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Molecular characterization and validation of commercially available methods for haptoglobin measurement in bottlenose dolphin

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ABSTRACT

Haptoglobin (Hp) is a positive acute-phase protein and a valuable marker of inflammation in both human and veterinary medicine. The aim of this study was to validate the molecular characterization of Hp in dolphins and to validate commercially available Hp measurement methods such as Hp-ELISA (originally designed for pigs) and Hp–hemoglobin (Hb) binding assay. The dolphin Hp (dHp) amino acid sequence appeared most similar to pig Hp by sequence homology and phylogenetic clustering. Amino acid sequence analysis revealed that dHp comprises the Hp1 form of α1 and β chains. The anti-pig Hp antibody cross-reacted with both recombinant dHp, expressed by *Escherichia coli*, and dHp from serum. The intra- and inter-assay levels of imprecision of pig Hp-ELISA and the Hp–Hb binding assay were found to be tolerable for the determination of Hp in dolphin, and there was no significant discrepancy between the two determination methods. The ability of the assay to differentiate between healthy and inflammation groups was investigated, and a significant increase in Hp concentration was detected in inflammatory conditions. Thus, Hp is a useful inflammation marker for dolphin, and the Hp concentration in dolphin serum samples can be reliably measured using commercially available pig Hp-ELISA and Hp–Hb binding assay.

1. Introduction

The routine health monitoring of captive cetaceans is performed based on changes in the factors associated with inflammatory responses, indicated by outward signs such as white blood cell counts (WBC), erythrocyte sedimentation rate (ESR) and body temperature (BT) [1,2]. However, timely diagnosis of inflammatory responses in cetaceans is often problematic; as such, initiation of treatment can be delayed and cetaceans can die rapidly while exhibiting minimal outward signs of disease. Currently available diagnostic tools for cetaceans are often insufficiently sensitive to detect disturbances of homeostasis. Thus, the identification of markers of inflammation that can be developed into high-sensitivity measurement methods are likely to be helpful for diagnosis of inflammation in cetaceans.

Acute phase proteins (APP) such as serum amyloid A (SAA), C-reactive protein and haptoglobin (Hp) can be used to detect dramatic changes in serum concentrations early in an inflammatory response [3]. The major site of APP synthesis is the liver and the dramatic fluctuations in the concentration of APP in blood reflect changes in proinflammatory cytokine concentrations [3]. As such, APP are thought be useful markers of inflammatory disease. Although APP responses differ from one species to the next, SAA and Hp levels have been known to increase 5- to 1000-fold in response to infection and inflammation in many animals, and have been measured using commercially available methods that cross-react with different species [3,4]. Thus, SAA and Hp have been used as highly sensitive, quantitative biomarkers of inflammation for general health screening in both human and veterinary medicine.

In cetaceans, many anatomical systems are highly modified for an aquatic lifestyle, and in this process of adaptation, cetacean APP such as SAA and Hp might have drifted evolutionally from terrestrial mammals. In fact, we have previously demonstrated that dolphin SAA shows different characteristics from many other animals and is not detected by a Multispecies SAA ELISA kit, which can quantify SAA from various species [5]. Thus, in dolphins, a specific detection system is required for determination of APP levels in blood samples.

In general, to quantify Hp in serum or plasma, Hp–Hb binding assay and ELISA are the used most common methods [4]. Although

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the Hp levels in serum samples from cetaceans have been measured using a commercially available Hp–Hb binding assay, the precision was not validated for clinical applications [6].

In this study, to evaluate the utility of Hp as an inflammation marker in dolphins, we carried out molecular characterization of dolphin Hp (dHp), validated a commercially available ELISA and Hp–Hb binding assay for dHp determination in clinical samples, and compared both assays.

2. Materials and methods

2.1. Sample

Bottlenose dolphins (*Tursiops truncatus*) were examined in this study. Liver samples were collected from one adult and two neonatal individuals that had accidentally died at Minamichita Beach Land Aquarium and Yokohama Hakkeijima Sea Paradise, respectively. Blood collection was carried out by the staff of Shinagawa Aquarium and Okinawa Churaumi Aquarium from 2010 to 2012. Serum samples were obtained using a vacutainer and assigned to either a healthy group ($n = 10$, sample = 46; WBC 3000–8000 cells/μl, ESR < 2 cm and BT 36.0–36.9 °C; normal clinical history and no macroscopic abnormality at blood sampling) or an inflammation group ($n = 5$, sample = 46; WBC $>$ 10,000 cells/μl, ESR > 2 cm and BT $>$ 37.0 °C; decreased appetite at blood sampling), and stored at −20 °C until analysis. Normal hematological ranges for bottlenose dolphins were obtained from the CRC Handbook of Marine Mammal Medicine, second edition [1]. The protein concentration of serum was determined using the Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan) using BSA as a standard.

