2× 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*. Since nucleotide sequences and sizes bracketed by the primers are specific to species, melting curve analysis of the amplified products may serve as a differential marker for hemoplasma speciation. Six ovine blood samples, Hitsuji1, Hitsuji5, Hitsuji6, Hitsuji7, Hitsuji8 and Hitsuji9, were positive in the real-time PCR. The *Tm* (mean ± SE.) values of the PCR products from these six ovine hemoplasma were estimated to 83.60 ± 0.09°C (Fig. 2).

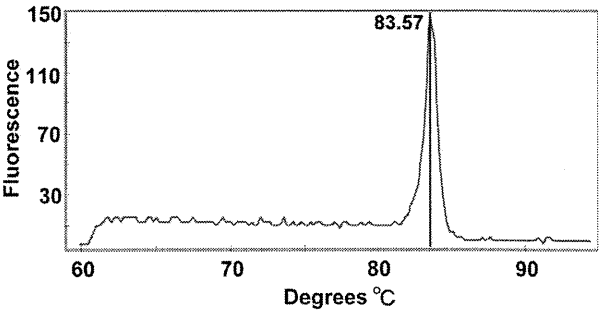

Fig. 2. Thermal melting curve of the PCR products depicted by using SYBR Green I. A characteristic melting temperature at 83.57°C was evident in '*Candidatus* M. haemovis' infection.

The positive samples were further examined by conventional PCR targeting the 16S rRNA gene for nucleotide sequencing. The conventional PCR was carried out with 50-

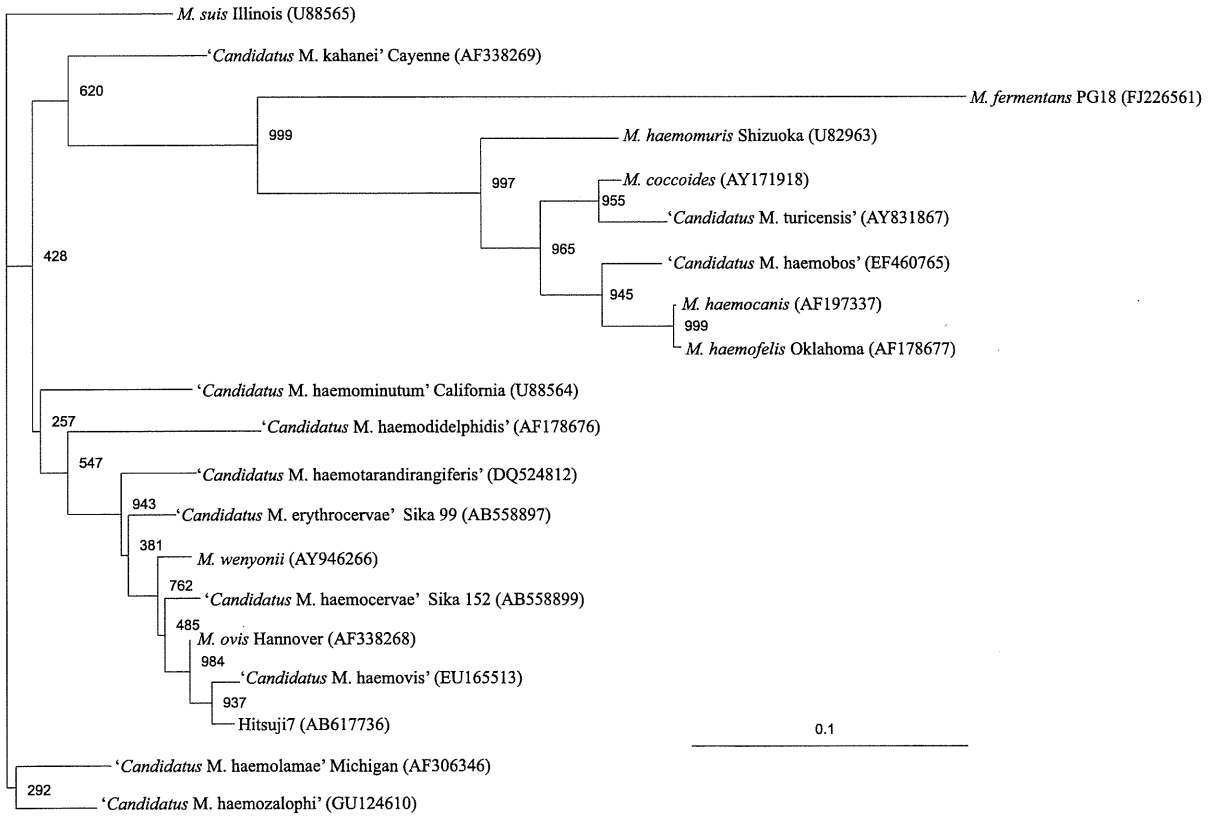

Fig. 3. A phylogenetic tree based on the 16S rRNA gene sequence comparison among mycoplasmas including 18 hemoplasma species (accession numbers are given in a parenthesis). Genetic distances were computed with CLUSTAL W [9]. Ovine strain Hitsuji7 representing Hitsuji1, Hitsuji5, Hitsuji6, Hitsuji8 and Hitsuji9 was included in the '*Candidatus* M. haemovis' clade. A nucleotide sequence of the 16S rRNA gene of *M. fermentans* PG18 strain with accession number FJ226561 was included as an out-group. Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications. Scale bar indicates the estimated evolutionary distance.

μl reaction mixtures containing 1 μl of DNA solution, 0.5 μl 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 (50 pmol/μl) of forward primers Ana-F1 (5'-GAGTTTGATCCTGGCTCAGG-3') or Hemo-F10 (5'- ATATTCCTACGGGAAGC AGC-3'), 0.2 μl (50 pmol/μl) of reverse primers Hemo-R11 or Hemo-R2 (5'-TACCTTGTTACGACTTAACT-3') 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 for 30 sec, annealing at 60 for 120 sec and extension at 72 for 60 sec in a thermal cycler.

The PCR products were fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts, Rockland, ME., U.S.A.) in TAE (40 mM Tris, pH8.0, 5 mM sodium acetate, 1 mM disodium ethylenediaminetetracetate) buffer at 50 volts for 60 min. After electrophoresis, the gels were stained in ethidium bromide solution (0.4 μg/m*l*) for 15 min and visualized under UV transilluminator. DNA was extracted by using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). In our experiments, results between the real-time PCR and conventional PCR were always consistent. The nucleotide sequences of the partial 16S rRNA gene have been deposited in the DDBJ, EMBL, GSDB and NCBI nucleotide sequence databases under the accession numbers AB617733 to AB617738. A single nucleotide substitution was apparent among these six sequences, suggesting a same lineage.

Nucleotide sequences of the 16S rRNA gene from the ovine strain along with the 19 established mycoplasma species were aligned using CLUSTAL W (version 1.83; DDBJ, Mishima, Japan) with further adjustment made manually by eyes as necessary [9]. A phylogenetic tree constructed by the algorithms implemented in the PHYLIP program (DDBJ, Japan) using the neighbor-joining method [7] indicated that the hemoplasma strain detected in sheep was classified as '*Candidatus* Mycoplasma haemovis' (Fig. 3). Besides, the nucleotide sequence '*Candidatus* M. haemovis' detected in the present study were most closely to *Mycoplasma* sp. TX1294-A (accession number GU230141) and TX1294-E (accession number GU383116) strains both detected from humans [8], suggesting an anthropozoonotic pathogen.

'*Candidatus* M. haemovis' was first demonstrated from a sheep flock with fatal hemolytic anemia in Hungary [3]. Prevalence of this hemoplasma species is currently unknown, because there has been no report on this particular species in other countries though *M. ovis* is prevalent throughout the world [1, 6]. We demonstrated this hemoplasma species in anemic sheep in Japan. Variations in the hematological values might be attributed to infection stages rather than differences in the hemoplasma strains, which were almost identical in the 16S rRNA sequences. It is most likely that the animals had been infected in the commercial farm, since the affected sheep in the present study was the only flock in the experimental farm of the Iwate University.

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ORIGINAL ARTICLE

Acidic environments induce differentiation of *Proteus mirabilis* into swarmer morphotypes

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ABSTRACT

Although swarmer morphotypes of *Proteus mirabilis* have long been considered to result from surfaced-induced differentiation, the present findings show that, in broth medium containing urea, acidic conditions transform some swimmer cells into elongated swarmer cells. This study has also demonstrates that *P. mirabilis* cells grown in acidic broth medium containing urea enhance virulence factors such as flagella production and cytotoxicity to human bladder carcinoma cell line T24, though no significant difference in urease activity under different pH conditions was found. Since there is little published data on the behavior of *P. mirabilis* at various hydrogen-ion concentrations, the present study may clarify aspects of cellular differentiation of *P. mirabilis* in patients at risk of struvite formation due to infection with urease-producing bacteria, as well as in some animals with acidic or alkaline urine.

Key words cytotoxicity, hydrogen-ion, *Proteus mirabilis*, swarmer cell.

