FULL PAPER *Laboratory Animal Science*

Low Levels of Haptoglobin and Putative Amino Acid Sequence in Taiwanese Lanyu Miniature Pigs

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(Received 31 August 2007/Accepted 18 December 2007)

ABSTRACT. Porcine haptoglobin (Hp) is an acute phase protein. Its plasma level increases significantly during inflammation and infection. One of the main functions of Hp is to bind free hemoglobin (Hb) and inhibit its oxidative activity. In the present report, we studied the Hp phenotype of Taiwanese Lanyu miniature pigs (TLY minipigs; n=43) and found their Hp structure to be a homodimer (β-α-α-β) similar to human Hp 1–1. Interestingly, Western blot and high performance liquid chromatographic (HPLC) analysis showed that 25% of the TLY minipigs possessed low or no plasma Hp level (<0.05 mg/m*l*). The Hp cDNA of these TLY minipigs was then cloned, and the translated amino acid sequence was analyzed. No sequences were found to be deficient; they showed a 99.7% identity with domestic pigs (NP_999165). The mean overall Hp level of the TLY minipigs (0.21 ± 0.25 mg/m*l*; n=43) determined by enzyme-linked immunosorbent assay (ELISA) was markedly lower than that of domestic pigs (0.78 ± 0.45 mg/m*l*; $p<0.001$), while 25% of the TLY minipigs had an Hp level that was extremely low (<0.05 mg/m*l*). In addition, the initial recovery rate (first 40 min) in the circulation of infused fluorescein isothiocyanate (FITC)-Hb was significantly higher in the TLY minipigs with extremely low Hp levels than those with high levels. This data suggests that the low concentration of Hp-Hb complex is responsible for the higher recovery rate of Hb in the circulation. TLY minipigs have been used as an experimental model for cardiovascular diseases; whether they can be used as a model for inflammatory diseases, with Hp as a marker, remains a topic of interest. However, since the Hp level varies significantly among individual TLY minipigs, it is necessary to prescreen the Hp levels of the animals to minimize variation in the experimental baseline. The present study may provide a reference value for future use of the TLY minipig as an animal model for inflammation-associated diseases.
KEY WORDS: amino acid sequence, evolution, haptoglobin concentration, hemoglobin, Lanyu minipig.

J. Vet. Med. Sci. 70(4): 379–387, 2008

Porcine haptoglobin (Hp) is an acute phase protein with a molecular weight approximate 120 kDa [11, 26, 36]. It possesses an electrophoretic mobility and quaternary structure similar to human Hp 1–1 and is a homodimer $(\alpha\beta)_2$ that is linked by disulfide bridges [12, 36]. Due to its homogeneity, porcine Hp can be conveniently isolated using a one-step high performance liquid chromatographic (HPLC) technique established in our laboratory [39].

A comprehensive list of functions has been proposed for Hp; one of the major functions is to scavenge and complex with plasma free hemoglobin (Hb), which possesses oxidative toxicity via iron-containing hemes [7, 35]. The Hp-Hb complex is then metabolized through a cycteine-rich receptor (CD163) on the macrophage [18]. It has bacteriostasis ability by attenuating the iron necessary for bacteria growth [2, 5]. Hp has also been proven to be an extremely potent antioxidant that directly inhibits Cu (II)-induced low density lipoprotein oxidation and reduces cell oxidative stress [38].

The plasma concentration of porcine Hp increases significantly during infection, inflammation and tissue damage [6, 9]. Pigs with lameness, tail biting, or diarrhea exhibit high levels of Hp [33]. Natural infection of pigs with porcine reproductive and respiratory syndrome virus (PRRS) [1] or with *Actinobacillus pleuropneumoniae* [9], *Mycoplasma hyorhinis* [28] or *Toxoplasma gondii* [13] results in significant elevation of their Hp levels. The protein level has therefore been used to identify both clinical and subclinical diseases [10, 11] as well as to monitor health status in pig production [33].

Human plasma Hp is classified into three phenotypes: 1–1, 2–1 and 2–2 (Fig. 1) [38, 40]. Hp 1–1 is a molecule of homodimer or $(\alpha\beta)_2$, whereas 2–1 is comprised of multiple forms including homodimer, trimer, tetramer and other linear polymers. Hp 2–2, on the other hand, consists of trimer, tetramer and other cyclic polymers. In non-human mammalians, both dimeric and polymeric forms of Hp exist. Thus far, polymeric forms analogous to human polymeric Hp have only been found in ruminant families of the Artiodactyla order [14]. TLY minipigs, classified as *Sus Scrofa*, are indigenous to Taiwan and have smaller ears than other breeds. The body weight of the TLY minipig is usually lower than 25 kg during the first six months of age compared with 80 kg for other domestic pigs [25]. Due to its low body weight and conserved population, the TLY minipig has been used as an experimental animal [25]. In the present study, we attempted to phenotype the Hp of TLY

