

Prevalence of Swine Hemoplasmas Revealed by Real-Time PCR Using 16S rRNA Gene Primers

Yusaku WATANABE^{1,2,4}, Masatoshi FUJIHARA^{1,2}, Jin SUZUKI¹, Fumina SASAOKA¹, Kazuya NAGAI³ and Ryô HARASAWA^{1,2}*

¹Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka, Iwate 020–8550, Japan

²Department of Applied Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Gifu 501–1193, Japan

³Cryobiofrontier Research Center, Faculty of Agriculture, Iwate University, Morioka, Iwate 020–8550, Japan

⁴Bremen Vet Center, Yahaba, Iwate 028–3602, Japan

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ABSTRACT. Hemoplasma is a tribal name for epierythrocytic mycoplasmas including *Mycoplasma suis* and *M. parvum* which are currently recognized in pigs as causative of porcine hemoplasmosis. Here, we report a real-time PCR assay for differential detection of these swine hemoplasma species by using allelic primers in the 16S rRNA gene, and its application to survey for hemoplasma infections in pigs. Universal primers and species-specific primers were designed and evaluated by using swine blood samples positive in hemoplasmas. *Mycoplasma suis* and *M. parvum* infections were both confirmed by universal primers, and mixed infections were clearly distinguished by species-specific primers. Further, we applied this real-time PCR assay to 120 swine blood specimens from clinically healthy pigs in eleven farms in Japan, and found six (5.0%) were positive for *M. suis* and 18 (15.0%) were positive for *M. parvum*, and three (2.5%) were mixed infection by both hemoplasma species.

KEY WORDS: hemoplasma, mycoplasma, swine.

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Hemoplasmas are epierythrocytic procaryotes that include mycoplasma species transferred from the *Eperythrozoon* and *Haemobartonella* genera, based on their similarity of the 16S rRNA sequences, and newly identified hemotropic mycoplasmas [2, 9]. They are uncultivable *in vitro* so far and causative of hemolytic anemia, resulting in icterus and pyrexia in various mammalian species. Two hemoplasma species, *Mycoplasma suis* and *M. parvum*, formerly *Eperythrozoon suis* and *E. parvum*, respectively, are currently known in pigs [14].

Mycoplasma suis is a causative agent of swine hemoplasmosis, previously called porcine eperythrozoonosis, of which symptoms are variable. In the acute form, anemia, icterus and anorexia are observed in sows [5], severe anemia and pyrexia in newborn and weaned piglets [2, 4]. Chronic infection following acute form may depress growth rate and increase susceptibility to other infectious diseases in feeder pigs, resulting to reproductive failures or immunosuppression in sows [4, 9, 18]. The clinical signs of *M. parvum* infection have not well been documented, despite severe anemia along with pyrexia in splenectomised pigs [1, 13].

Diagnosis of swine hemoplasmosis has been based on microscopic observation of the organisms on the surface of erythrocytes in Giemsa-stained blood smears, but this

method is limited in acute infection, because the parasites are not always apparent unless the parasitemia is developed [10]. Serological tests including indirect hemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) have also been applied for *M. suis* detection [8, 10], but they are not common because of difficulty in obtaining specific antigens. Polymerase chain reaction (PCR) using specific primers targeting the 16S rRNA gene has most widely been used for screening various hemoplasmas [6, 12, 15–17]. Species identification of *M. suis* is usually depending on PCR [3, 7], but the identification of *M. parvum* has long been hampered due to lack of established strains maintained *in vivo* so far. This particular species was detected by real-time and end-point PCR from clinically healthy pigs in a commercial farm, and the nucleotide sequences of almost entire region of the 16S rRNA and RNase P RNA genes were recently determined [17].

In the present study, we evaluated universal and species-specific primers for PCR to detect swine hemoplasmas by applying to six swine blood specimens, designated A through F, shown positive in hemoplasma infection in our previous study [17]. To evaluate the primers for real-time PCR, we examined three sets of primers, consisting of an universal primer set Pig 16S and two species-specific primer sets, Parvum 16S specific for *M. parvum* and Suis 16S specific for *M. suis* (Table 1). The six swine blood specimens infected with hemoplasmas were subjected to real-time PCR by using these primers as described previously [17].

All the six blood specimens were positive for hemoplasma infection by the real-time PCR using the universal Pig 16S primer set. The PCR products showed the melting

*CORRESPONDENCE TO: HARASAWA, R., Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka, Iwate 020–8550, Japan.

e-mail: harasawa-tky@umin.ac.jp

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Table 1. Primer sets and sequences of primer used in real-time and end-point PCR assays