Proteus species, Gram-negative bacteria in the family *Enterobacteriaceae*, cause urinary tract infections. Affected patients with long-term urinary catheters in place or structural abnormalities of the urinary tract have an increased risk of struvite and carbonate apatite formation, due to increase in their urinary pH and direct damage to the uroepithelium by ammonia (1–3). *Proteus* infections are frequently persistent and difficult to treat and can lead to complications such as acute or chronic pyelonephritis. Additionally, *Proteus* species are the most common bacilli associated with the formation of bacteria-induced bladder and kidney stones (about 70% of all bacteria isolated from such urinary calculi) (4, 5).

Proteus mirabilis may also form a bull's-eye pattern on an agar plate because it sometimes swarms over the entire surface of the agar medium. Differentiation into swarmer cells, which are characterized by a 10 to 40-fold increase in cell length, a drastic increase in the number of flagella and production of higher amounts of specific virulence factors, is considered to be closely related to establishment of infections (6, 7). Swarmer cell differentiation is initiated upon contact with a solid surface, by inhibition of flagellar rotation, or by cell–cell signaling (8–11), but *P. mirabilis* is usually unable to differentiate into swarmer cells on minimal media (12). Jones *et al.*, however, have reported that biofilms in artificial urine (pH 6.5) produce greater numbers of swarmer cell populations than those observed in Luria broth, and suggested that swarmer cells have an important role in urinary tract biofilms (13). In this study, we found that broth cultures containing urea under acidic conditions induced a few swarmer morphotypes of *P. mirabilis* L1N2.

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List of Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDH, lactate dehydrogenase; *P. mirabilis Proteus mirabilis*; SE, standard error; T5, 7 or 9, Trypto-soy broth pH 5, 7 or 9; UT5, 7 or 9, urea containing Trypto-soy broth pH 5, 7 or 9.

Little is known about the behavior of *P. mirabilis* in alkaline urine in patients infected with urease producing bacteria. Moreover, urinary pH varies among animal species. For example, carnivores void acidic urine (*Felis catus*: pH 5~7) but herbivores void alkaline urine (*Bos taurus*: pH 7~9). Although *P. mirabilis* is mainly a human uropathogen, it also infects the urinary tracts of carnivores and other omnivores. In contrast, infections of the urinary tract of herbivores are rare, though *P. mirabilis* has been isolated from the respiratory and genital tracts of cattle (14, 15). Therefore, in the present study, we examined the behavior of *P. mirabilis* L1N2 at several pHs of Trypto-soy broth with or without urea.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Proteus mirabilis strain L1N2, kindly supplied by Professor S. Igimi of the National Institute of Health Sciences in Tokyo, was grown in Trypto-soy broth or agar (Eiken Chemical, Tokyo, Japan). Trypto-soy broth was adjusted to pH 5.0, 7.0 or 9.0, supplemented with 25 g/L urea (designated UT5, UT7 or UT9, respectively) or without urea (T5, T7 or T9, respectively), and filter-sterilized. Because growth rates vary according to the pH conditions, bacterial numbers were adjusted by inoculating about 10^5, 10^2 or 10^3 bacterial cells into 1 mL broth at pH 5.0, 7.0 or 9.0, and incubating them at 37°C for 12 hr. For controls, swarmer cells were harvested with a platinum loop from the margins of colonies grown on Trypto-soy agar at 37°C and suspended in PBS. Bacterial cells were harvested from the bacterial suspension by centrifugation at 12,000 × *g* for 1 min and used for the following experiments.

Cell morphology

Bacterial cells collected by centrifugation were resuspended to a concentration of $10^{9\sim10}$/mL in PBS containing 1 μg/mL of FM1–43FX (Invitrogen, Eugene, OR, USA) and 2 μg/mL of DAPI (Dojindo, Kumamoto, Japan). The bacterial cells were examined with an Eclipse TE2000-U fluorescence microscope (Nikon, Tokyo, Japan) with mercury lamp. Fluorescent signals were observed by using the phase contrast objective UplanF1100X and a U-WIBA filter cube (an FM1–43 excitation filter for 460–490 nm and a barrier filter for 515–550 nm) or a U-NUA filter cube (a DAPI excitation filter for 360–370 nm and a barrier filter for 420–460 nm). Images were captured and submerged by using a VB-7010 digital camera (Keyence, Osaka, Japan). Bacterial cells longer than 10 μm were defined as swarmer morphotypes (16).

Bacterial flagella were observed with an H-800 transmission electron microscope (Hitachi, Tokyo, Japan). Aliquots of bacterial suspension fixed with 10% neutral-buffered formalin, were placed on a formvar-coated grid and negatively stained with 1% uranyl acetate.

Urease activity

Bacterial cells washed twice with 50 mM HEPES buffer (pH 7.5) and sonicated on ice to release soluble proteins served as a crude urease preparation. Following centrifugation at 12,000 × *g* for 2 min, the supernatant fluids were placed on ice. The concentrations of the crude enzyme extracts were determined by Coomassie brilliant blue protein assay solution (Nacalai, Kyoto, Japan), according to the manufacturer's protocol. BSA was used as a standard.

The urease activity of the extracts was estimated by measuring the amount of ammonia molecules released from urea in a phenol-hypochlorite assay (17). Briefly, the extracts were added to the urease buffer (50 mM HEPES buffer, pH 7.5, plus 25 mM urea) in a 330 μL final volume and incubated at 37°C for 20 min. The reaction was stopped by adding to a cuvette with 500 μL of solution A (containing 10 g of phenol and 50 mg of sodium nitroprusside per liter). An equal volume (500 μL) of solution B consisting of 5 mg/mL NaOH and 0.044% (v/v) NaClO was added and mixed well. Following incubation at 37°C for 30 min, the absorbance at 625 nm was measured in a Bio-Spec-mini spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). Ammonium chloride was used as a standard to convert the absorbance at 625 nm to nanomoles of ammonia. The specific activity of urease was defined as mm of ammonia produced per min per mg of protein. Values were expressed as the mean ± SE of four to five independent cultures in triplicate assays.

Cytotoxicity assay

A transitional human bladder carcinoma cell line T24 obtained from the Japanese Collection of Research Bioresources (Osaka, Japan) was routinely maintained in Dulbecco's modified Eagle medium (Nissui, Tokyo, Japan) supplemented with 10% FBS (Invitrogen), 2 mM glutamine and penicillin/streptomycin (50 U/mL and 50 μg/mL) at 37°C in a humidified 5% carbon dioxide atmosphere. Cytotoxicity was examined by using the Cytotoxicity Detection Kit (Roche Applied Science, Indianapolis, IN, USA) which measures LDH release from the cytosol of damaged T24 cells into the supernatant fluids. Two controls were included for calculation of the cytotoxicity rate (%). Low controls consisted of supernatant fluids from T24 cells with no exposure to bacteria. High controls were from cells treated with lysis solution for 15 min. LDH activity was examined three times in a 96-wells plate, according to the manufacturer's protocol. Cultured cells were infected with about 1.0×10^6

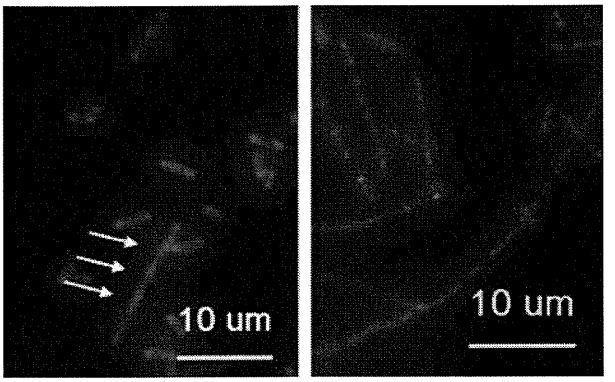

Fig. 1. DAPI and FM1-43FX staining of bacterial cells grown in UT5 (left hand photo)and on agar plates (right hand photo). These photos depict elongated multinuclear cells (arrows in UT5).

($0.7 \sim 1.5 \times 10^6$) of *P. mirabilis* grown in each medium for 30 min in incubation solution (HBSS- minimal medium-0.2M Tris buffer, pH 7.5, 80:10:10% v/v) as described by Peerbooms *et al.* (18). Cytotoxicity was expressed as the mean percentage ([experimental value–low control]/[high control–low control] $\times$ 100 $\pm$ SE) for three independent examinations.

Statistical analysis

All values are presented as the mean $\pm$ SE. Statistical analysis was performed by JMP software (SAS Institute, Cary, NC, USA) using one-way analysis of variance followed by the Turkey-Kramer test.