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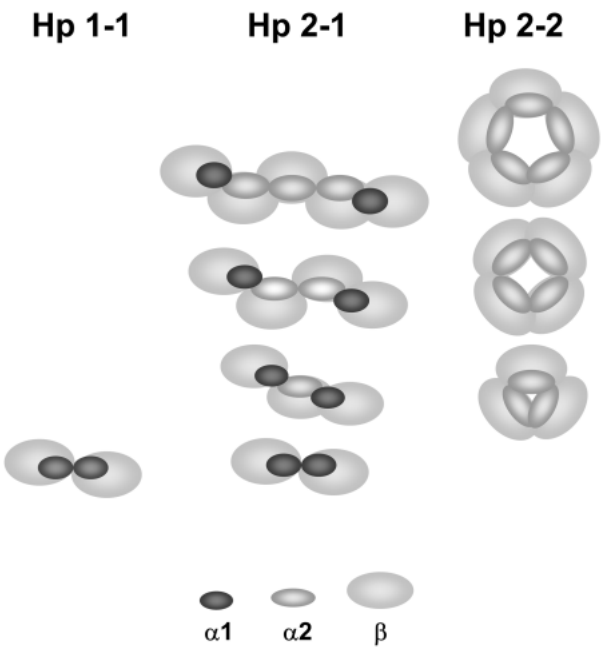

Fig. 1. Schematic drawing of molecular arrangement in human Hp phenotypes. Hp 1–1 exclusively possesses only the basic dimer $(\alpha 1\beta)_2$, whereas Hp 2–1 is comprised of polymeric structures: dimer $(\alpha 1\beta)_2$, trimer $(\alpha 1\beta)_2(\alpha 2\beta)$ and other linear polymers. Hp 2–2 is comprised of a trimer $(\alpha 2\beta)_3$ and other cyclic polymers. The amino acid residue lengths of α1, α2 and β were 83, 142 and 245, respectively, and α2 was similar to α1, differing only by the additional insertion of a repeat identical to 3/4 of α1. Owing to the extra Cys-74 in α2, Hp 2–1 and 2–2 are able to form complicated polymers.

minipigs; during phenotyping, we noticed that many of the animals possessed extremely low plasma levels of Hp. The cDNA nucleotide sequence of TLY Hp was determined to identify whether this was due to an Hp gene defect. It is of interest to observe that there was no direct relationship between the putative amino acid sequence of Hp and its levels. The animal's amino acid sequences were almost completely identical to those of domestic pigs (except Val-65→Leu–65). Finally, a non-competitive enzyme-linked immunosorbent assay (ELISA) was employed to determine the plasma Hp levels. On average, the mean Hp level for the TLY minipigs (0.21 ± 0.25 mg/m*l*) was significantly lower (4X) than that of domestic pigs (0.78 ± 0.45 mg/m*l*; $p<0.001$). Furthermore, 25% of the TLY minipigs exhibited a level markedly lower than 0.05 mg/m*l*. The present study may provide a reference value for future use of the TLY minipig, in terms of the Hp levels, as an animal model for inflammatory, cardiovascular and infectious disease studies.

MATERIALS AND METHODS

Materials: Purified porcine Hb and rat polyclonal antibodies against porcine Hp were prepared according to methods described previously [30, 39]. Goat anti-rat IgG was purchased from Chemicon (Temecula, CA, U.S.A.). A sepharose-12 HR column was obtained from Pharmacia (Uppsala, Sweden). A total RNA extraction kit was purchased from Geneaid (Taipei, Taiwan). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and were not subjected to any further purification. The buffers used in this report were all filtered through a 0.45-μm filter before use (Millipore, MA, U.S.A.).

Preparation of plasma samples: Forty-three TLY minipigs (23 females and 20 males) that were 1 to 2 years old were raised at Taitung Animal Propagation. Thirty-five domestic pigs (12 Duroc, 15 Landrace, and 8 Yorkshire) that were 6–8 months old were used in this study and were obtained from the Animal Technology Institute, Taiwan. All of the pigs were reared in natural lighting environments. Management and medical treatment were conducted according to the instructions established by the National Science Council of Taiwan. The pigs were vaccinated against swine enzootic pneumonia, hog cholera, pseudorabies, atrophic rhinitis, picornavirus and actinobacillus pleuropneumoniae. All selected animals were free of adverse signs of disease after a clinical examination of the herd. Blood samples were collected via jugular puncture using a 21-gauge needle and were drew into tubes containing 0.1% ethylenediamine tetraacetic acid (EDTA). Plasma was obtained by centrifugation at 2,000 × g for 20 min and stored at –20°C until analysis.

Phenotyping of Hp: A 7% native polyacrylamide gel electrophoresis (native-PAGE) was conducted according to the Laemmli method using 5% polyacrylamide (w/v) as the a stacking gel [20]. Eight μ*l* of plasma was incubated with 16 μg of porcine or human Hb for 30 min at room temperature and subsequently mixed with a loading buffer [12 mM Tris-HCl, pH 6.8, 5% glycerol (v/v), 2.88 mM of 2-mercaptoethanol and 0.02% bromophenol blue (w/v)] in a final volume of 15 μ*l*. Following electrophoresis, the gel was briefly washed and then immersed in freshly prepared 3,3'-diaminobenzidine (DAB) solution (0.125 g of DAB dissolved in 0.5 m*l* dimethyl sulfoxide (DMSO) with an addition of 30 m*l* phosphate-buffered saline (PBS) containing 0.05% H_2O_2) with gently shaking to develop the pattern of the Hp-Hb complex based on the peroxidase activity of Hb.

