear (Gene Linx International), mounted with a coverslip using Eukitt mounting medium (Kindler GmbH, Freiburg, Germany), and observed under an Olympus BX 60 light microscope (Olympus Europa GmbH, Hamburg, Germany).

Isolation

Individual conjunctival swabs were inoculated into pleuropneumonia-like organism (PPLO) broth prepared using a commercial base (Difco Laboratories, Detroit, Michigan, USA), enriched with yeast extract, yeast autolysate, equine serum, glucose, tryptose, thallium acetate, and penicillin. The inoculated broths were incubated at 37 C in microaerophilic conditions in 3.5-l jars with 10% CO_2 (CampyGen; Oxoid LTD, Basingstoke, Hampshire, UK). After 3 days of incubation, broths were filtered through a membrane with 0.45-µm pores (Seitz, Avondale, Pennsylvania, USA) and incubated again, streaking at the same time some drops onto PPLO agar (Difco), prepared as the broth and adding 3% agar. Pleuropneumonia-like organism plates were incubated for up to 8 days and monitored every second day for signs of *Mycoplasma* growth. Ocular swabs were also inoculated on blood agar and McConkey agar plates in parallel, in order to determine the presence of other bacterial agents causing eye pathology, such as *Staphylococcus* species and *Moraxella* species.

Electron microscopy

Pellets of bacterial colonies were prefixed in Karnowsky's fixative in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 3 hr at 4 C. After postfixation in 1% OsO_4 for 1.5 hr at 4 C, samples were washed in a corresponding buffer, dehydrated through an ethanol series, transferred to propylene oxide, and embedded in Epon 812 resin. Thin sections (80 nm) were stained with saturated uranyl acetate, followed by Reynolds lead citrate and examined under a Zeiss EM900 (Carl Zeiss AG, Göttingen, Germany) and Philips CM 10 (Philips Research, Eindhoven, Netherlands) transmission electron microscopes at 80 kV.

Polymerase chain reaction

DNA was extracted from a 1-ml aliquot of stationary-phase culture using the Genelute genomic DNA kit according to the manufacturer's instructions (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK). *Mycoplasma conjunctivae* detection was performed by a specific PCR (Baker et al., 2001). The amplicons were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet illumination.

Polymerase chain reaction denaturing gradient gel electrophoresis (DGGE)

The bacterial strains *M. ovipneumoniae*, *M. conjunctivae* (from Arrhus collection), *M. conjunctivae* (NCTC 10147), *Mycoplasma arginini*, *Mycoplasma agalactiae*, *M. mycoides* subsp. *capri* (formerly LC), and *MmmSC* were used as controls for the PCR/DGGE method with the test strain 283F08. In addition to the type strains used in this study, a number of field strains were tested using DGGE to ensure that there was intraspecific stability of DGGE profiles. Briefly, *Mycoplasma* DNA was extracted as stated above for the PCR. The PCR and DGGE were performed as described by McAuliffe et al. (2005).

Analysis of the nucleotide sequences

In order to obtain virtually complete 16S rDNA gene sequence a fragment was PCR-amplified from extracted DNA using the primer pair 5′ AGTTTGATCCTGGCTCAG 3′ and 5′ ACCTTGTTACGACTT 3′. Sequencing was undertaken by standard approaches using a series of internal sequencing primers and an ABI PRISM 310xl genetic analyzer (Perkin-Elmer Corporation, Norwalk, Connecticut, USA) to obtain a 1,445-bp sequence contig. The 16S rDNA nucleotide sequence of the ibex strain 283F08, first presented in this paper, has been deposited in the DNA Data Bank of Japan, European Molecular Biology Laboratory and GenBank nucleotide sequence databases under accession number GQ409970. A phylogenetic tree based on the 16S rDNA was constructed from sequence alignment with ClustalX (Thompson et al., 1997) using the neighbor-joining method (Saitou and Nei, 1987).

RESULTS

From the monitoring program for IKC undertaken by CeRMAS in the northern Italian region of Valle d'Aosta, several cases of clinical disease have been reported during the 3-yr survey. All cases were submitted for gross pathologic, histologic, and bacteriologic examination. A confirmed diagnosis of *Mycoplasma* species was only successful in one of the 86 samples tested.