RESULTS

Cell morphology

The final pH values of broth media T5, T7, T9, UT5, UT7 and UT9 were 4.9, 6.0, 8.0, 8.7, 9.2 and 9.2, respectively. The percentage of swarmer morphotypes was 0.6% in T5, 0.2% in T7, less than 0.1% in T9, 1.8% in UT5, 0.8% in UT7 and 0.1% in UT9. Swarmer morphotypes appeared in acidic rather than alkaline broth, irrespective of the addition of urea. Elongated and multinucleated cells grown in broth media, which were morphologically similar to swarmer cells grown on agar plates, were stained with DAPI (Fig. 1). More than 70% of the *P. mirabilis* L1N2 cells on agar plates were longer than 10 μm. Two other *P. mirabilis* strains, NBRC 3849 and NBRC 105697, both purchased from the National Institute Technology and Evaluation (Osaka, Japan), behaved similarly, the incidence of swarmer morphotypes of these two strains in broth cultures without urea being less than 0.1%. The incidence of swarmer morphotypes of NBRC 3849 was 1.1, 0.3 and 0.1% in UT5, UT7 and UT9, respectively. Similarly, the incidence of swarmer morphotypes of NBRC105697 was 2.6, 1.2 and 0.4% in UT5, UT7 and UT9, respectively.

Electron microscopic photos of bacterial cells grown in each broth are shown in Figure 2. Elongated and hyperflagellated cells, as well as short swimmer cells expressing many flagella, were evident in UT5. Similarly, bacterial cells grown in T7, T9 and UT7 also expressed peritrichous flagella. The number of flagella on bacterial cells grown in T5 and UT9 was markedly decreased.

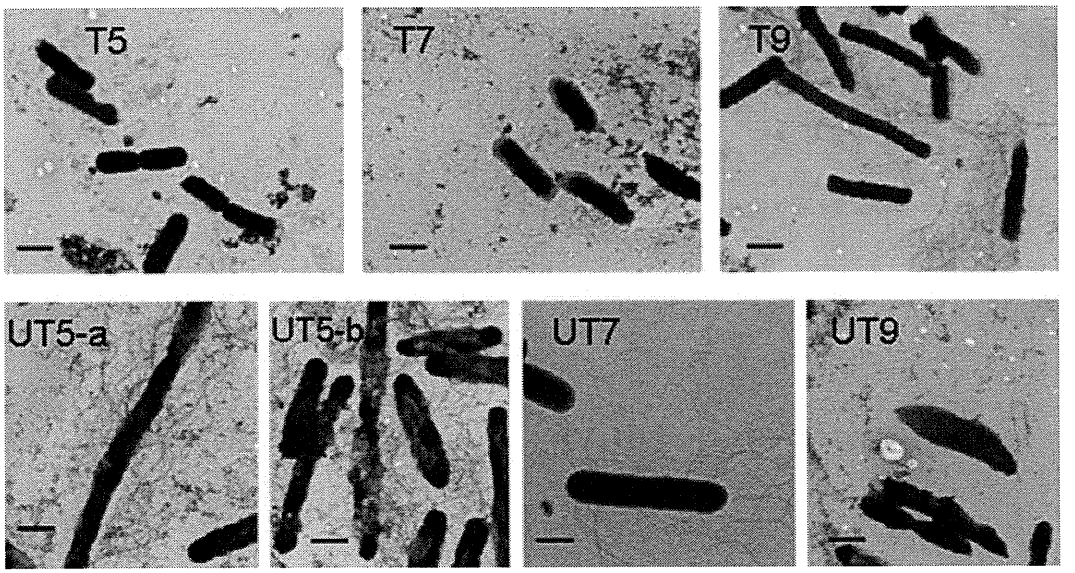

Fig. 2. Electron micrographs of bacterial cells grown in each broth. Many peritrichous flagella are visible on the elongated cells (UT5-a) and short rods (UT5-b) in UT5. The bacterial cells in T7, T9 and UT7 are also expressing peritrichous flagella, but those in T5 and UT9 show small number of flagella. Bar represents 1 μm.

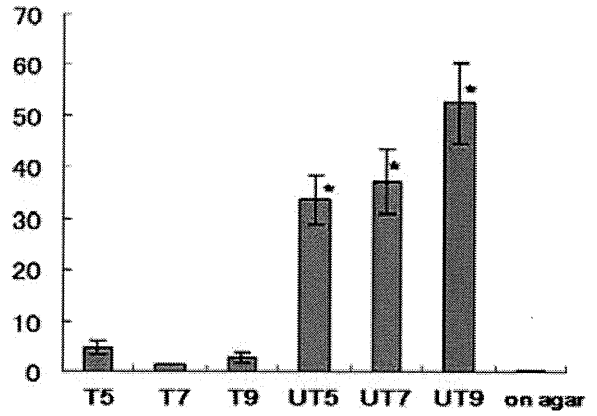

Fig. 3. Urease activities in each broth and on agar plates. The specific activity of urease was defined as μ mol of ammonia produced /min/mg of protein. Values are expressed as the mean ± SE for four to five independent cultures, with each assay made in triplicate. Asterisks shown above the columns indicate a significant difference from the cells grown in T5, T7, T9 and on agar plates ($P < 0.05$).

Urease activity

Urease activities for each broth and agar medium are shown in Figure 3. Urease activities in broth containing urea were around tenfold higher than in broth without urea, but no significant change was evident in broths of different pH. Urease activities (expressed as μmol of ammonia/min/mg of protein) in the broth media were 4.72 ± 1.47 (mean ± SE) in T5, 1.34 ± 0.13 in T7, 2.75 ± 1.20 in T9, 32.34 ± 5.94 in UT5, 33.45 ± 6.70 in UT7, 52.26 ± 10.11 in UT9. The urease activity in swarmer cells grown on agar plates was 0.26 ± 0.06.

Cytotoxicity

Results of cytotoxicity tests are shown in Figure 4. Swarmer cells on agar palates showed the highest cytotoxicity (90.56 ± 1.29% [mean ± SE]). Bacterial cells grown in UT5 showed 11.12 ± 0.17% cytotoxicity, whereas cells grown in the other broths showed less than 5% cytotoxicity (the high controls were taken as 100%).

DISCUSSION

Infections by urease-producing bacteria increase urinary pH due to hydrolysis of urea into carbon dioxide and ammonia. During the process of infection, the alkaline environment might increase the pathogenicity of *P. mirabilis*. For example, the optimum pH for subtilase-like protease activity in toxic agglutinin is 8.5–9.0, which is also optimal for expression of toxic agglutinin encoded by PMI2341 of *P. mirabilis* (19). However, in the present studies, 1.8% of cells of *P. mirabilis* L1N2 grown in UT5 differentiated into hyperflagellated swarmer morphotypes, thus enhancing cytotoxicity, though the inoculum size was changed because of the slow growth rate in UT5, and the pH was increased by urease activity after 12 hr of incubation. On the other hand, shorter rods appeared in UT9; these were less flagellated and had lower cytotoxicity. The cytotoxicity of the other strains, NBRC 3849 and NBRC 105697, in UT5 was also higher than in other broth media (data not shown). These results pose questions about the optimum pH for expression of other virulence factors and swarmer cell differentiation.

Differentiation into swarmer cells is considered to be linked to expression of virulence factors such as hemolysin and IgA metalloprotease (20, 21). Hemolysin activity is known to be responsible for the cytotoxicity of *P. mirabilis* (22). Urease activity, which we did not examine, is also increased during swarming on urea-containing agar plates (6). Further examination is necessary to confirm the virulence of swarmer cells and their temporal behavior *in vivo*, since swimmer morphotypes have predominantly been previously demonstrated in mouse models of ascending urinary-tract infection (16). Our results suggest that *P. mirabilis* cells differentiate into swarmer morphotypes at an early phase of infection when the host's urine is acidic, but they dedifferentiate into swimmer morphotypes when the urinary pH is increased.

Allison *et al.* have reported that the swarming phenomenon of *P. mirabilis* is responsible for the ability to invade into Vero cells and two human uroepithelial cell lines (22). They showed that swarmer cells invade within

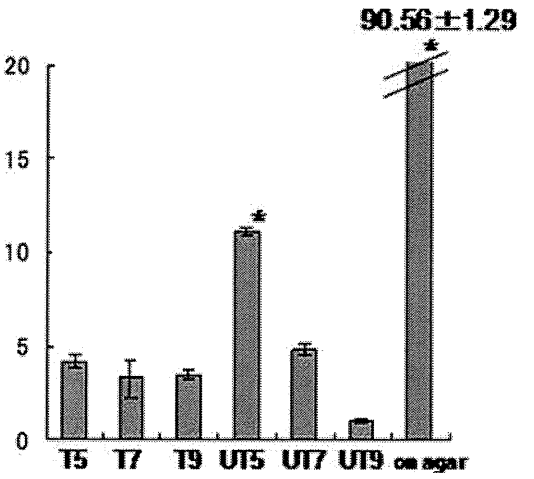

Fig. 4. Cytotoxicity (%) of *P. mirabilis* cells grown in each broth and on agar plates. LDH activities were determined in triplicate wells of a 96 well plate. High controls were used as a standard (100%) and values are expressed as the mean ± SE for three independent cultures, with each assay made in triplicate. Asterisks shown above the columns indicate a significant difference from the cells grown in other conditions. ($P < 0.05$)

30 mins, the invasion rate being about 15- to 20-fold greater after 2 hr as compared with invasion by swimmer cells, which are internalized more slowly (22). In their study, non-swarming mutants were 25-fold less invasive than wild-type cells. To the contrary, in our study invasion was seen within 30 min in all the broth conditions, though no significant differences were apparent among the broth conditions. The invasive rates of swarmer cells grown on agar plates were not determined because of their high cytotoxicity and detachment of T24 cells (data not shown).