Western blot: Following separation of proteins by sodium dodecyl sulfate (SDS)-PAGE, the gel was electrically transferred to a nitrocellulose membrane attached to a 3 MM filter paper presoaked in a transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS (w/v) and 20% methanol (v/v), pH 8.3. The rest of the procedures for Western blot were similar to those described previously [22, 39]. Rat polyclonal antibody prepared against the β-chain of porcine Hp was used for determination of the presence of Hp.

HPLC gel filtration chromatography: Plasma from 3 individual pigs (1 Yorkshire and 2 TLY minipigs with normal and low Hp levels) was used for analysis of the Hp elution profile. Each plasma sample was fractionated via 50% saturated ammonium-sulfate. After gentle stirring for 30 min at room temperature, the mixture was centrifuged at

10,000 × g for 15 min at 4°C. The supernatant was subsequently dialyzed against 4 *l* of PBS at 4°C overnight with three changes. The sample was then chromatographed onto a Superose-12 HR column (30 × 1.0 cm) pre-equilibrated with PBS using a Waters 600 HPLC controller system (Millipore, Billerica, MA). The flow rate was 0.5 m*l*/min, and PBS was used as the mobile phase.

Analysis of the cDNA sequences of Hp from the TLY minipigs: Total RNA was isolated from whole blood containing leukocytes using a Total RNA Mini Kit according to the manufacture's instructions. An oligo (dT) 18 mer primer was used for the reverse transcription step. The first strand cDNA was synthesized with M-MLV reverse transcriptase at 37°C for 50 min. A gene fragment coding for porcine Hp was amplified by polymerase chain reaction (PCR) using proofreading DNA-polymerase and oligonucleotide primers as described previously [21]. The primer design was based on the published nucleotide sequence of porcine Hp (NM_214000). The cDNA primers prepared were 5'-CGCGC CCTGG GAGCT GT-3' (forward) and 5'-CAGTT GGCAG CTATG GTTGT CTGAA CCA-3' (reverse). The PCR product was then cloned and transformed into *Escherichia coli* JM109 via a TA-cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). After sequencing, the putative amino acid sequence alignment between the TLY minipig and domestic pig (NP_999165) was determined. The identity/divergence percentage was analyzed using DNAStar software (DNAStar, Madison, WI, U.S.A.).

Determination of plasma Hp using ELISA: The porcine Hp concentration was determined by Pig Hp ELISA kit (Immunology Consultants Laboratory, Newberg, OR, U.S.A.) according to the manufacture's instructions. In brief, plasma samples were first diluted to 1:10,000 with a sample dilution buffer. Either 100 μl of the diluted samples or calibrator were added to each microtiter well containing immobilized anti-Hp IgG and then incubated for 30 min at 24°C. After washing them thoroughly, 100 μl of horseradish peroxidase (HRP)-conjugated anti-porcine Hp IgG was added, and the samples were incubated for 15 min. Finally, the plate was washed three times and developed in 100 μl of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate for 15 min. The reaction was stopped by adding 100 μl of stop solution (1 N HCl) and was read at 450 nm.

Uptake (recovery) rate of Hb infused in TLY minipigs: Porcine Hb was purified using a procedure described previously [22]. Sixty mg fluorescein isothiocyanate (FITC) dissolved in 120 μl DMSO was gently mixed with 1 g of porcine Hb in 30 m*l* of $NaHCO_3$ buffer (pH 9.0; 60 μg FITC per mg Hb) at 37°C for 1 hr. The reaction mixture was then dialyzed against 4 *l* of PBS at 4°C overnight (in dark) with three changes. Before use, the dialyzed FITC-Hb was sterilized by filtration through a 0.22-μm filter. The FITC-Hb was then infused into 3 TLY minipigs with low Hp levels (0.043, 0.0064 and 0.091 mg/m*l*) and 3 TLY minipigs with high levels (approximately 0.4 mg/m*l*) through the ear vein at a dosage of 1.5 mg of Hb per kg body weight. Approximately 2 m*l* of blood was collected from each TLY minipig into a vacuum tube containing 0.1% EDTA by venipuncture from the jugular vein at a time point between 0 and 480 min. Because the fluorescence intensity was significantly influenced by pH, 800 μl of the tested plasma samples was buffered to pH 7.4 by addition 200 μl of 500 mM HEPES [27, 34]. FITC-Hb standards ranging from 0 to 0.02 mg/m*l* were prepared by spiking the desired amount of FITC-Hb into plasma. Eventually, the fluorescence intensity was measured with an excitation wavelength of 485 nm and read with an emission wavelength of 538 nm using a fluorescence spectrophotometer (F-7000, Hitachi, Tokyo, Japan) in order to determine the remaining levels of FITC-Hb.

RESULTS

Phenotyping of domestic pigs and TLY minipigs: In non-human mammalians, both dimeric and polymeric forms of Hp exist. The dimeric structure or $(\alpha\beta)_2$ is similar to the human Hp 1–1 present in the plasma of most mammalians. Thus far, the polymeric forms that mimic human Hp have only been found in ruminant families of the *Artiodactyla* order [14]. From the phylogenetic tree, pigs belong to the *Artiodactyla* order; we attempted to address whether the TLY minipig might possess Hp polymeric forms. We identified their Hp phenotype based on formation of the Hp-Hb complex on a 7% native-PAGE. Human plasma Hp 2–1 or 2–2 was clearly demonstrated by the presence of polymeric forms. Seventy-eight pigs were screened, and no Hp polymers were observed in any of the animals. A typical example of phenotyping among domestic (n=35) and TLY (n=43) pigs exhibiting as Hp 1–1 is shown in Fig. 2. Interestingly, we found some TLY minipigs whose Hp 1–1 levels were considerably lower than those of domestic pigs based on Hp-Hb complex formation (Fig. 2). Hypothetically, some TLY minipigs may have an Hp deficiency or may have a structural alteration of Hp.