2.2. Determination of N-terminal amino acid sequence

Serum samples from the healthy ($n = 3$) and inflammation ($n = 3$) groups were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% polyacrylamide gels under reducing conditions. The serum samples were applied at 25 μg protein/lane. After separation, semi-dry Western blotting onto an Immobilon-P Transfer Membrane (Millipore, Bedford, MA, USA) was carried out for 1 h at room temperature. The membrane was stained with EzStain AQua (ATTO Corporation, Tokyo, Japan) and then destained with 7.5% acetic acid and 50% methanol. The protein band corresponding to about 35 kDa was excised and submitted to N-terminal amino acid sequencing using a protein sequencer (Applied Biosystems, model 492).

2.3. Cloning and sequence analysis of dolphin haptoglobin (dHp) cDNA

Total RNA was isolated from liver samples using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The primers used in this study are shown in Table 1. PCR primers were designed based on published cattle and pig Hp cDNA sequences (GenBank accession numbers: NM_001040470 and NM_214000, respectively). First-strand cDNA synthesis and amplification of partial dHp cDNAs were performed as previously described [7]. Firstly, 5′ and 3′ RACE cDNAs were generated using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. These RACE reactions were followed by PCR using dHp-specific primers in addition to the universal primer mix included in the SMART RACE kit. Nucleotide sequences of the PCR products were determined by direct sequencing using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The signal peptide and mature protein sequences were predicted using SMART (http://smart.embl-heidelberg.de/). Multiple alignments of dHp from other animals were generated and analyzed using ClustalW [8]. Phylogenetic trees were created by the neighbor joining (NJ) method with MEGA4.0 software.

2.4. Expression and purification of recombinant dHp protein (rdHp) from Escherichia coli

To express the mature form of dHp as a recombinant protein, the deduced sequence encoding the signal peptide was excluded from the target PCR product. The primer pair used to generate this product is shown in Table 1. The PCR product was inserted into the pET100 vector (Invitrogen, Carlsbad, CA, USA) and rdHp was expressed as a His-tagged fusion protein. This expression plasmid was transformed into *E. coli* strain, BL21 Star (DE3) (Invitrogen). Transformants were isolated and grown overnight in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin. Overnight cultures were diluted 1:20 in LB medium containing 100 μg/ml ampicillin and grown to an optical density (OD_{600}) of 0.7. Expression of the recombinant fusion protein was induced in cultures for 6 h at 37 °C using 1 mM isopropyl β-D-thiogalactosidase (IPTG). The medium was centrifuged at 3000*g* for 10 min and the induced cells were suspended and sonicated, followed by centrifugation at 9000*g* for 30 min at 4 °C. The pellet containing the inclusion bodies was washed three times with 50 mM Tris–HCl, 200 mM NaCl, and 2% Triton X-100, pH 8.0. Finally, the pellet was washed twice with 50 mM Tris–HCl and 200 mM NaCl. The inclusion bodies were dissolved and gently stirred in 50 mM Tris–HCl, 150 mM NaCl, 5 mM imidazole and 8 M urea, pH 8.0, for 1 h at 4 °C and then centrifuged at 9000*g* for 30 min at 4 °C. The remaining soluble supernatant was passed through a syringe filter (0.45 μm) and then refolding was initiated by 25-fold dilution in 50 mM Tris–HCl and 150 mM NaCl carried out with constant slow stirring for 1 h at room temperature. To isolate rdHP, the remaining soluble supernatant was purified by immobilized metal affinity chromatography on a His trap HP column (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions. The purified recombinant protein was assessed by SDS-PAGE using 15% polyacrylamide gels. The gels were stained with EzStain AQua (ATTO Corporation) and destained with distilled water. The protein concentration was determined using the Protein Assay CBB Solution (Nacalai Tesque) and BSA as a standard.

2.5. Western blotting

To evaluate the specificity of the anti-pig Hp polyclonal antibody conjugated to horseradish peroxidase (HRP) included in the commercially available Hp-ELISA kit designed for pig (pig Haptoglobin ELISA kit; Immunology Consultants Lab Inc., Portland, OR, USA), Western blotting was carried out. This antibody reacts to the α- and β-chains of pig Hp. SDS-PAGE was performed using 15% polyacrylamide gels. The rdHp and serum samples from healthy ($n = 3$) and inflammation ($n = 3$) groups were applied at 2 μg protein/lane. After separation, semi-dry Western blotting onto an Immobilon-P Transfer Membrane (Millipore) was carried out for 1 h at room temperature and the membrane was blocked in 20 mM Tris–HCl, 150 mM NaCl and 0.1% (v/v) Tween 20, pH 7.6 containing Block Ace (Dainihon Pharmaceutical Co., Ltd., Osaka, Japan) for 1 h at room temperature. After blocking, the membrane was incubated with anti-pig Hp antibody (1:100 dilution) in 20 mM Tris–HCl and 150 mM NaCl, and 0.1% (v/v) Tween 20, pH 7.6 containing Block Ace (Dainippon Pharmaceutical Co., Ltd.) for 1 h at room temperature and then washed three times with 20 mM Tris–HCl, 150 mM NaCl, pH 7.6 containing 0.1% (v/v) Tween 20 at room temperature. The visualization of immunoreactive bands was performed using the AE-9300 EZ-capture chemiluminescence imaging system (ATTO Corporation) with an enhanced chemiluminescence (ECL) prime Western blotting detection reagent (GE Healthcare, Buckinghamshire, UK).