In conclusion, 1.8% cells of *P. mirabilis* L1N2 differentiated into hyperflagellated and multinucleated swarmer morphotypes in acidic broth containing urea, and these cells exerted higher cytotoxicity against T24, though no significant differences were evident in urease activity among the different pH conditions. On the other hand, *P. mirabilis* grown in alkaline broth containing urea showed few elongated cells, and low levels of flagella production and cytotoxicity. Our study may help in the understanding of the cellular differentiation of *P. mirabilis* in the acidic urine of meat eating animals and in the alkaline urine of patients infected with urease producing bacteria or of grass eating animals.

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NOTE *Bacteriology*

Rapid Identification of Hemoplasma Species by Palindromic Nucleotide Substitutions at the GAAA Tetraloop Helix in the Specificity Domain of Ribonuclease P RNA

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ABSTRACT. We examined secondary structures of the ribonuclease P RNA sequences obtained from DNA databases, and identified a determinative prototype of the P12 helix peculiar to each species of hemoplasmas. This key structure will provide a rapid means for species identification of these uncultivable pathogens without making a phylogenetic tree based on alignments of nucleotide sequences. This procedure based on palindromic nucleotide substitutions at the stem portion of the P12 helix provide clear information such as the level of heterogeneity within a species, the relatedness between species, or facilitating the characterization and clustering of specific strains. In conclusion, the PNS analysis is based on the evaluation of only the strategic and highly conserved genomic region in the specificity domain of RNase P RNA.

KEY WORDS: GAAA tetraloop, hemoplasma, mycoplasma, ribonuclease P RNA.

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Hemoplasmas, hemotropic mycoplasmas, are causative of infectious anemia not only in animals but also probably in humans [14, 17]. Microbiological identification of hemoplasmas has been hampered by lack of an appropriate means to grow them *in vitro*. Thus the diagnosis of hemoplasma infections has largely been depending on cytological examinations and/or PCR. PCR is most preferably used since the cytological identification of the organisms in blood smear has a low diagnostic sensitivity and may instead detect Howell-Jolly bodies. Only two conserved nucleotide sequences, the 16S rRNA and ribonuclease (RNase) P RNA genes, are widely used in specific PCR diagnosis for hemoplasma infections, and hemoplasmas have been divided into haemominutum and haemofelis clusters based on phylogenetic analyses of these RNA sequences [12]. Although the 16S rRNA gene has long been used for identification and classification of prokaryotes, it is sometimes difficult to distinguish between closely related species, such as *Mycoplasma haemofelis* and *M. haemocanis* [11]. These 2 species have been distinct in the RNase P RNA sequence, though they have been grouped together consistently in phylogenetic analysis of the 16S rRNA sequence [1, 16]. In the present study we found a genetic marker for identification of hemoplasma species by an in-depth investigation of the secondary structure of the RNase P RNA molecules. This genetic marker allows rapid identification of hemoplasma species without aligning nucleotide sequences or making phyologenetic trees.

RNase P RNA, one of the first catalytic RNA molecules, is RNA moiety of RNase P that has been identified in all three domains of life, Bacteria, Eukarya and Archaea [7]. Bacterial RNase P RNA consists of two independently folding domains, a specificity domain and a catalytic domain [9]. The RNase P RNA of mycoplasmas has been assigned to class B architecture represented by *Bacillus subtilis*, including low G+C content Gram-negative bacteria [4]. We have examined the RNase P RNA genes of hemoplasmas and found a useful secondary structure in the specificity domain, that is peculiar to each hemoplasma species. Nucleotide sequences of RNase P RNA were obtained from the DNA databases and referred by accession numbers in the present study.

Prokaryotic RNase P RNA is a ribozyme that is responsible for processing the 5' end of tRNA by cleaving a precursor and leading to tRNA maturation. Several secondary structure modules in RNase P RNA molecules have been predicted from the phylogenetic comparison, which play the interaction with a defined structural motif [15]. The GAAA tetraloop at the top of P12 helix has high affinity for the motif called tetraloop receptor of 11 nucleotides in P10.1 helix of the RNase P RNA class B molecules [8, 10]. *M. fermentans* is the only *Mycplasma* species that lacks the GAAA terminal loop at the P12 helix [13]. Alignments of short nucleotide sequences including the P12 and P10.1 helices of the haemominutum and haemofelis clusters are separately shown in Fig. 1. The GAAA tetranucleotide was conserved at the terminal loop of the P12 helix in all the species of hemoplasmas. Typical 11-nucleotide (5'-TCTAAG.....TATGA-3') motif at the P10.1 portion, that is a putative region for a crossing-pair with the GAAA tetraloop, was well conserved in the haemominutum clusters. Secondary structures of the P12 portions were pre-

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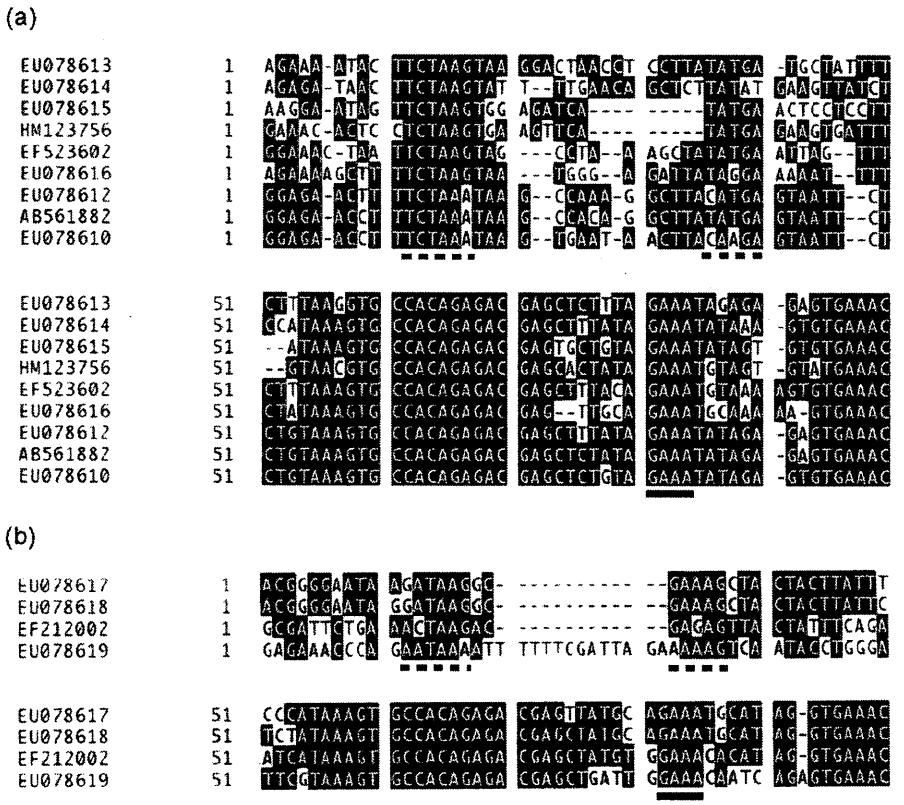

Fig. 1. Nucleotide sequence alignments for partial RNase P RNA genes from the (a) haemominutum and (b) haemofelis clusters in hemoplasmas. Sequences are designated according to accession numbers in the DNA databases as follows, EU078613 for '*Candidatus* M. haemolamae', EU078615 for '*C.* M. kahanei', HM123756 for '*C.* M. aoti', EF523602 for M. suis, EU078616 for '*C.* M. haematoparvum', EU078614 for '*C.* M. haemominutum', EU078612 for *M. ovis*, AB561882 for '*C.* M. haemocervae' EU078610 for *M. wenyonii*, EF 212002 for '*C.* M turicensis', EU078617 for *M. haemofelis*, EU078618 for *M. haemocanis*, and EU078619 for '*C.* M. coccoides'. The GAAA motif at the P12 helix was underlined. Tentative P10.1 portion was marked by a dotted underline. Nucleotides that are identical two out of three sequences are shown as inverted characters. Dashes represent spacers between adjacent nucleotides introduced for maximum matching. The nucleotide numbers are given from a consensus alignment.

dicted according to the algorithm of Zuker and Stiegler [19]. Nucleotide base-pairings at the stem region were variable among hemoplasma species, but capable to form a stable secondary structure to minimize free energy, by G-T wobble as well as canonical Watson-Crick base pairings (Fig. 2). The minimum free energy for the each GAAA tetraloop module was calculated by the method of Freier *et al.* [3]. Size of the stem portion was variable from 5 to 7 base-pairings. Most stems of the P12 helix of hemoplasmas were consisting of seven base-pairings. The shortest stem was appeared in '*Candidatus* M. haematoparvum', but it showed a substantially negative free energy to maintain a stable helix. Sequence divergence at the stem regions was resulted from coordinated mutations of complementary paired nucleotides in base-pairings. Such point mutations, also called palindromic nucleotide substitutions (PNS), may correspond to radical evolutionary changes, and can universally generate new species [2, 5, 6]. The P12 helices of *M. haemofelis* and *M. haemocanis* were almost identical except for a single base-pairing at the inception of the stem structure in terms of PNS. The former showed T-G or T-A pairing at this position but the latter was distinct by transitional substitution, which can be a genetic marker discriminating these two species. Thus, analysis of the secondary structures may provide a clear picture for species identification without making a phylogenetic tree, since nucleotide fluctuation at this particular portion was not found among nine hemoplasma strains (AF407210, AF407212, AY150987, AY150991, DQ859006, DQ859008, DQ859011, EU078611, EU078617) within a single species of *M. haemofelis* (data not shown). The PNS method provides results comparable with other taxonomical procedures by using phylogenetic analysis based on the primary structure, but it differs from them in that only the strategic and highly conserved portions in P12. Point mutations occur continuously and at random through the prokaryotic genome at