Evaluation of the presence of Hp in TLY minipigs by Western blot and HPLC: To further determine the plasma Hp levels in the TLY, we analyzed their Hp content on a Western blot using a rat polyclonal antibody against porcine Hp β-chain [39]. Approximately, 25% of the TLY minipigs exhibited little or no Hp compared with the domestic pigs. A typical example of Western blotting is shown in Fig. 3. In order to rule out the possibility that structural alterations in the Hp of the TLY minipigs are responsible for the low immunoreactivity, we conducted chromatography using an established gel filtration method [39] to identify the presence of plasma Hp. Figure 4 shows a typical chromatographic profile from those TLY with "Western-blot negative" plasma; we were unable to find the peak corresponding to Hp for this pig, normal TLY and domestic pigs. Thus, the "absence" of Hp in some TLY minipigs was confirmed.

Analysis of porcine Hp mRNA expression in the leukocytes of TLY minipigs: Next, we cloned the cDNA of Hp from TLY minipigs who possessed low or no levels of plasma Hp. There were two reasons for this experiment.

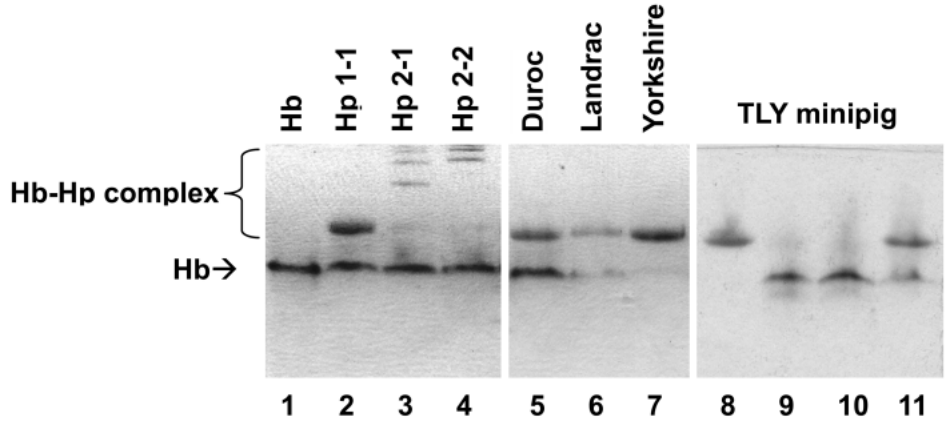

Fig. 2. Phenotyping of human and porcine Hp on 7% native-PAGE. Briefly, each plasma (8 μl) was incubated with hemoglobin (Hb; 16 μg in 4 μl) at room temperature for 30 min. Following electrophoresis, the gel was directly reacted with a freshly prepared DAB solution containing 0.05% H_2O_2 as a developer for the endogenous peroxidase activity of hemoglobin. Lane 1: Hb alone. Lanes 2–4: Human plasma Hp 1–1, 2–1 and 2–2 complexed with Hb. Lanes 5–7: Plasma from three species of domestic pigs. Lanes 8–11: Plasma from four individual TLY minipigs.

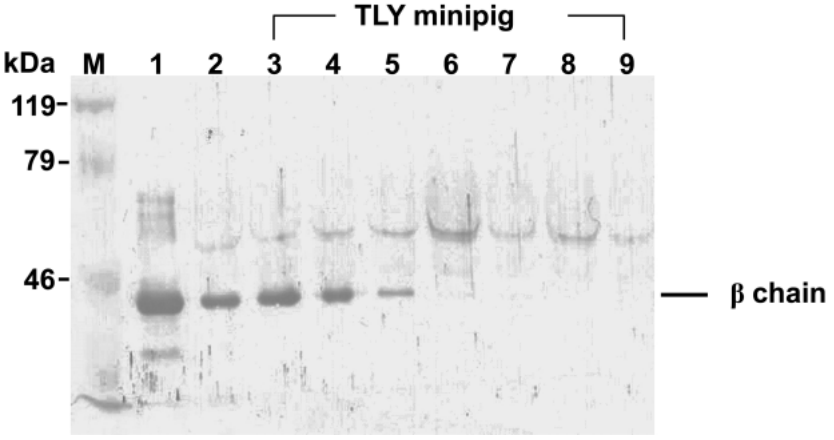

Fig. 3. Western blot of plasma Hp using rat polyclonal antibody prepared against porcine Hp β-chain. Porcine plasma (1 μl) and purified Hp (3 μg) containing 1% 2-mercaptoethanol was loaded onto a 8% SDS-PAGE. Lane M: Molecular markers. Lane 1: Purified porcine Hp. Lane 2: Yorkshire plasma. Lanes 3–9: Plasma from seven individual TLY minipigs with high and low Hp levels. The weak bands showing a molecular weight greater than the Hp β chain in each lane represent those from non-specific binding between the secondary antibody (goat anti-rat IgG) and porcine IgG.