Table 1
PCR primers used in this study

Primer name	Primer sequence (5′→3′)	Use
HpF	CTCCTGCTCTGCGGGC	Partial cDNA cloning
HpR	CCCACATAGAGTCTTAAAGTAGGAG	Partial cDNA cloning
vedHpF	CACCCCACAACTGCCAC	Protein expression
vedHpR	TTAGTTGTCAGCTATGGTTTTCT	Protein expression

2.6. Hp-ELISA and Hp–Hb binding assay

ELISA was performed with the pig Haptoglobin ELISA kit (Immunology Consultants Lab Inc.), and the dHp concentration in serum was quantitated by a standard curve obtained using rdHp. Following manufacturer's instructions, prior to the assay, serum samples were diluted 1:10000 with sample dilution buffer. Then, 100 μl of the diluted sample or standard solution, in duplicate, was added to each well and incubated for 15 min at room temperature. After a thorough washing with wash buffer, 100 μl of enzyme–antibody conjugate (anti-pig Hp polyclonal antibody conjugated to HRP) was added to each well and incubated for 15 min at room temperature. The plate was washed with the same buffer and reacted with 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for 10 min at room temperature. The reaction was stopped by adding 100 μl of stop solution. The results were read at 450 nm using a spectrophotometer.

The Hp–Hb binding assay was carried out with the PHASE RANGE Haptoglobin Assay Kit (Tridelta Development, Greystones, Ireland). Following manufacturer's instructions, 7.5 μl of each prepared calibrator along with the serum samples, in duplicate, were transferred to a microplate. Then, 100 μl of reagent 1 (stabilized hemoglobin) was added to each well. After gentle mixing, 140 μl of reagent 2 (a mixture of chromogen and substrate) was added to each well. The mixtures were incubated for 5 min at room temperature. The results were read immediately at 630 nm using a spectrophotometer.

2.7. Comparison of assay performances

Analytical validation was determined by intra- and inter-assay coefficient of variation (CV). Serum pools from dolphins ($n = 10$, sample $= 20$) in the healthy group and from dolphins ($n = 5$, sample $= 20$) in the inflammation group were used as sources of low and high Hp concentrations, respectively. The intra-assay CV was calculated by measuring the pools six times in a single assay, while the inter-assay CV was assessed by measuring the same pools on 5 different days. All samples were frozen in aliquots and only vials needed for each analytical run were thawed in order to prevent potential variation due to repeated freeze–thaw cycles. The CVs were assessed using standard descriptive procedures.

The limit of detection (LOD) was calculated as LOD $= 3.3$ SD$/S$. The SD of the response can be determined based on the SD of the blank, while S is the slope of the calibration curve in the initial stages [9].

Trueness was assessed by a comparison experiment. Briefly, serum samples from the healthy ($n = 10$, sample $= 46$) and inflammation ($n = 5$, sample $= 46$) groups were analyzed with the pig Hp-ELISA and Hp–Hb binding assay, and regression analysis was used to evaluate differences between the two methods. Agreement between results obtained using the two methods was evaluated using a Bland–Altman difference plot and Wilcoxon signed rank test [10]. Significance was set at $P < 0.05$.

2.8. Clinical validation of the Hp-ELISA and Hp–Hb binding assay

Hp concentrations in serum samples from the healthy ($n = 10$, sample $= 46$) and inflammation ($n = 5$, sample $= 46$) groups were determined by pig Hp ELISA and Hp–Hb binding assay. The comparison was made using the Mann–Whitney test and the probability level of $P < 0.05$ was considered significant.

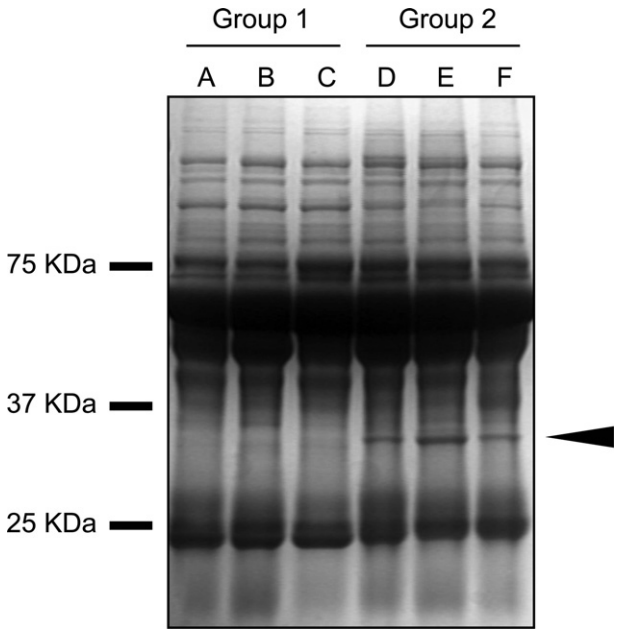

Fig. 1. SDS-PAGE of serum samples from group 1, dolphins in healthy group (Lanes A, B and C), and group 2, dolphins in inflammation group (Lanes D, E and F). The band indicated with a solid arrowhead was determined to be haptoglobin by N-terminal amino acid sequence analysis. The protein amount loaded per lane was 25 μg.