Primer set	Primer name	Primer sequence (5' to 3')	Position	size
Pig 16S	Pig 16S F	5'-ttt tag tgg caa acg ggc ga-3'	60–79**	247 bp
	Pig 16S R	5'-tca atc cca ttg cgg ctg tt-3'	287–306**	
Parvum 16S	Parvum 16S F	5'-aac aca tat tta act tgc tc-3'	100–119*	137 bp
	Parvum 16S R	5'-cat att cct att cat ccg cg-3'	217–236*	
Suis 16S	Suis 16S F	5'-aac gca tac tta act tac tt-3'	82–101**	138 bp
	Suis 16S R	5'-cat act cct att tac ccg ct-3'	200–219**	
Parvum 16S-2	Parvum 16S F	5'-aac aca tat tta act tgc tc-3'	100–119*	484 bp
	Hemo 16S R	5'-cct acg ctt cct tta cgc cc-3'	546–565**	
Suis 16S-2	Suis 16S F	5'-aac gca tac tta act tac tt-3'	82–101**	483 bp
	Hemo 16S R	5'-cct acg ctt cct tta cgc cc-3'	546–565**	

* Relative to the *M. parvum* Morioka 9 (AB610850) sequence. ** Relative to the *M. suis* Illinois (U88565) sequence.

temperature (T_m) at $84.62 \pm 0.33^\circ\text{C}$ in the melting experiments. Specimens A, B and C were positive in the real-time PCR using the species-specific Parvum 16S primer set, and the T_m was $80.38 \pm 0.13^\circ\text{C}$. All the specimens except B were positive in the real-time PCR using species-specific Suis 16S primer set, with T_m at $81.36 \pm 0.14^\circ\text{C}$ (Fig. 1).

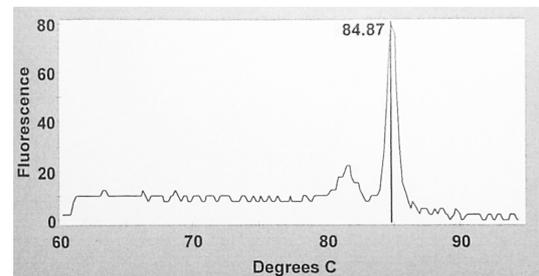
Then, we performed end-point PCR by using the same primer sets as real-time PCR. The PCR consisted of $5 \mu\text{l}$ of DNA solution, $5 \mu\text{l}$ of $10\times$ Buffer for KOD-Plus-Ver.2, $5 \mu\text{l}$ of 2 mM dNTPs, $3 \mu\text{l}$ of 25 mM MgSO_4 , $0.3 \mu\text{l}$ of forward primer, $0.3 \mu\text{l}$ of reverse primer ($50 \text{ pmol}/\mu\text{l}$ each), $1 \mu\text{l}$ of KOD -Plus- ($1 \text{ U}/\mu\text{l}$) and water to a final volume of $50 \mu\text{l}$. After the denaturation at 98°C for 2 min, the reaction was carried out 30 cycles with denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The amplified products were fractionated by 2.5% agarose gel electrophoresis with a Quick-Load 100 bp DNA Ladder marker (New England BioLabs Inc., Ipswich, MA, U.S.A.), and photographed by the GelDoc-It Imaging system (UVP, Upland, CA, U.S.A.) after staining with $0.4 \mu\text{g}/\text{ml}$ ethidium bromide.

By using the primer set Pig 16S, all the specimens produced a relevant sized band in the end-point PCR. In the case of Parvum 16S primer set, specimens A, B and C produced a relevant sized band, and in the case of Suis 16S primer set, specimens A, C, D, E and F produced a relevant sized band. All the end-point PCR results were consistent with real-time PCR (Fig. 2). In addition, the PCR products obtained by using species-specific primer sets were too small to determine the nucleotide sequence in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

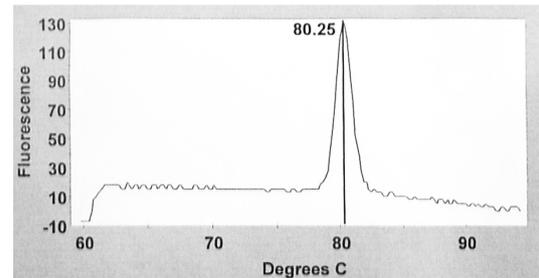
Next, we explored two other primer sets, Parvum 16S-2 and Suis 16S-2, consisting of species-specific forward primers and a common universal reverse primer bracketing about 500 bp portion of the 16S rRNA gene (Table 1), by subjecting all the six blood specimens to end-point PCR as described previously [16]. The amplified products were fractionated by 1.0% agarose gel electrophoresis with a 200 bp ladder DNA size marker (TaKaRa Bio., Otsu, Japan), and photographed as described above.