First, we attempted to address whether their Hp gene was genetically defect. Second, we sought their putative amino acid sequence for comparison with that of domestic pigs. Using RT-PCR for the Hp $\alpha\beta$ chain, we demonstrated that the TLY minipigs with extremely low or no Hp levels (n=4) were capable of expressing Hp mRNA in their leukocytes (Fig. 5). The putative amino acid sequence of the TLY minipigs deducted from the nucleotide sequence of the $\alpha\beta$ chain is shown in Fig. 6. Alignment of the amino acid sequence of the TLY minipigs with that of # NP_999165 (domestic pig from Genbank of NCBI) shows that there is only one substitution at the N-terminus (Val-65→Leu-65). This reveals that the divergence between TLY minipigs and domestic pigs is less than 0.6% (Table 1).

Determination of the plasma Hp concentrations of TLY minipigs and domestic pigs using ELISA: The fact that the Hp mRNA expression sequences of the TLY minipigs were almost identical suggests that a gene deficiency was not responsible for the little or no Hp levels found in some of the animals. The Hp concentrations were then measured using a noncompetitive ELISA. The plasma Hp levels (mean ± SD) of the TLY minipigs (n=43) and domestic pigs (Duroc, Landrace and Yorkshire; n=35) were 0.21 ± 0.25 and 0.78 ± 0.45 mg/m*l*, respectively. The levels of the Duroc (n=12), Landrace (n=15) and Yorkshire (n=8) were 0.60 ± 0.18, 0.78 ± 0.51 and 1.05 ± 0.55 mg/m*l*, respectively (Fig. 7 and

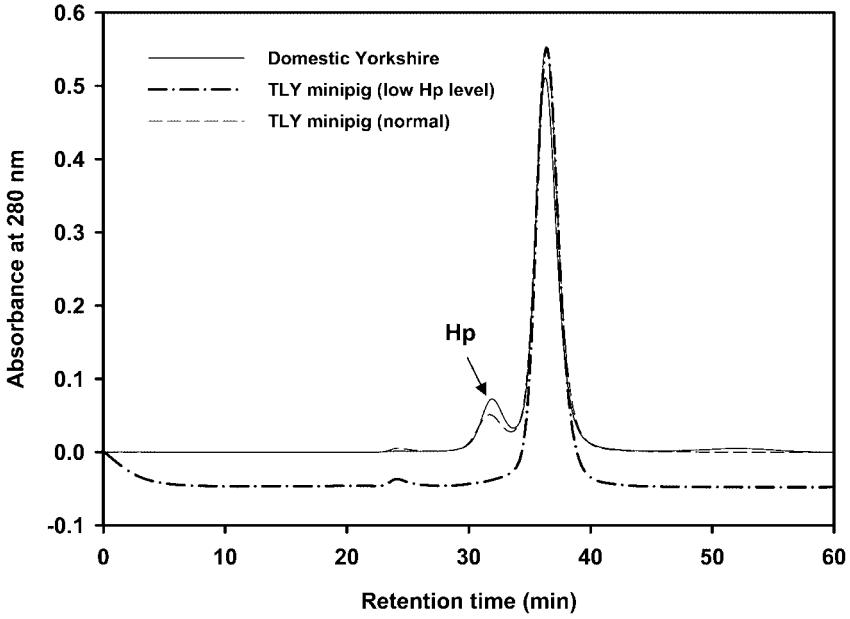

Fig. 4. Typical HPLC profiles of ammonium-sulfate fractionated porcine plasma with normal or low Hp levels on a Superose-12 gel-filtration column. Porcine plasma fractionated from 50% saturated ammonium-sulfate (top fraction) was applied onto previously established HPLC system [39]. Chromatography was conducted at a flow rate of 0.5 m*l*/min for 60 min at room temperature. PBS containing 0.01% NaN_3 was used as mobile phase.

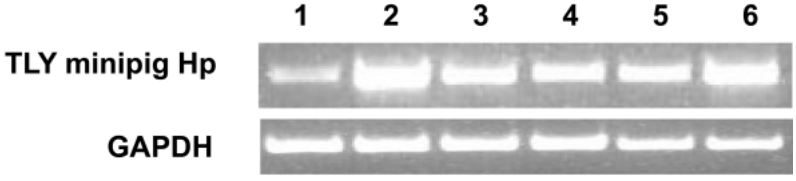

Fig. 5. Expression of Hp mRNA in TLY minipigs using RT-PCR. Briefly, cDNA was obtained by reverse transcription of mRNA from the leukocytes of the minipigs. One μg of total cDNA was amplified by PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Hp specific primers. The PCR products were analyzed on an ethidium bromide-stained agarose gel (1.2%). Lanes 1–4: Four individual TLY minipigs with low or no Hp levels. Lanes 5–6: Two TLY minipigs with normal Hp concentrations.

Table 2). The population distribution (histogram) of the TLY minipigs and domestic pigs according to Hp level is depicted in Fig. 8. In the TLY minipigs, 40% of the animals had Hp levels that were lower than 0.1 mg/m*l* (0.046 ± 0.030 mg/m*l*), while 25% of them had levels that were lower than 0.05 mg/m*l* (0.026 ± 0.02 mg/m*l*).