3. Results

3.1. Determination of N-terminal amino acid sequence

Fig. 1 shows the SDS-PAGE results of serum samples from clinical healthy dolphins ($n = 3$) and dolphins with inflammation ($n = 3$). A protein band with a molecular mass of about 35 kDa was clearly detected in the inflammation group. This band was subjected to N-terminal amino acid sequence analysis and was found to comprise exclusively the sequence GGSLDAKGSFPWQAK, corresponding to the Hp β-chain from UniProt numbers Q2TBU0 (cattle), Q8SPS7 (pig), P19007 (rabbit), Q61646 (mouse), E3UTY9 (cat) and G1K2D9 (dog).

3.2. Cloning of dHp cDNA

The open reading frame of the gene encoding dHp was composed of 1038 nucleotides encoding a predicted protein of 345 amino acids (GenBank accession number: AB775208). Identical nucleotide sequences were found in cDNA samples from three different dolphins. dHp comprises an 18-residue signal peptide (aa 1–18), an 81-residue α-chain (aa 19–99) and a 245-residue β-chain (aa 101–345). The molecular masses calculated from the sequences of the α- and β-chains are 9.2 kDa and 27 kDa, respectively (Fig. 2). The dHp α-chain does not have the repeat region observed in human Hp2, cattle Hp and deer Hp, while it has a two-codon deletion corresponding to

Table 2
Comparative homology between the deduced amino acid sequence of dolphin haptoglobin (Hp) and Hp of other mammals

	Amino acid homology (%)		
	Overall	α-chain	β-chain
Human Hp1	79.8	72.5	82.8
Rabbit	78.3	72.5	80.8
Mouse	75.7	70.5	77.9
Cat	77.2	66.6	81.6
Dog	77.8	63.3	83.6
Pig	81.8	71.5	86.1
Human Hp2	68.2	47.4	82.8
Cattle	68.0	45.9	81.2
Deer	69.0	44.8	84.0

UniProt numbers: Q0VAC5, human Hp1; P19007, rabbit; Q61646, mouse; E3UTY9, cat; G1K2D9, dog; Q8SPS7, pig; Q8SPS7, human Hp2; Q2TBU0, cattle and B6D985, deer.

residues 11 and 43 (pig Hp numbering). The dHp α- and β-chains have cysteine residues at positions 14, 33, 66 and 70 / 105, 148, 179, 190 and 220, respectively, and are conserved among mammalian Hps except for cat and dog at position 15 on the α-chain. The dHp β-chain contains two potential N-glycosylation sites at positions 23–25 and 49–51, and two lysine residues involved in Hb binding as well as residues homologous to the catalytic triad of serine proteinase conserved in all Hps, including dHp, at positions 136 and 218 / 41, 85 and 194, respectively. Table 2 shows homology between the deduced dHp amino acid sequence and Hp from several mammals. Hps with the highest identity with the α-chain were human Hp1 (72.5%) and rabbit Hp (72.5%), and with the β-chain were pig Hp (86.1%); pig Hp had the highest identity overall (81.8%). A phylogenetic tree based on the β-chain revealed that dHp is the shortest phylogenetic distance from pig Hp (Fig. 3).

3.3. Recombinant dHp protein

The purified rdHp fusion protein obtained by immobilized metal ion affinity chromatography corresponded to a single 39 kDa band after SDS-PAGE (Fig. 4).

3.4. Cross-reactivity between dHp and anti-pig Hp antibody

The anti-pig Hp antibody recognized bands of about 39 kDa and 35 kDa from rdHp and a serum sample from the inflammation group, respectively, but no band was detected from the serum sample of the healthy group (Fig. 5).

3.5. Comparison of assay performances

The results of the precision study are shown in Table 3. Intra- and inter-assay CVs for ELISA were 3.2–3.8%, and 9.7–15.8%, respectively; those for the Hp–Hb binding assay were 3.3–3.5%, and 10.4–21.7%, respectively, with the highest CVs in serum samples having low Hp. The LOD for the pig Hp-ELISA designed and Hp–Hb binding assay were 0.047 ng/ml and 0.068 mg/ml, respectively. The linear regression model between pig Hp-ELISA and the Hp–Hb binding assay yielded a *y*-intercept at the 95% confidence interval not different from 0 (0.17, range 0.07–0.26), and a slope at the 95% confidence interval not different from 1 (1.01, range 0.97–1.04). The difference between both methods seemed to increase with increasing dHp concentrations in serum, but this difference was not significant ($P = 0.23$) (Fig. 6 A and B).