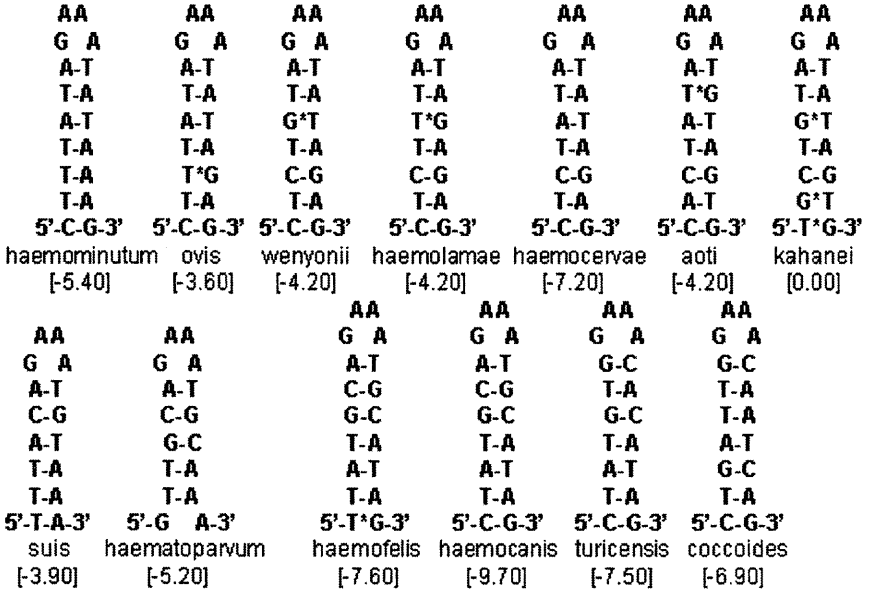

Fig. 2. Secondary structures predicted for the P12 portions in RNase P RNA genes. Watson-Crick base-pairing is indicated by a dash, and a G-T wobble pairing tolerated in the secondary structure by an asterisk. Species designation is shown by specific epithet alone. The folding energy of the each GAAA tetraloop module, which showed substantially negative free energy, except for '*C.* M. kahanei', is expressed in Kcal/mol in brackets.

every multiplication phase. Although point mutation must occur in both the translated and untranslated regions at the same rate, incidence of some nucleotide substitutions, observed in the prokaryotic ribozymes for housekeeping molecules, are biased by the selection of lethal mutation. These lethal mutations are not obvious in critical regions of the RNase P RNA gene. Therefore, comparison of the secondary structures may be more meaningful than the phylogenetic analysis based solely on an alignment of the RNase P RNA sequences. In conclusion, the PNS method based on the evaluation of only the strategic and highly conserved genome region in the specificity domain of RNase P RNA provides clear information at the level of heterogeneity within a species, the relatedness between species, or facilitating the characterization and clustering of specific strains.

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NOTE *Virology*

Epidemiological Survey of Border Disease Virus among Sheep from Northern Districts of Japan

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ABSTRACT. The first epidemiological survey of *Border disease virus* (BDV) was undertaken in small ruminants in Japan. Ovine sera, collected from the northern prefectures of Hokkaido, Aomori and Iwate, were examined for the presence of antibodies against BDV using the neutralization peroxidase-linked antibody test. Twenty-nine (17.6%) of one hundred and sixty-five samples were seropositive for BDV. Results were specific, excluding cross-reactions with *bovine viral diarrhea virus* (BVDV). Only one sample (0.6%) was positive for BVDV, and was negative for BDV. Despite serological evidence of virus circulation, there have been no clinical cases of border disease in sheep in Japan. Although no diagnostic measures were performed, the infection did not appear to be associated with a reduction in ewe fertility nor with lamb mortality.

KEY WORDS: Border disease virus, epidemiology, Japan, *Pestivirus*, sheep.

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Border disease virus (BDV) is a recognized species in the genus *Pestivirus* of the family *Flaviviridae* [7]. Border disease (BD) affects mainly sheep and goats, and has substantial loss-related economic implications worldwide. In addition to cattle, goats, sheep and pigs, ruminant pestiviruses have been isolated from many other wild ruminant species, and serologic surveys have demonstrated infection with pestiviruses in more than 40 species worldwide [3, 24].

Sheep and goat farming is a relatively minor sector in Japan. Land dedicated to small ruminant farming is limited because the terrain mainly consists of mountains unsuitable for pasture use. The diversity of farms is consequently particularly reduced, with few available hectares, and economic problems associated with keeping animals. Furthermore, the winter climate conditions necessitate food supplementation, resulting in increased costs. The small ruminant population consists mainly of goats (21,000) and sheep (11,000) (data from Japan Livestock Industry Association 2000). In 1994, sheep numbered 24,000 and goats 30,000. This marked decrease probably arose because of a lack of adequate support for the optimization of animal farming and the development of production technologies. Ovine and caprine breeds are little studied, either from the animal husbandry or veterinary health aspect. Currently, the production of wool, milk and milk products is practically nonexistent. Only isolated and specific, but promising, studies are being undertaken at an embryonic stage.

At present in Japan, only Caprine Arthritis Encephalitis virus, Scrapie and Visna Maedi have been examined in small ruminant populations, having recently been the subject of epidemiological studies [10, 12, 13, 16, Giangaspero *et al.*, unpublished]. No previous epidemiological surveys on BDV have been undertaken in small ruminants in Japan. Furthermore, no clinical cases of BD have been reported among sheep flocks. The other genetically related *Pestivirus* species, namely bovine viral diarrhea virus (BVDV) type 1, BVDV type 2, and classical swine fever, have been previously reported in the country [8, 15, 19].

In order to verify the presence of BDV infection and to obtain a preliminary indication of its epidemiology, a serological survey of antibodies to BDV was carried out. This was to determine the prevalence of the virus in the northern prefectures in Japan, i.e., Hokkaido, Aomori and Iwate, where the largest concentrations of sheep, a total of 4,775 sheep (43%), are bred. The survey was performed on sheep raised both commercially and traditionally. Farmers were interviewed regarding flock management, productivity and losses, referring also to previous years, to define possible factors influencing the epidemiology of BDV infection, and to explore the potential impact of disease on sheep productivity.

Fourteen sheep flocks from Hokkaido, Aomori and Iwate were sampled from September 2007 to January 2008. The

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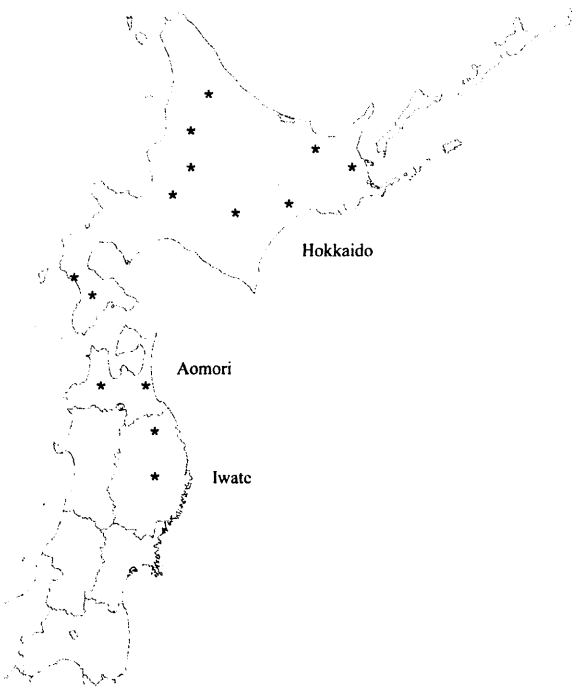

Fig. 1. Northern prefectures of Japan. Gray line: prefecture boundaries; light gray line: municipal boundaries; * sampling locations in Hokkaido, Aomori and Iwate prefectures.

number of flocks was identified according to the animal population of each prefecture and was representative of the livestock production systems in the country as a whole. Ten flocks were sampled in the Hokkaido prefecture, considering that about 37% of sheep breeding in Japan is concentrated in this region. The 10 flocks were arbitrarily chosen to include flocks from different parts of Hokkaido prefecture. The samples also included two flocks from Aomori prefecture and two from Iwate prefecture in the Tohoku region (Fig. 1).