By using the primer set Parvum 16S-2, specimens A, B and C produced a relevant sized band to *M. parvum* in the end-point PCR. In the case of the Suis 16S-2 primer

Fig 16S : B



Parvum 16S : B



Suis 16S : D

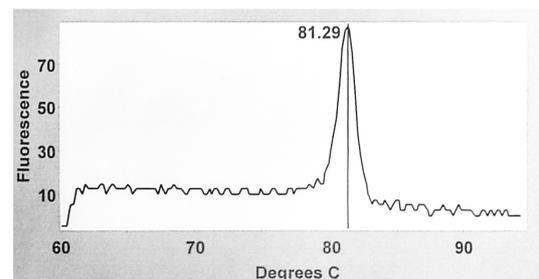


Fig. 1. Representative melting curves using primer sets Pig 16S (top: specimen B), Parvum 16S (middle: specimen B) and Suis 16S (bottom: specimen D).

set, specimens A, C, D, E and F produced a relevant sized band to *M. suis*, though the band of specimen A was faint to compare to others (Fig. 3). These PCR products were

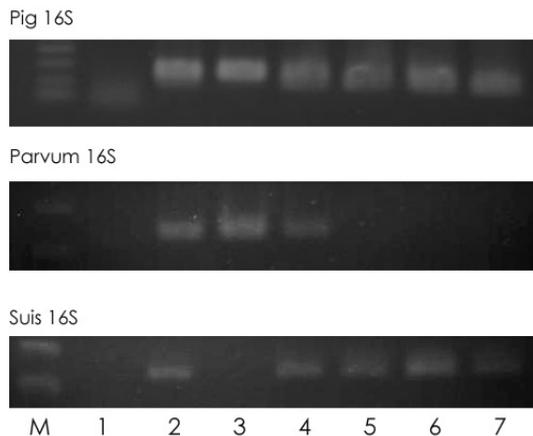


Fig. 2. Agarose gel electrophoresis of the PCR products amplified from six blood samples infected with hemoplasma using three primer sets, Pig 16S, Parvum 16S and Suis 16S. Lane M, DNA size marker (100 bp DNA ladder); lane 1, negative control; lane 2, A; lane 3, B; lane 4, C; lane 5, D 1; lane 6, E; lane 7, F.

further subjected to direct sequencing as described above. All the nucleotide sequences of PCR products obtained by using Parvum 16S-2 primer set were identical to *M. parvum* sequence. On the other hand, the nucleotide sequences of the PCR products obtained by using Suis 16S-2 were identified as *M. suis*, though sequencing of the specimen A was failed in our experiment. Taken together these results, existence of both *M. parvum* and *M. suis* was confirmed in specimen C, and this indicates the existence of mixed infection of both swine hemoplasmas that was not revealed by the universal primers. End-point PCR using Parvum 16S-2 and Suis 16S-2 primer sets were not sensitive as compared to real-time and end-point PCRs using Parvum 16S and Suis 16S primer sets for detection of swine hemoplasmas.

Our data support that the universal primers allow to detection of hemoplasma infection by real-time PCR as well as end-point PCR, but they were not able to distinguish the mixed infection of swine hemoplasmas.

Each hemoplasma species was detected by both real-time and end-point PCRs by using Parvum 16S or Suis 16S primer sets, but the real-time PCR with melting experiments was much more convenient than the end-point PCR. Thus, the real-time PCR using species-specific primers seemed more useful to evaluate the epidemiology of swine hemoplasmas.

Lastly, we applied real-time PCR in the same condition using species-specific primer sets to investigate the prevalence of swine hemoplasmas in 120 blood samples collected from clinically healthy pigs including 108 feeders and twelve sows raised in eleven different commercial swine farms in Japan. Of them, 18 (15.0%) pigs in six (54.5%) farms were positive for *M. parvum*, and six (5.0%) pigs in one (9.1%) farm were positive for *M. suis*. Mixed infections with both hemoplasma species were detected in three (2.5%) blood samples.

Our results first demonstrated the existence of mixed

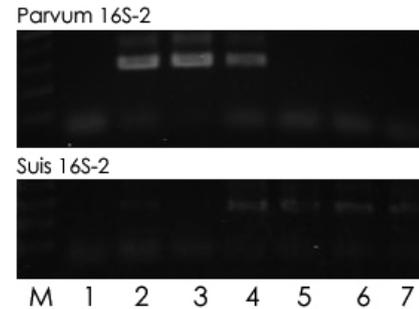


Fig. 3. Agarose gel electrophoresis of the PCR products amplified from six blood samples infected with hemoplasma using two primer sets, Parvum 16S-2 and Suis 16S-2. Lane M, DNA size marker (200 bp DNA ladder); lane 1, negative control; lane 2, A; lane 3, B; lane 4, C; lane 5, D 1; lane 6, E; lane 7, F.

infection with *M. parvum* and *M. suis* in swine, and also revealed the prevalence of swine hemoplasma infections in Japanese farms nowadays. *Mycoplasma suis* infection causes panleucopenia in both piglets and sows and immunosuppression under the stressed conditions by parturition for sows or weaning for piglets [4]. This may lead to increased infections of respiratory and enteric diseases [6], and also it pertinent to consider this organisms affect as etiologic agent with recent swine disease complex associated with emergent infectious diseases due to *Porcine circovirus 2* infection etc. Besides, human infection with *M. suis* has been reported among swine farm workers in China [18] and should be considered in aspects of public hygiene. Although *M. parvum* has been thought relatively less pathogenic to swine [13], a clinical episode has been recorded in Japanese commercial swine farm in 1984 [11]. Thus, an in-depth investigation of swine disease complex is extremely urgent, because of high incidence of hemoplasma infection in the modern swine operation of this country.

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