3.6. Clinical validation of the pig Hp-ELISA and Hp–Hb binding assay

The results of this analysis are shown in Fig. 7. In the pig Hp-ELISA and Hp–Hb binding assay, the mean ± SD mg/ml (median; min–max) dHp concentrations were 0.59 ± 0.62 mg/ml (0.34; 0.04–1.96) and 0.58 ± 0.55 mg/ml (0.35; 0–1.56) in serum samples from the healthy group and 3.96 ± 1.35 mg/ml (3.68; 2.21–8.58) and 3.52 ± 1.17 mg/ml (3.21; 2.12–7.32) in serum samples from the inflammation group, respectively. The values determined by both methods were not significantly different ($P = 0.92$; healthy group and $P = 0.09$; inflammation group), while dHp concentrations were significantly higher in the inflammation group than in the healthy group ($P < 0.05$).

Table 3
Intra- and inter-assay variation in determination of haptoglobin (Hp) concentration in bottlenose dolphin serum

	ELISA (mg/ml)		Hp–Hb binding assay (mg/ml)	
	Mean (SD)	CV (%)	Mean (SD)	CV (%)
Intra-assay	4.65 (0.15)	3.2	4.23 (0.14)	3.3
	1.06 (0.04)	3.8	0.86 (0.03)	3.5
Inter-assay	4.75 (0.46)	9.7	4.43 (0.46)	10.4
	0.95 (0.15)	15.8	0.83 (0.18)	21.7

4. Discussion

In this study, we observed that dHp levels in the serum increase under inflammatory conditions. We evaluated, for use in dolphins, commercially available Hp measurement methods consisting in a Hp-ELISA designed for pigs and a Hp–Hb binding assay. In addition, the ability of the assay to differentiate between clinically healthy and inflammatory conditions was investigated.

Hp comprises α- and β-chains, and is known to be synthesized as a single polypeptide chain, which is then processed to yield the mature form upon release of an arginine residue located at the junction between α- and β-chains [11]. The α-chain is distinguished as α1 or α2; the α1 chain is observed in many animals except for ruminants such as cattle and deer, and the α2 chain, which contains an additional repeat region to α1, exists in human as well as ruminants and the α- and β-chains form the mature Hp, classified as Hp1 or Hp2, corresponding to the formation of two polypeptide chains, α1β or α2β, respectively [12–14]. We determined the dHp cDNA sequence from liver because, in general, Hp is synthesized in this organ [3]. There was no polymorphism detected in the deduced amino acid sequence analysis of dHp from three dolphins, while a difference of two amino acid residues in the corresponding sequences between our study (accession number: AB775208) and a simple protein search (Protein ID: ENSTTRT00000001911) from the Ensemble webpage was observed. The signal peptide, α-chain and β-chain contain 18, 81 and 245 amino acid residues, respectively. The α-chain of dHp appears to be an α1-chain because it does not include a repeat region. Thus, the mature form of dHp appears to be the Hp1 observed in many species. This result corresponds to the results of examinations on the size of the α-chain in dolphins [14]. However, the α-chain of dHp has a two-codon deletion compared to that of other animals. This unique mutation may have occurred during the unique evolution of cetaceans when their ancestors shifted from a terrestrial to an aquatic habitat [15]. A total of nine cysteine residues in the α- and β-chains are highly conserved: cysteine-70 on the α-chain and cysteine-105 on the β-chain participate in the monomer by disulfide bridge linking, and dimerization of two monomers occurs via disulfide bridges at cysteine-15 on the α-chain of each monomer [12,16,17]. Dog and cat Hp lack the cysteine-15 residue and are dimerized by non-covalent hydrophobic interactions [18]. Lysine residues at positions 136 and 218 on the β-chain are involved in Hb binding [19]. These two lysine residues are conserved across all Hps, including the bottlenose dolphin protein. Thus, dHp is thought to possess Hb-binding capacity. One feature of the amino acid sequence of Hp is that even though the β-chain is