At necropsy of the adult male ibex (*Capra ibex*) (CeRMAS register 130104/2005), we observed bilateral ocular discharge with diffuse inflammation, severe corneal involvement at the left eye (Fig. 1) and mild corneal opacity at the right eye. Histologic evaluation revealed different

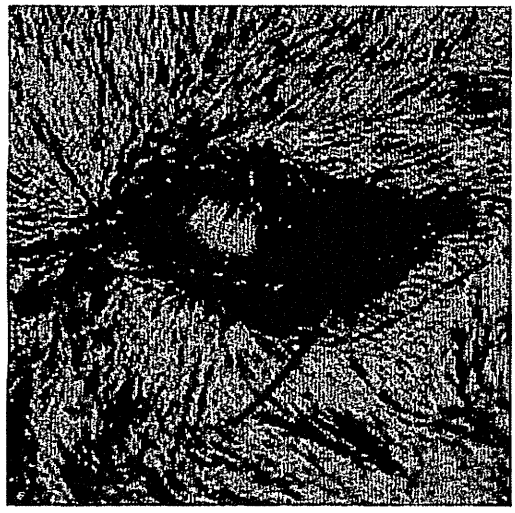

FIGURE 1. Severe corneal involvement in the left eye in an adult male ibex (*Capra ibex*) recorded under number 130104/2005 at the National Reference Center for Wild Animal Diseases, Aosta, Italy. The animal was euthanized by forest rangers in Val Veny (Valle d'Aosta Region) due to severe blindness.

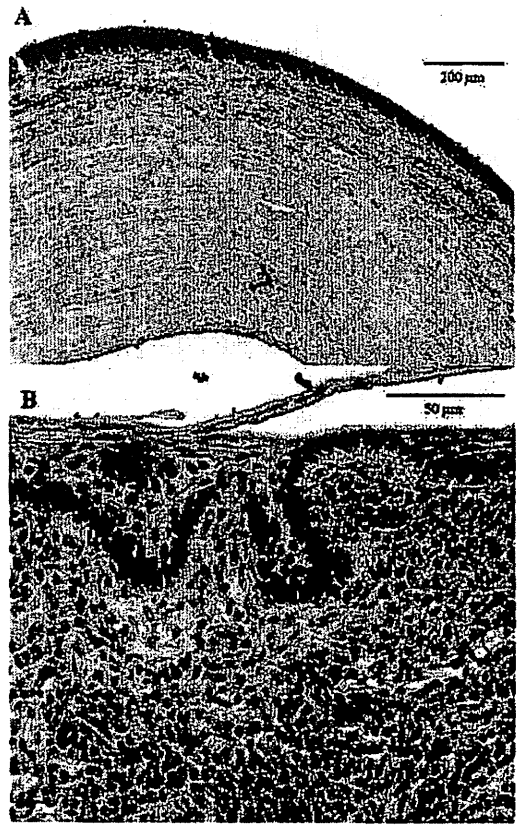

FIGURE 2. Histologic lesions associated with strain 283F08 (130104/2005, ibex isolate). Keratoconjunctivitis characterized by lymphocytic and plasmacellular infiltration: cornea (A), conjunctiva (B). H&E stain.

stages of the ocular lesions on the cornea and conjunctiva (Fig. 2). The keratoconjunctive flogosis was characterized by lymphocytic and plasma cellular infiltration, which was particularly intense at the level of the irido-corneal angle, and associated with small areas of corneal ulceration with neutrophilic invasion.

Bacteriologic examination on selective PPLO agar showed growth of small "fried egg"–shaping colonies, ascribable to *Mycoplasma* sp. Colonies showed slightly darker granulations, which varied depending on culture conditions (e.g., change in the substrate or percentage of CO_2). After 8 days growth the fully developed colonies were 1 mm in diameter. No other bacterial agents were isolated on blood agar or McConkey agar plates.

Transmission electron microscope micrographs of strain 283F08 (130104/2005, ibex isolate) showed the undefined morphology of the bacterium. The cells were round, 0.6–0.9 µm, and showed electron-dense aggregations in the cytoplasm. At higher magnification, the cytoplasm appeared limited by a single triple-layered unit membrane. This ultrastructure of bacterial bodies corresponded to the typical architecture of *Mollicutes*, characterized by lack of a cell wall. Fine granulations were evident on the cell surface.

A *M. conjunctivae*–specific PCR was performed on the ibex isolate. The test was negative and no evidence of the presence of *M. conjunctivae* was found in this sample.