Uptake of Hb in TLY minipigs: To determine the possible effect of low Hp level on the uptake or recovery rate of Hb in the circulation, minipigs with high (n=3) and low Hp levels (n=3) were infused with FITC-labeled Hb (FITC-Hb). Prior to this experiment, the linear relationship between the concentration of FITC-Hb and intensity of fluorescence was established with a regression coefficient of 0.98 (data not shown). The recovery rate of infused FITC-Hb was then determined over time by fluorescent intensity transcribed to the Hb concentration using a standard curve. Interestingly, the initial recovery rate of FITC-Hb in the low Hp-level group was significantly greater than the group with high levels within the first 40 min ($p<0.05$); the overall clearance rate following the plateau (120–480 min) was almost identical in the two groups except for the last time point (500 min, $p>0.1$; Fig. 9).

DISCUSSION

Porcine Hp is consisted of -SH covalently linked for α and β chains similar to the human Hp 1–1 phenotype as $(\alpha\beta)_2$. In the absence of a reducing reagent, the protein shows a major band at 120 kDa on SDS-PAGE [11, 26, 36]. Interestingly, the $\alpha\beta$ of the dog, cat and bear are joined by

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Domestic   MRALGAVVALLLCGQLFAAETGNEATDATADSCPKPPEIPKGYVEHMYRYHCQTYYKLRT  60
Lanyu      MRALGAVVALLLCGQLFAAETGNEATDATADSCPKPPEIPKGYVEHMYRYHCQTYYKLRT  60

Domestic   AGDGVYTLDSNKQWTNKYTGEKLPECEAVCGKPKNPYDQYQRIMGGSLDAKGSFPWQAKM  120
Lanyu      AGDGLYTLDSNKQWTNKYTGEKLPECEAVCGKPKNPYDQYQRIMGGSLDAKGSFPWQAKM  120

Domestic   ISHHNLTSGATLINEQWLLTTAKNLRLGHKNDTKAKDIAPTLRLYYGKKQEVEIEKVIFH  180
Lanyu      ISHHNLTSGATLINEQWLLTTAKNLRLGHKNDTKAKDIAPTLRLYYGKKQEVEIEKVIFH  180

Domestic   PDNSTVDIGLIKLKQKVPVNERVMPICLPSKDYVNVGLVGYVSGWGRNANLNFTEHLKYV  240
Lanyu      PDNSTVDIGLIKLKQKVPVNERVMPICLPSKDYVNVGLVGYVSGWGRNANLNFTEHLKYV  240

Domestic   MLPVADQEKCVQYYEGSTVPEKKTPKSPVGVQPILNEHTFCAGLSKYQEDTCYGDAGSAF  300
Lanyu      MLPVADQEKCVQYYEGSTVPEKKTPKSPVGVQPILNEHTFCAGLSKYQEDTCYGDAGSAF  300

Domestic   AVHDKDDDTWYAAGILSFDKSCRTAEYGVYVRVTSILDWIQTTIADN               347
Lanyu      AVHDKDDDTWYAAGILSFDKSCRTAEYGVYVRVTSILDWIQTTIADN               347
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Fig. 6. Alignment of the putative amino acid sequence between the domestic pig and TLY minipig. Alignment was preformed using DNAStar software. The amino acid sequence of the domestic pig was obtained from Genbank, NCBI, and its accession number was NP_999165. There was only one variation at the N-terminus (Val-65→Leu-65). Identity and similarity were 99.7% (346/347) and 100%, respectively.

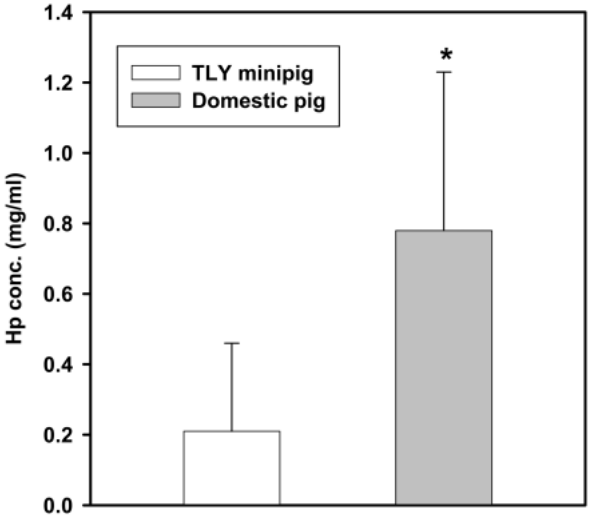

Fig. 7. Plasma Hp concentrations of the TLY minipigs and domestic pigs. Porcine Hp levels were determined by a pig Hp ELISA kit according to the manufacture's instructions as described in Materials and Methods. The levels (mean ± SD) of Hp in the TLY minipigs (n=43) and domestic pigs (n=35) were 0.21 ± 0.25 and 0.78 ± 0.45 mg/m*l*, respectively. The Hp concentrations of the TLY minipigs were significantly lower than those of the domestic pigs ($p<0.001$).

non-covalent interaction [19, 32, 37]. In the present study, we analyzed the Hp phenotype of several species of porcine Hp including TLY minipigs and domestic pigs (Duroc, Landrace and Yorkshire). All of them belong to Hp 1–1.