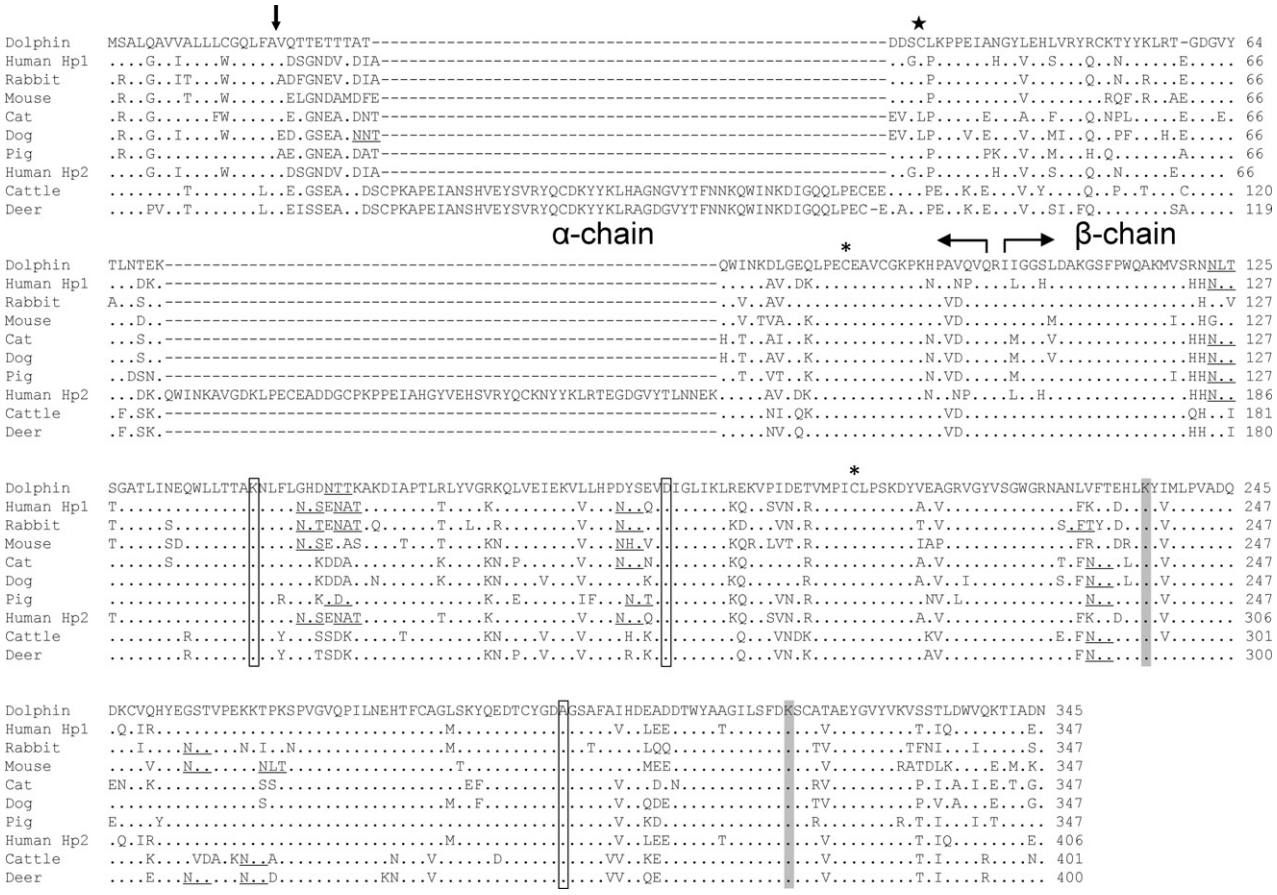

Fig. 2. A comparison of the deduced amino acid sequence of dolphin haptoglobin (Hp) with Hp from other species (UniProt numbers: Q0VAC5, human Hp1; P19007, rabbit; Q61646, mouse; E3UTY9, cat; G1K2D9, dog; Q8SPS7, pig; Q8SPS7, human Hp2; Q2TBU0, cattle; B6D985, deer). Dots (.) indicate conserved amino acid residues. Gaps in the alignment are indicated by a dash (-). The arrow indicates the signal peptide cleavage site. Asterisks indicate binding sites of α- and β-chains for forming monomers by disulfide bridges. The star indicates the binding site of monomers for forming dimers by disulfide bridges. Gray boxes indicate the lysine residues involved in hemoglobin binding. Boxes indicate the residues homologous to the catalytic triads characteristic of serine proteinases.

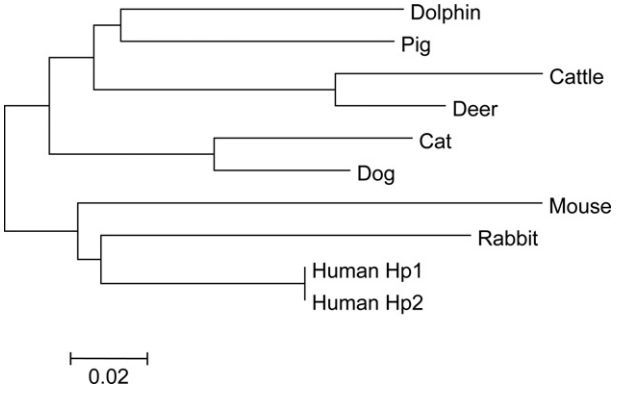

Fig. 3. Phylogenetic tree constructed according to the amino acid sequences of the β-chain. For the branch length, the distance given on the scale represents 0.02 amino acid substitutions per site. The accession numbers of the sequences used are given in Fig. 2.

highly homologous to the mammalian serine proteases of the chymotrypsin family, Hp does not have any proteolytic activity [16,19]. This is due to the fact that two of the catalytic triads characteristic of serine proteinases, histidine-57 and serine-195 (UniProt number: P00766, bovine chymotrypsinogen A numbering), are replaced by lysine-41 and alanine-194, respectively, on the β-chain [17]. On the other hand, asparagine acid-102 on bovine chymotrypsinogen A and asparagine acid-85 on the β-chain are conserved among all animals.

