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 erythrocytes, 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'-ATATTCCTACGGAAGCAGC-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 hemoplasma 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 (*T_m*) levels. *T_m* (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 end-point 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 Morioka1, Morioka4, and Morioka9 in cluster B was successfully amplified by end-point PCR with forward primer [5'-TATTTAAAGTAGAG-GAAAGTC-3' equivalent to nucleotide numbers 10 to 30 of *M. suis* (EF523602)] and reverse primer [5'-GAGGAGTT-TACCGCGTTT-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 CLUSTAL 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 CLUSTAL 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 pathogenicity [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 phylogenetic 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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