Using the 16S rDNA PCR and separation of the PCR amplicon by DGGE, strain 283F08 (130104/2005, ibex isolate) was positive for *MmcLC* serovar (Fig. 3). The ibex isolate did not correspond to any of the other *Mycoplasma* profiles used as controls in the test. The diagnosis as *MmcLC* by PCR/DGGE was confirmed by sequence

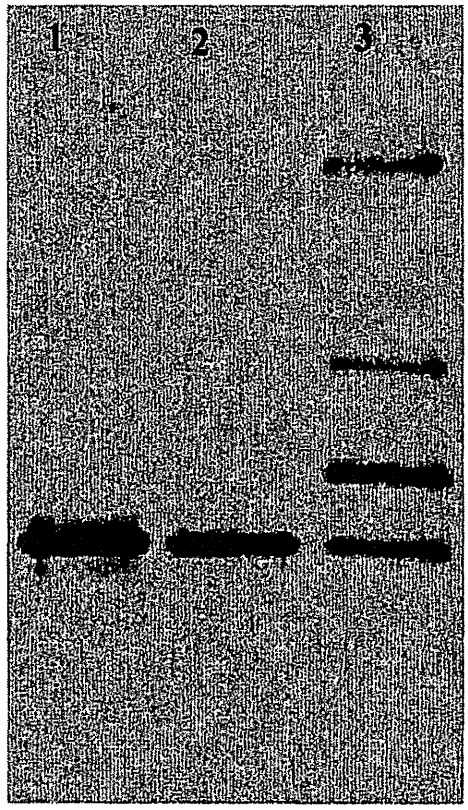

FIGURE 3. Determination of *Mycoplasma* species based on PCR of the 16S rDNA gene with *Mycoplasma*-specific primers and separation of the PCR product using denaturing gradient gel electrophoresis: Lane 1, strain 283F08 (130104/2005, ibex isolate); lane 2, *Mycoplasma mycoides* subsp. *capri* large-colony type; lane 3, *Mycoplasma mycoides* subsp. *mycoides* small-colony type.

analyses. Using a BLAST search (GenBank), the test sequence matched the *MmcLC* 16S ribosomal DNA gene sequence (accession number EU040177) showing nucleotide identities of 99%. The construction of phylogenetic trees based on the 16S genomic region comparison, using neighbor-joining, suggested a taxonomic position of the ibex isolate, strain 283F08 in the *M. mycoides* cluster, *MmcLC* serovar (Fig. 4).

DISCUSSION

Mycoplasma species isolation was only successful in one of the 86 samples tested. In this case the animal was euthanized and promptly submitted for laboratory analyses. In general, histologic examination is important to exclude postmortem degenerations, which may confuse anatomopathologic findings. In particular, slight corneal opacity may develop rapidly following death. This may suggest pathologic events that when evaluated microscopically will not match the degenerative lesions with the inflammatory processes. An exhaustive description of the microscopic lesions observed at different stages of IKC in Alpine ibex has been reported by Mayer et al. (1997). Based on macroscopic and histologic findings, eye lesions were classified into stages I to IV (from mild to severe). According to this classification, the lesions observed in the present case corresponded to stage II.

Mycoplasma diseases may not be diagnosed solely on the basis of clinical signs, pathologic lesions, or serologic tests because of the close relatedness between *Mycoplasma* species. Identification of the causative organism is therefore required to confirm diagnosis. However this requires specialized laboratorians with experience with these fastidious organisms.

Isolation and cultivation of a *Mycoplasma* strain is laborious and not always successful, possibly due to the stage of infection, type of sample, and length of time between sampling and testing. In this study only one animal was culture-positive for *Mycoplasma* despite the report of several suspected clinical cases of IKC. The use of molecular tests directly on clinical specimens may improve the sensitivity of testing as the tests are not reliant on containing viable bacteria. However, the use of specific PCRs, such as the *M. conjunctivae* PCR and the *Mycoplasma "mycoides* cluster" and *Mycoplasma "mycoides* subcluster" PCRs coupled with restriction fragment length polymorphisms (Bashiruddin et al., 1994) will only detect and identify the specific *Mycoplasma* species the investigator is targeting and requires the use of several different tests. The recently introduced PCR/DGGE