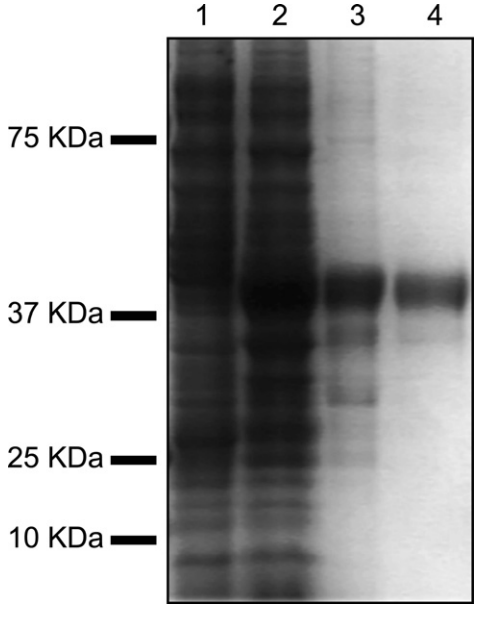

Fig. 4. SDS-PAGE of recombinant dolphin haptoglobin (rdHp) expressed in *E. coli*. Lane 1: rdHp protein from uninduced cultures. Lane 2: rdHp protein from cultures induced with isopropyl β-D-thiogalactosidase (IPTG). Lane 3: inclusion bodies including rdHp after washing. Lane 4: His-tagged rdHp purified by affinity chromatography on a His-trap column.

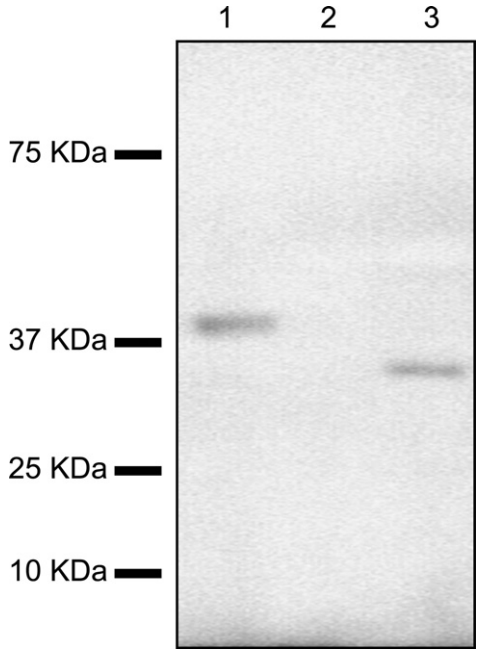

Fig. 5. Western blot analysis of recombinant dolphin haptoglobin (rdHp) and serum samples from healthy and inflammation groups using the detection antibody included in the pig Hp ELISA kit. Lane 1: rdHp expressed in *E. coli*. Lane 2: serum sample from dolphins in healthy group. Lane 3: serum sample from dolphins in inflammation group. The protein amount loaded per lane was 2 μg.

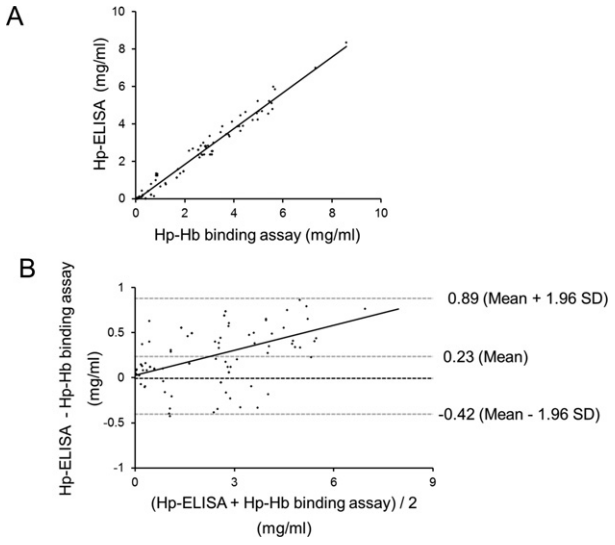

Fig. 6. Trueness was assessed by a comparison experiment using an ELISA designed for measuring pig haptoglobin (Hp) (Hp-ELISA) and a Hp–hemoglobin (Hb) binding assay with serum samples from healthy (n = 10, sample = 46) and inflammation (n = 5, sample = 46) groups. (A) The solid line represents the regression equation: Hp-ELISA (mg/ml) = 0.97 × Hp–Hb binding assay (mg/ml) + 0.16. The y-intercept at the 95% confidence interval was not different from 0 (0.16, range 0.07–0.24), the slope at the 95% confidence interval was not different from 1 (0.97, range 0.94–0.99), and the correlation coefficient was r = 0.97. (B) Bland–Altman plot for Hp concentration. The solid black line indicates the linear regression line for the difference = 0.092 × Mean + 0.029 (r = 0.25), the dashed grey lines indicate mean of the difference and the 95% limits of agreement (mean of the differences ± 1.96 SD of the mean difference) and the dashed black line indicates zero bias.

The amino acid sequence of dHp was most similar to pig Hp, phylogenetically clustering closest to this species. These results suggest that although there is a minor unique change in the structure of dHp, the molecular structure of dHp is similar to that of other animals, in particular pig.