Twenty sheep from each flock were selected for sampling, according to the national standard of flock composition (number of rams, ewes and yearlings). All age categories, from 1 to 12 years of age, were sampled. The majority of the sampled animals were Suffolk and Suffolk cross-breed. Lambs were not sampled to avoid interpretation difficulties arising from the potential presence of maternal antibodies. In 2 of the 14 flocks, only 11 and 16 sheep, respectively, were available for sampling, and therefore, a total of 267 serum samples were collected. All the sera were stored at –20°C prior to examination. The collected ovine sera were subsequently transported to laboratories in Italy and the United Kingdom for further analyses. The sera were imported legally into these countries, under the authorization of the Department of Public Veterinary Health, Nutrition and Food Safety, Ministry of Health, Rome, Italy, and license AHZ/963/93/4 issued under the terms of the Importation of Animal Pathogens Order by the Department for the Environment, Food and Rural Affairs, London, U.K.

Taking into account the genetic relatedness between BDV and BVDV, and the potential for cross-reactivity, detection of BDV antibodies was accompanied by anti-BVDV antibody screening. The presence of BDV antibodies was determined using the neutralization peroxidase-linked antibody (NPLA) test as described by Hyera *et al.* [11]. The sera underwent a serum neutralization test, followed by immunoperoxidase staining. Sera were double-diluted to a 1/40 and tested using BDV isolate S137/4 [23] and bovine turbinate cells. Cell lines were determined to be negative for pestivirus contamination. Peroxidase staining was performed using a mixture of monoclonal antibodies [WB103, WB105 & WB112 as provided by Animal Health and Veterinary Laboratories Agency (AHVLA), Surrey, U.K.] [5, 6, 17]. Testing for neutralizing antibodies against BVDV was performed by serum neutralization assay as described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organisation for Animal Health – Office International des Épizooties [25]. The BVDV CP strain NADL obtained from the American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.) [1] was grown on cell substrate bovine turbinate cells (Central Veterinary Laboratory, Surrey, U.K.).

Concerning flock production, the annual lambing rate was calculated as number of lambs born per ewes exposed to a ram, and was based on the lambing season occurring from February to April, with an exception being made for one farm where the reproductive cycle was related to three breeding seasons. Average annual wool yield referred to uncleaned material from shearing from April to May.

Percentages of sheep positive for antibodies to BDV among different age categories were compared using Fisher's exact probability test. Pearson's correlation coefficients were calculated for a possible relationship between the prevalence of BDV infection and production parameters such as annual lambing rate, annual lamb mortality rate, annual adult mortality rate, and annual wool yield. Differences were considered to be significant at $P<0.05$.

Out of the 267 serum samples, 16 had insufficient aliquots for testing, 18 were contaminated with fungi or bacteria, and 68 showed cytotoxic reactions, and therefore, all these samples (n=102) were excluded. As a result, a total of 165 samples were examined in the NPLA test, and 29 samples (17.6%) were observed to be positive for anti-BDV immunoglobulins (Table 1). Titers ranged from 20 to >40. Toxicity in the samples was indicated by cell death, probably caused by the sub-optimal condition of the samples.

BDV infection was detected in nine out of the 14 sampled flocks. Infection was found in flocks from Hokkaido and Iwate prefectures, but not from Aomori prefecture. The prevalence of BDV infection was found to vary between prefectures (Table 2). Sera showed positivity in 8 of the 10 flocks from Hokkaido and in 1 of the 2 flocks from Iwate prefecture. One flock from Hokkaido could not be evaluated because of cytotoxicity and insufficient serum quantity.

Table 1. Neutralization peroxidase-linked antibody (NPLA) test. Results of serological screening for antibodies to Border disease virus in sheep from prefectures of northern Japan

Flock No.	Prefecture	NPLA titer					Contaminated	Toxic	NE	Total /folck
		Negative	20	30	40	>40				
1	Hokkaido	19	0	1	0	0	0	0	0	20
2	Hokkaido	18	1	0	0	0	1	0	0	20
3	Hokkaido	15	0	1	0	2	1	0	1	20
4	Hokkaido	0	0	0	0	0	0	6	14	20
5	Hokkaido	12	3	2	0	3	0	0	0	20
6	Hokkaido	13	1	2	0	0	1	3	0	20
7	Hokkaido	13	1	3	0	0	3	0	0	20
8	Hokkaido	8	2	0	0	0	3	6	1	20
9	Hokkaido	14	0	0	0	0	0	6	0	20
10	Hokkaido	5	2	0	2	0	0	2	0	11
11	Iwate	6	0	0	0	0	2	12	0	20
12	Iwate	4	2	1	0	0	6	7	0	20
13	Aomori	8	0	0	0	0	1	11	0	20
14	Aomori	1	0	0	0	0	0	15	0	16
Total		136	12	10	2	5	18	68	16	267

NE: not executed because of insufficient aliquots for testing.

Table 2. Comparison between different prefectures of northern Japan for the percentage of sheep positive for antibodies to Border disease virus

Prefecture	Sampled flocks	% Positive	Samples	Samples not considered*	% Positive
Hokkaido	10	88.9	191	48	18.2
Iwate	2	50.0	40	27	23.1
Aomori	2	0	36	27	0
Total	14	69.2	267	102	17.6

* Samples showing toxicity (n=68), contamination (n=18) or not tested because of insufficient serum quantity (n=16).

None of the sera collected from Aomori prefecture were found to be positive. The percentage of sheep with positive sera was 18.2% in Hokkaido prefecture and 23.1% in Iwate prefecture. The mean prevalence of seropositive animals in individual herds ranged from 5.0 to 42.9% in the nine sampling groups from seropositive flocks.

Results were specific, and showed no cross-reactions with BVDV. Only one serum sample (0.6%) was positive for BVDV, and was negative for BDV. The sample was from a sheep in a flock from Hokkaido, showed a serum-neutralization titer of 1:256 against BVDV.

Comparison between different age categories showed that the highest percentage of sheep positive for antibodies to BDV was in animals 3 years old (45.5%), with another peak (33.3%) in animals 6 years of age (Table 3), but there was no statistically significant difference in the prevalence among different age categories.

Evaluation of the possible impact of BDV infection on production in the sampled flocks did not reveal any correlation with BDV seropositivity.

This survey has demonstrated that infection with BDV is present in sheep in the northern prefectures of Japan, and provides the first serological evidence of the occurrence of the disease in the country. Antibodies against BDV were found in two prefectures (Hokkaido and Iwate), out of three tested, where 43% of Japanese sheep are bred. This first report is based on specific serological results. Taking into account genetic relatedness among species in the genus *Pestivirus*, BVDV was investigated to exclude cross-reactivity. Only one serum sample was positive for BVDV, and was negative for BDV. Interestingly, the sample came from a sheep flock on a farm mainly focused on dairy cattle breeding, thus being in close contact with a herd of 700 Japanese Black cows, a possible source of the BVDV.

Despite serological evidence of virus circulation, no clinical cases of BD have been reported among sheep flocks in Japan. Furthermore, although no diagnostic measures were performed, the infection was not related to a reduction in sheep production, as determined by fertility, lambing rate and mortality in lambs.

The relatively high number of newly deposited sequences of BDV isolates from domestic and wild animals, and the recent evidence of novel "atypical" pestivirus sequences, in particular recent reports of atypical strains from clinical cases of BD from domestic and wild small ruminants [2, 4, 20–22], indicate that laboratory diagnosis of BD has to be expanded and it should not be limited in domestic ruminants but also include wild ruminants. Recent reports

Table 3. Comparison between different age categories for the percentage of sheep positive for antibodies to Border disease virus

Age category (year)	No. animals	No. excluded	No. tested	No. BDV positive	BDV positive: breed	%*
1	16	10	6	1	1 ewe: Corriedale	16.7
2	27	13	14	1	1 ewe: Cross breed	7.1
3	32	21	11	5	3 ewe: Corriedale; Suffolk 2 rams: Corriedale	45.5
4	57	14	43	8	7 ewes: Cross breeds Suffolk × Cheviot; Suffolk 1 ram: Cross breeds Suffolk × Cheviot	18.6
5	34	16	18	2	2 ewes: Suffolk; Cross breeds South down × Poll Dorset	11.1
6	26	9	17	6	6 ewes: Suffolk; cross breeds Suffolk × Cheviot; cross breed	35.3
7	33	11	22	3	3 ewes: Cross breeds Suffolk × Cheviot; Cross breeds South down × Poll Dorset	13.6
≥8	20	8	12	2	2 ewes: Suffolk; cross breeds South down × Poll Dorset	16.7
Not known	22	0	22	1	1 ewe: Suffolk	4.5
Total	267	102	165	29	29	17.6

* Percentage excluding samples with toxicity, contamination or not tested because of insufficient serum quantity.

demonstrated confirmed cases with isolates of ruminant pestivirus and disease with a significant population impact on free-range animals [14, 18, 22]. An outbreak of a previously unreported disease, associated with a new pestivirus belonging to the BDV virus cluster, was reported in Southern chamois (*Rupicapra pyrenaica*) in the Catalan Pyrenees (northeast Spain) in 2001 and 2002, and was supposedly responsible for a population decrease of 40–45% in the most affected areas [14]. In Japan, evidence for pestivirus infection in free-living Japanese serows (*Capricornis crispus*) has also been reported [9]. These recent cases indicate the potential for a disease to emerge in wild animal populations, with a possible severe impact on farmed populations. Furthermore, the role of wild animals as a reservoir for spreading the disease in domestic populations could increase the risk of mutation, resulting in increased virulence.