In newborn domestic piglets, the plasma Hp levels are increase dramatically (2-fold) after weaning [31]. This increase is thought to be associated with pathogen induced infection. In adults, infections of porcine reproductive and respiratory syndrome virus [1], *Actinobacillus pleuropneumoniae* [9], *Mycoplasma hyorhinis* [28] and *Toxoplasma gondii* [13] significantly elevate Hp concentrations, but depend on the pathogen. For example, the Hp levels may increase by up to 15-fold when animal is infected with *Streptococcus suis* [36]. In general, for a conventional herd, the average plasma Hp level is approximately 0.9 mg/m*l*, and for a SPF herd, it is 0.3 mg/m*l* [33].

In the present study, 43 TLY minipigs from an undeclared SPF herd were all clinically healthy, their plasma Hp levels (0.21 ± 0.25 mg/m*l*) were approximately 4-fold lower than those of domestic pigs (0.78 ± 0.45 mg/m*l*; $p<0.001$). About 25% of the TLY minipigs had Hp levels that were extremely low (<0.05 mg/m*l*). In humans, there is an inverse relationship with severity of hemolysis. Hypohaptoglobinemia or anhaptoglobinemia are found in malaria and anemia patients and is associated with hemolysis. However, we did not observe any hemolysis in the TLY herd. It has

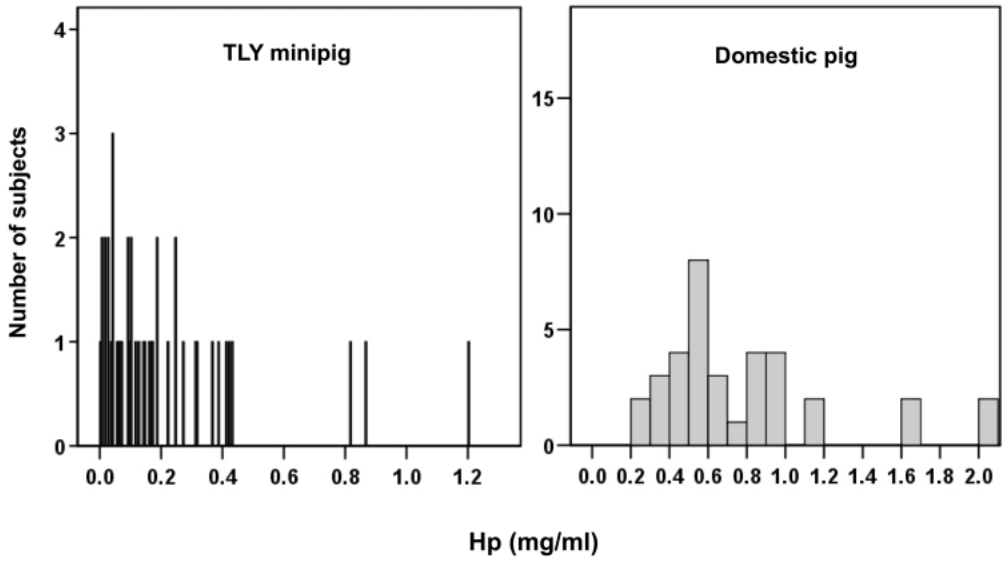

Fig. 8. Population distribution (histogram) of the Hp levels of the TLY minipigs and domestic pigs. The TLY (n=43) and domestic (n=35) subjects were sorted based on an interval of 0.005 and 0.1 mg/m*l*, respectively.

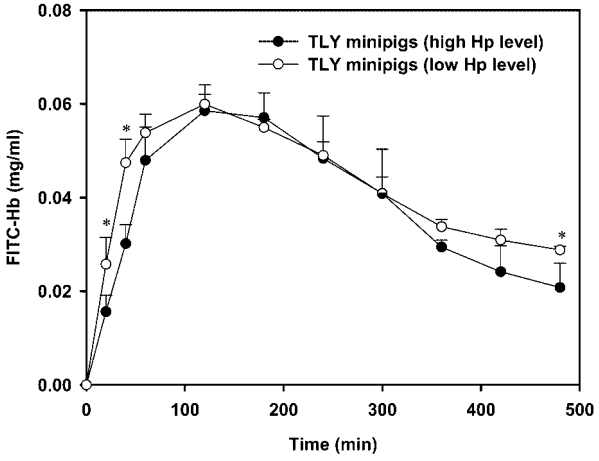

Fig. 9. Uptake rate of infused FITC-Hb. FITC-Hb was infused into TLY minipigs with high Hp (approximately 0.4 mg/m*l*; n=3) and low Hp levels (approximately 0.05 mg/m*l*) (n=3) via intravascular injection from the jugular vein. The fluorescence intensity was measured with an excitation wavelength of 485 nm and read with an emission wavelength of 538 nm using a fluorescence spectrophotometer. * P<0.05.

been reported that the hypohaptoglobinemia or anhaptoglobinemia (Hp^0 phenotype) of some patients is of genetic origin [4, 8, 29]. A silent allele Hp^0 has been identified as a complete Hp gene deletion from the upstream of the promoter region of Hp gene [16, 17]. However, the Hp mRNA expressed in TLY minipigs with "hypohaptoglobinemia" are similar to other normal minipigs (Table 1) indicating that the extremely low Hp levels of TLY minipigs are not associated with the Hp gene deficiency. In healthy cattle, the mean concentration of plasma Hp is about 0.084 mg/m*l* [3]; the low plasma Hp levels found in our minipigs may mimic the characteristics of cattle.