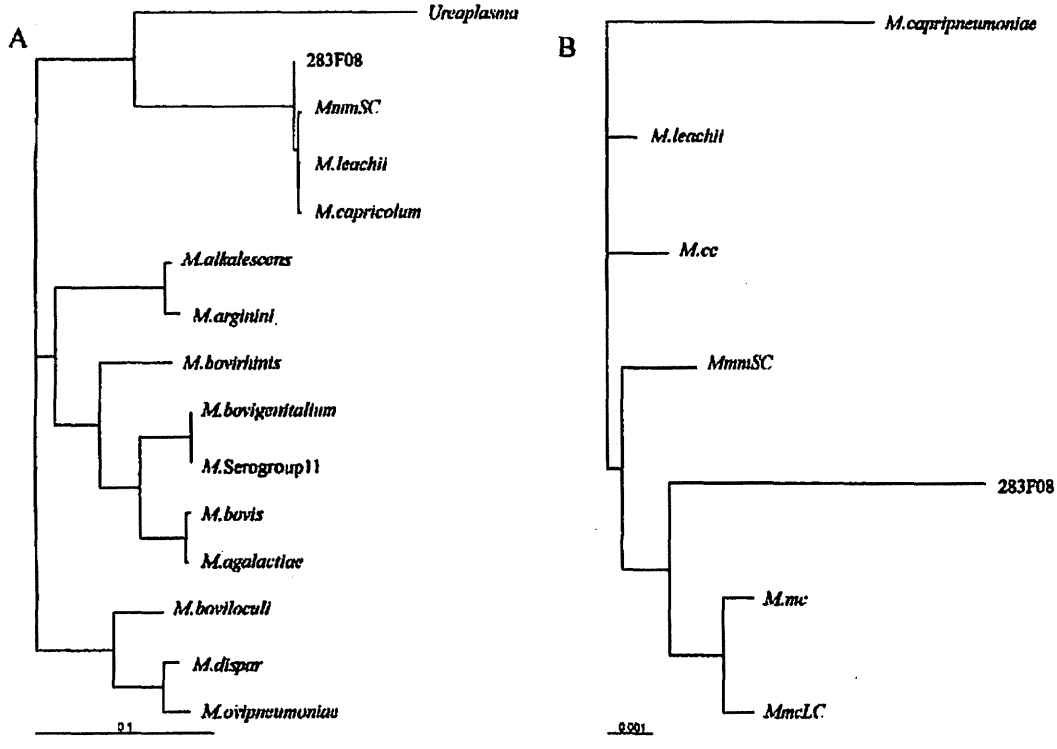

FIGURE 4. Phylogenetic trees based on the 16S genomic region comparison, suggesting a taxonomic position of the ibex isolate 283F08 (accession number GQ409970) in the *Mycoplasma mycoides* cluster. Comparison with nucleotide sequences of the reference *Mycoplasma* strains of bovid ruminants (A), and with *Mycoplasma* strains of the *Mycoplasma mycoides* cluster (B). The nucleotide sequences of 16SrDNA regions were obtained from the published data for reference *Mycoplasma* strains. Nucleotide sequences of the reference *Mycoplasma* strain of bovid ruminants, obtained from the DNA databases, are as follows [accession number is given in square brackets]: *Mycoplasma bovigenitalium* strain PG11 [AY121098], *Mycoplasma bovirhinis* PG43 [U44766], *Mycoplasma bovoculi* M165/69 [U44768], *Mycoplasma bovis*, strain Donetta [U44767], *Mycoplasma dispar* 462/2 (T) [AF412979], *Mycoplasma ovipneumoniae* strain Y-98 [U44771], *Mycoplasma alkalescens* PG51 [U44764], *Mycoplasma arginini* strain G230 (T) [AF125581], *Mycoplasma agalactiae* strain 847.121 [AF332744], *Mycoplasma* sp. ovine/caprine serogroup 11 isolate 126SR99 [AY121100], and *Ureaplasma diversum* A417 [D78650]. Accession numbers of the *Mycoplasma* strains of the *Mycoplasma mycoides* cluster are as follows: *Mycoplasma mycoides* subsp. *mycoides* small-colony type, strain PG1 [U26039], *Mycoplasma mycoides* subsp. *capri* large-colony (LC) type, strain Y-goat [U26044], *Mycoplasma mycoides* subsp. *capri* PG3 [U26037], *Mycoplasma capricolum* subsp. *capripneumoniae* 4/2LC [U26052], *Mycoplasma capricolum* subsp. *capricolum* G5 [U26041], and *Mycoplasma* sp. Group 7, strain PG50 [U26054]. Distances were computed with ClustalX using the neighbor-joining method. Numbers at branches refer to bootstrap values of 1,000 replications. Scale bar indicates 10 nucleotide substitutions per 100 nucleotides.

method (McAuliffe et al., 2005) is a sensitive single generic test capable of detecting and differentiating mycoplasmas to the species level. By DGGE, the ibex isolate was observed to be a *Mmc* serovar. The use of PCR/DGGE represents a significant improvement on current tests as diagnosis of *Mycoplasma* infection can be made directly from clinical samples in less than 24 hr and mixed *Mycoplasma* infections can also be detected and identified in this single test. The use of molecular testing has limitations, in that sometimes it is essential to obtain live organisms for research and other purposes, such as antibiotic sensitivity testing.

In this study, the growth in microaerophilic condition, on selective PPLO agar, of typical small "fried egg"–shaped colo-