The demonstration of BDV circulation in sheep flocks in the prefectures of Hokkaido and Iwate, based on serological analysis, advances the knowledge on pathogens affecting domestic sheep in Japan. These interesting findings deserve further evaluations in order to examine the full extent of the problem in small ruminant populations, taking into account that BDV infection has a potential negative impact on reproduction. The complexity of the topic suggests the need for lager, more detailed epidemiological studies in the animal population and, considering the genetic heterogeneity of pestiviruses, molecular studies should be undertaken to extend the serological findings. Furthermore, investigations should include wild ruminants, because of concern for the protection of the valuable ruminant wild fauna present in Japan.

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NOTE *Bacteriology*

Two Genetic Clusters in Swine Hemoplasmas Revealed by Analyses of the 16S rRNA and RNase P RNA Genes

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ABSTRACT. Only two hemoplasma species, *Eperythrozoon parvum* and *Mycoplasma suis*, have been recognized in pigs. Here we demonstrate the genetic variations among six hemoplasma strains detected from pigs, by analyzing the 16S rRNA and RNase P RNA (*rnpB*) genes, and propose a novel hemoplasma taxon that has not been described previously. Phylogenetic trees based on the nucleotide sequence of the 16S rRNA gene indicated that these six hemoplasmas were divided into two clusters representing *M. suis* and a novel taxon. We further examined the primary and secondary structures of the nucleotide sequences of the *rnpB* gene of the novel taxon, and found it distinct from that of *M. suis*. In conclusion, we unveiled a genetic cluster distinct from *M. suis*, suggesting a new swine hemoplasma species or *E. parvum*. Our findings also suggest that this novel cluster should be included in the genus *Mycoplasma*.

KEY WORDS: Eperythrozoon, hemoplasma, mycoplasma.

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Hemoplasma is a trivial name for the group of hemotrophic prokaryotes that lack a cell wall, but have never been cultured *in vitro* [10]. This group is composed of former *Eperythrozoon* and *Hemobartonella* species as well as newly identified hemotrophic mycoplasmas. Currently only two hemoplasma species, *Eperythrozoon parvum* and *Mycoplasma suis* previously known as *E. suis*, are recognized in pigs (*Sus scrofa domestica*) [11, 12, 18]. Hemoplasmas parasitize the cell surface of swine erythrocyes, where it causes membrane deformation and damage [10, 25]. Damaged erythrocytes are subsequently removed from the blood circulation, resulting in hemolytic anemia and icterus in pig [10].

The clinical signs of *M. suis* infection are variable, but in the acute form, sows usually develop anemia, icterus and anorexia for a few days [6], and it causes severe anemia in newborn and weaned piglets [5]. Chronic carrier states have been associated with reproductive failures in sows and decreased weight gains in piglet [26]. Mycoplasmas have host specificity to some extent, but human infections with *M. suis* have been reported in China, suggesting a zoonotic pathogen [23, 24]. Pathogenicity of *E. parvum* has not well been documented, but it also causes severe anaemia accompanied with fever on splenectomised pig [1].

In the present study, we examined the genetic variations among swine hemoplasmas detected from a commercial farm in Japan. Heparin-anticoagulated blood samples collected from 12 pigs in Morioka (latitude 39.7N and longitude 141.1E), Japan in December 2010, were stored at –80°C prior to analysis. Total DNA was extracted from 200 μl of the Heparin-anticoagulated whole blood samples by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, eluting into 200 μl of buffer AE, and stored at –20°C until examination in the PCR assay.

For the preliminary screening of hemoplasma infections by real-time PCR, 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 [22]. After real-time PCR, melting experiment was performed as described previously [4]. Six (50%) out of the 12 blood samples tested by real-time PCR were found to be positive for hemplasma infection. No mixed infection with both the hemoplasma species was found in this screening test. The six samples designated Morioka1, Morioka4, Morioka5, Morioka6, Morioka8, and Morioka9 were divided into two clusters, A and B, according to the melting temperature (*Tm*) levels. *Tm* (mean ± SE) of the cluster A consisting of Morioka5, Morioka6, Morioka8 showed lower temperature at 82.40 ± 0.08°C, and the cluster B including Morioka1, Morioka4 and Morioka9 showed higher temperature at 83.33 ± 0.22°C.

These six positive samples were further subjected to endpoint PCR to amplify entire region of the 16S rRNA gene by using universal primers as described previously [22]. DNA extracted from PCR products was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Six nucleotide sequences of the almost entire region of the 16S rRNA gene have been deposited to the DDBJ, EMBL, GSDB and NCBI nucleotide sequence databases under the accession numbers AB610845 to AB610850.

A phylogenetic tree constructed with the neighbor-joining method [16] from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model [7]

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divided the six swine hemoplasmas into two clusters, A and B, as same as the *Tm* values in the real-time PCR experiments (Fig. 1). Nucleotide sequences of Morioka5, Morioka6 and Morioka8 in cluster A were identical and closely related to *M. suis* (more than 99% homology) in the phylogenetic tree of the 16S rRNA gene sequences. On the contrary, the three strains, Morioka1, Morioka4 and Morioka9, in cluster B were distinct from the former sequences (less than 95% homology), suggesting a different taxon in the hemoplasma taxonomy according to the cutoff value of the 16S rRNA gene sequence identity for species definition [2].

We have further examined the nucleotide sequences of the RNase P RNA (*rnpB*) gene of the cluster B hemoplasmas, a distinct taxon revealed by 16S rRNA sequences, to compare with other hemoplasma species [14]. The *rnpB* gene has been shown to be suitable for phylogenetic discrimination of closely related taxonomic groups when examined by the 16S rRNA sequence comparison [20]. The *rnpB* gene of only the strains Morika1, Morika4, and Morioka9 in cluster B was successfully amplified by endpint PCR with forward primer [5'-TATTTAAAGTAGAGGAAAGTC-3' equivalent to nucleotide numbers 10 to 30 of *M. suis* (EF523602)] and reverse primer [5'-GAGGAGTTTACCGCGTTT-3' equivalent to nucleotide numbers 206 to 223 of *M. suis* (EF523602)]. Reaction was the same as used for the amplification of the 16S rRNA gene except for the annealing temperature at 56°C instead of 58°C. PCR product was then subjected to direct sequencing as described previously and the nucleotide sequences of the *rnpB* gene of these strains, which were identical, have been deposited to the DNA database under the accession numbers AB641639 to AB641641. A phylogenetic tree was constructed with the *rnpB* gene sequences of these three strains with other hemoplasma sequences from DNA databases (Fig. 2). The *rnpB* gene sequence of the three strains in cluster B showed less than 90% homology to *M. suis*, and it included deletion of three serial nucleotides in the gene, supporting the notion