While the mechanisms related to the low levels of Hp in TLY minipigs are unknown, it should be mentioned that these minipigs were first found on Lanyu Island in southeastern Taiwan. In 1980, 4 boars and 16 sows were imported to Taiwan and randomly mated to produce offspring. The coats of the offspring appeared to have a few white spots on solid black coats and were thus named spotty Lanyu minipigs. The spotty minipigs were isolated, and subsequently, some of their offspring had white coats. These offspring are now bred for use as animal models in our laboratories [25]. Hypothetically, the large amount of "hypohaptoglobinemia" in TLY minipigs might be a consequence of close inbreeding.

Hp-Hb complex leads hemolyzed Hb clearance by CD163/HbSR through endocytosis of macrophages or liver parenchymal cells to attenuate undesired glomerular filtration of Hb. Interestingly, the initial uptake or recovery rate (first 40 min) of injected FITC-Hb in TLY minipigs with low levels of Hp was significantly greater than those of TLY minipigs with high Hp levels (Fig. 9). The higher recovery rate in the circulation could be explained by the limited level of Hp that is responsible for the Hb clearance. However, the overall clearance rate of FITC-Hb (downward slope) in these pigs was almost the same between the two groups. It remains unclear as to why both groups reached the same plateau at 120 min. This might be due to the limited level of Hp being sufficient for Hp clearance for the amount we injected (1.5 mg/kg body weight). On the other hand, a previous report has shown that Hp knockout (–/–) mice are able to clear Hb through altered by pass pathways utilizing the liver, spleen and kidney [15]. However, during severe intravascular hemolysis, Hp deficient mice suffer serious renal

Table 1. Amino acid identity/divergence of Hp between domestic pigs and TLY minipigs

		Percentage of identity (%)						
		Domestic	TLY					
		NP_999165[a)]	104–2[b,c)]	113–1[b,c)]	113–2[b,d)]	126–1[b,c)]	153–4[b,d)]	153–5[b,d)]
Divergence (%)	Domestica	–	99.1	98.8	98.8	98.8	99.1	99.1
	104–2[b,c)]	0.9	–	99.7	99.7	99.7	100	100
	113–1[b,c)]	1.2	0.3	–	99.4	99.4	99.7	99.7
	113–2[b,d)]	1.2	0.3	–	–	99.4	99.7	99.7
	126–1[b,c)]	1.2	0.3	0.6	0.6	–	99.7	99.7
	153–4[b,d)]	0.9	0.0	0.3	0.3	0.3	–	100
	153–5[b,d)]	0.9	0.0	0.3	0.3	0.3	0.0	–

a) Hp amino acid sequence from GenBanks NCBI (NP_999165).
b) Hp amino acid sequence of TLY minipigs analyzed in our laboratory.
c) TLY minipigs with low Hp levels.
d) TLY minipigs with high Hp levels.

Table 2. Mean plasma Hp concentrations (mean ± SD) of TLY minipigs and domestic pigs, including Duroc, Landrace and Yorkshire pigs

Trait	n	Hp (mg/ml)
TLY minipig	43	0.21 ± 0.25
Duroc (D)	12	0.60 ± 0.18
Landrace (L)	15	0.78 ± 0.51
Yorkshire (Y)	8	1.05 ± 0.55
(D)+(L)+(Y)	35	0.78 ± 0.45

damage resulting in poor recovery and regeneration of renal tissue [23, 24].

The original characteristics of TLY minipigs are a dark black heavy coat and ears that are smaller than those of most domestic breeds. They weigh about 25 kg when sexually matured compared with 100 kg for other domestic pigs. The morphologic characteristics of TLY minipigs are distinct from other breeds; however, we showed that the amino acid sequence of Hp of TLY minipigs does not diverge from that of domestic species in the present study. There was only one amino acid residue that was consistently converted (Val-65→Leu-65) and the identity and similarity in comparison with domestic pigs were 99.7% and 100%, respectively. The TLY minipigs may be a relevant animal model for pre-clinical studies of such things as atherosclerosis, restenosis and heart transplantation due to the similarities between their cardiovascular system and that of humans [25]. Whether TLY pigs can be used as a model for infectious diseases (using Hp as a marker) has not been explored and remains a subject of interest. However, since there was significant individual variation of the Hp level among the TLY minipigs, the Hp levels should be prescreened to minimize the variation in baseline levels, especially in relation to inflammatory, infectious or other related diseases. The present study may provide a reference value for future use of the TLY minipig as an animal model related to inflammation-associated diseases.

ACKNOWLEDGMENTS. This work was supported by NSC 95–2313-B-009–003-MY2 from the National Science Council, Taiwan, ROC. We especially thank Yang Ting Hsiao, Chun Wei Hung, Shen Yu Hung, Hung Shen Chang, Tsung Min Hsu and Tung Yueh Li of the Taitung Animal Propagation Station for technical assistance and performance of the animal pathological examinations. We also thank James Lee for his editing effort in preparing this manuscript.

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