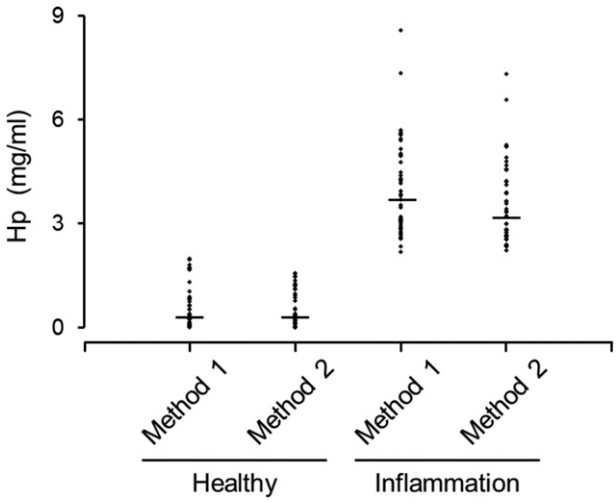

Fig. 7. Haptoglobin (Hp) values assayed by Hp-ELISA designed for pigs and Hp–hemoglobin (Hb) binding assay in healthy dolphins (n = 10, sample = 46; WBC 3000–8000 cells/μl, ESR < 2 cm and BT 36.0–36.9 °C) and dolphins with inflammation (n = 5, sample = 46; WBC > 10,000 cells/μl, ESR > 2 cm and BT > 37.0 °C). Horizontal lines show median values. Method 1 and Method 2 indicate Hp-ELISA designed for pigs and Hp–Hb binding assay, respectively.

To validate the specificity of the anti-pig Hp antibody included in a commercially available pig Hp-ELISA for dHp, we performed Western blotting. Although this antibody reacts to the α- and β-chains of pig Hp, a single band was detected with both rdHp and serum samples from dolphins with inflammation. In general, the Hp in serum or plasma separates into an α-chain (~9 kDa) and a β-chain (~35 kDa) under reducing conditions [11]. In the present study, we produced rdHp from *E. coli*, and non-cleavage of rdHP into α- and β-chains in *E. coli* was expected since the C1r-like protease, responsible for the cleavage in mammalian cells, is not present in *E. coli* [20,21]. On the other hand, the single band observed in serum from dolphins with inflammation was 35 kDa, which corresponds to the β-chain. Thus, it was concluded that this antibody cross-reacts with only the β-chain of dHp.

It is generally accepted that the CV must be lower than 10% for analytical determinations. However, in the case of immunological assays such as ELISA, CVs could be acceptable upto 15% or 20% if the concentrations are very low and close to the detection limit of the assay [22]. The intra- and inter-assay imprecision (CVs) of a commercially available pig Hp-ELISA and a Hp–Hb binding assay were found to be acceptable for determination of dHp in serum. The inter-assay imprecision of the Hp–Hb binding assay was >20% when the Hp content was low, but this problem could be resolved by performing all analyses with the same batch. The LOD of the ELISA was extremely low compared to that of the Hp–Hb binding assay (more than 1000-fold more sensitive). Thus, the pig Hp-ELISA is more sensitive for dHp determination in serum samples than the Hp–Hb binding assay. In the comparison experiment, the high correspondence between pig Hp-ELISA and the Hp–Hb binding assay was demonstrated with a linear regression model and differences between the results obtained by the two methods had a tendency to increase with increasing dHp concentrations in serum, but these differences were not significant. Thus, agreement between both techniques indicates that the results were not due to non-specific reactivity, and dHp concentrations in serum samples can be reliably measured using these methods.

Dolphins with inflammation displayed a significant elevation in serum dHp concentration compared with healthy dolphins (about 10-fold). Thus, dHp appears to be a helpful inflammation marker. The mean dHp concentrations in healthy dolphins ± SD mg/ml (median; min–max) were 0.59 ± 0.62 mg/ml (0.34; 0.01–1.96) and 0.58 ±

0.55 mg/ml (0.35; 0–1.56) by pig Hp-ELISA and Hp–Hb binding assay, respectively. Corresponding data reported by Cray et al. was 0.13 ± 0.11 mg/ml (0.10; 0.01–0.5) by Hp–Hb binding assay [6]. Cray et al. and we completed this assay by the automated method and manual method, respectively, using different criteria to determine healthy individuals. Thus, the discrepancy in Hp concentrations in healthy dolphins may have been caused by the differences in measuring methods and/or the criteria for health between the two studies.

The present results indicate that dHp concentration in serum samples can be reliably measured using a commercially available pig Hp-ELISA and Hp–Hb binding assay, and dHp appears to be a helpful marker for clinical diagnosis of inflammation in dolphins. In this study, we did not clarify whether inflammation could be detected at an early stage because we used dolphins with obvious inflammatory symptoms. Finally, we propose that the measurement of dHp alone appears to be insufficient to monitor dolphin health, and helpful for diagnosis of inflammation only in combination with traditional methods such as WBC and ESR.

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