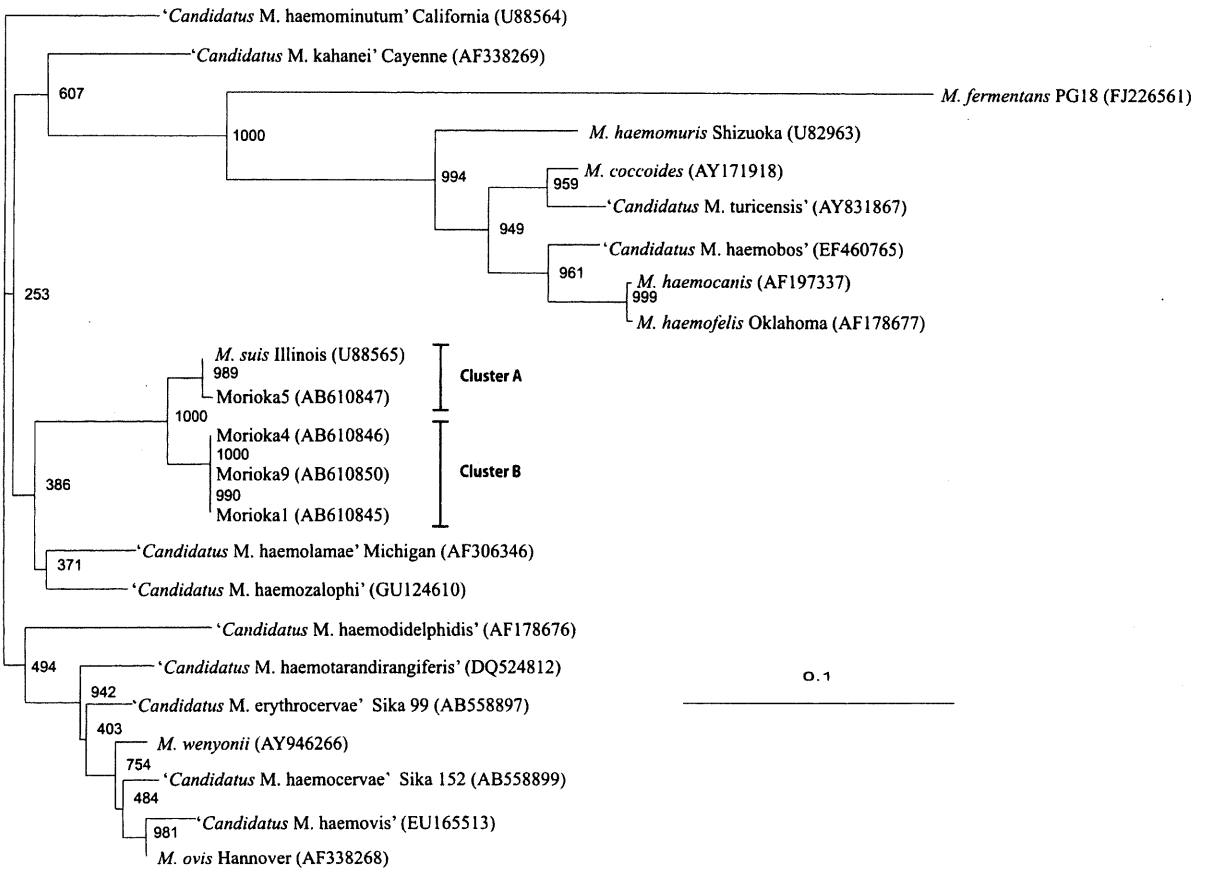

Fig. 1. A phylogenetic tree based on the 16S rRNA gene comparison among mycoplasmas including 18 hemoplasma species (accession numbers are given in a parenthesis) and putative taxa created by clusters A and B. Genetic distances were compared with CLUTAL W [21]. Morioka5, representative of morioka6 and Morioka8 in cluster A was identified as *M. suis*. Morioka4, Morioka9 and Morioka1 in cluster B were distinct from *M. suis*. Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications. Scale bar indicates the estimated evolutionary distance.

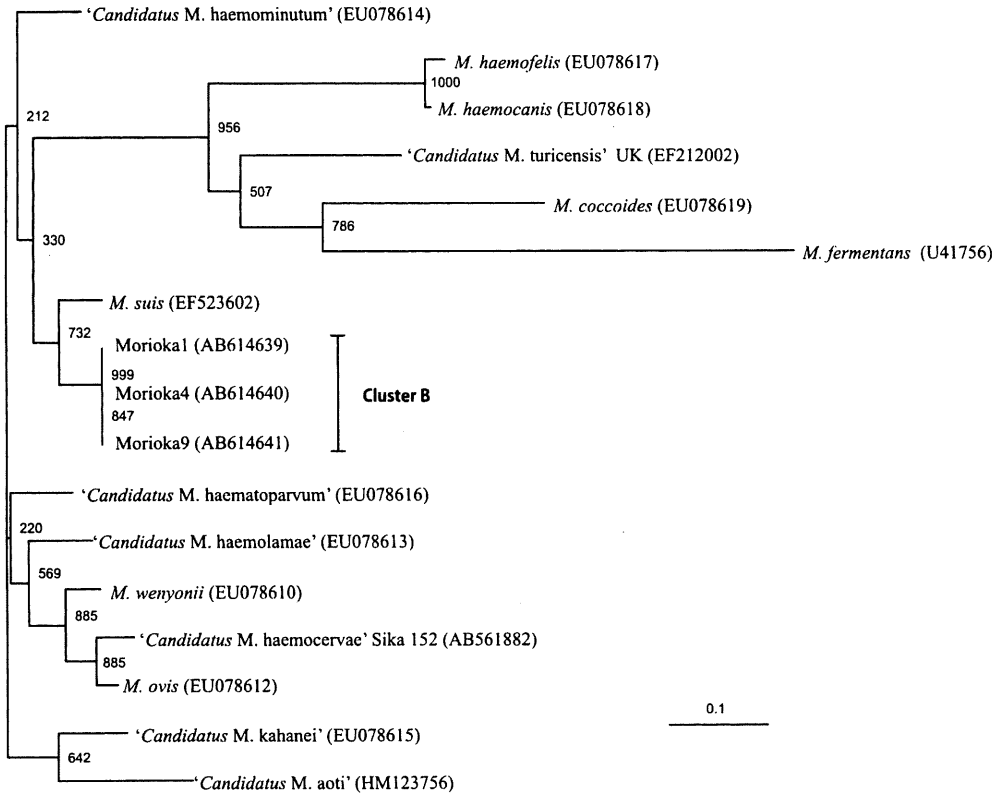

Fig. 2. A phylogenetic tree generated by partial *rnpB* gene sequences among mycoplasmas including 13 hemoplasma species (accession numbers are given in a parenthesis) and a putative taxon as cluster B. Genetic distances were compared with CLUTAL W [21]. Hemoplasma strains Morioka1, Morioka4 and Morioka9 in cluster B were detected from a commercial swine farm 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.

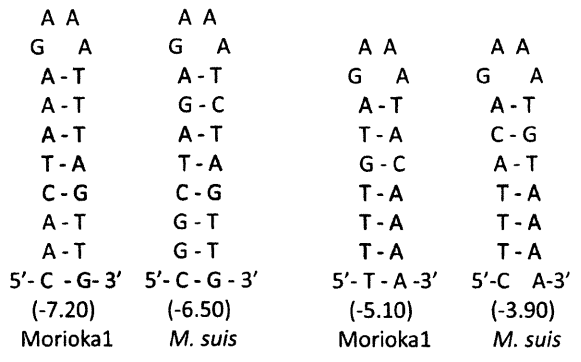

Fig. 3. Comparison of the representative secondary structures containing the GAAA tetraloop modules in the *rnpB* gene. *M. suis* sequences in the left and right structures correspond to the nucleotide numbers 93–112 and 189–206, respectively, of the accession number EF523602. Homologous sequences between *M. suis* and Morioka1 are shown on a gray background. Minimum free energy (Kcal/mol) is given in a parenthesis.

that the cluster B was distinct from *M. suis*.

As an additional means of assessing genetic relatedness between *M. suis* and cluster B, we compared the secondary structures of *rnpB* predicted according to the algorithm of Zuker and Stiegler [27]. Two copies of the GAAA tetraloop module were allocated in the *rnpB* gene in the cluster B as well as *M. suis*. Transitional substitutions between pyrimidines and purines were evident in the stem regions of the GAAA tetraloop modules (Fig. 3).

The GAAA tetraloop has high affinity for the motif called tetraloop receptor and exerts an important function in ribozyme activity [8, 9]. Nucleotide base-pairings at the stem region were variable between these two hemoplasma taxa, but were capable to form a stable secondary structure to minimize free energy. The folding energy of the each GAAA tetraloop module showed substantial negative free energy [3]. Palindromic nucleotide substitutions in base-pairings correspond to radical evolutionary changes, which can generate new genotypes or species. Several secondary structure modules in *rnpB* molecules have been predicted from the phylogenetic comparison, which play the interac-

tion with a defined structural motif. Analysis of the secondary structures may provide a clear picture for species identification. Point mutations occur continuously and at random through the prokaryotic genome at every multiplication phase. Although point mutations occur in both the translated and untranslated regions at the same rate, incidence of some nucleotide substitutions, observed in the controlling regions of prokaryotic genomes, are biased by the selection of lethal mutation. These lethal mutations are not obvious in critical regions of the prokaryotic *rnpB* gene that is responsible for processing the 5' end of tRNA by cleaving a precursor and leading to tRNA maturation. The secondary structure differences delineated the differences between the two species more clearly than the nucleotide sequence alignments, which only showed a small number of differences and some of these are common to both species. Therefore, comparison of the secondary structures may be more meaningful than the phylogenetic analysis of the *rnpB* nucleotide sequences.

There have only been two hemoplasma species, *M. suis* and *E. parvum*, recognized in swine [18], that have been distinguished by morphology and pathogenecity [17, 19]. In the present study, we demonstrated two genetic clusters among swine hemoplasmas. One of them was classified *as M. suis* according to nucleotide sequence homology of the 16S rRNA gene. The nucleotide sequence of the 16S rRNA gene of *M. suis* was first determined on the Illinois strain which had been maintained by serial passage in pigs, and deposited in DNA databases under the accession number U88565 [15]. However, *E. parvum* has not been maintained *in vivo* so far, and this has long hampered to identification of this particular species. The accumulated data in the present study suggested the cluster B strains, distinct from *M. suis* by phylogenic analysis, were thus a new hemoplasma species or most likely to be *E. parvum*, which is the only hemoplasma species remaining in the genus *Eperythrozoon* [13]. All the other recognized species in this genus have already been transferred to the genus *Mycoplasma* [11, 12]. Our results indicated that the novel cluster was genetically close to the *Mycoplasma* species, suggesting that this taxon should be included in the genus *Mycoplasma*. In conclusion, the present study reports the existence of two genetically distinct clusters among swine hemoplasmas, representing *M. suis* and a provisional *M. parvum*.

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