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# Local identification of porcine haptoglobin in salivary gland and diaphragmatic muscle tissues

**A.M. Gutiérrez[1], J. Yelamos[2], F.J. Pallarés[3], J. Gómez-Laguna[4]* and J.J. Cerón[1]**

[1]Department of Animal Medicine and Surgery, University of Murcia, Espinardo, Murcia, Spain, [2]Department of Immunology, Cancer Research Program, IMIM-Hospital del Mar, Barcelona, Spain, [3]Department of Anatomy and Comparative Pathological Anatomy, University of Murcia, Espinardo, Murcia, Spain and [4]Department of Anatomy and Comparative Pathological Anatomy, University of Cordoba, Cordoba, Spain

*Present address: CICAP, Pozoblanco, Cordoba, Spain

**Summary.** In order to clarify the origin of the haptoglobin (Hp) quantified in saliva and meat juice samples, the extrahepatic localization of Hp in salivary gland and in diaphragmatic muscle, as part of the systemic acute phase response in pigs, was studied by immunohistochemistry. For this purpose a specific monoclonal antibody (mAb) produced by immunising mice with purified porcine Hp was used. Reactivity of the mAb was assessed by direct ELISA and by western blot, which showed the ability and specificity of the mAb to identify porcine haptoglobin as a purified antigen or in porcine serum in a native or denatured but non-reduced state. Five healthy and five diseased pigs were sampled at slaughter for serum and tissue procurement. Hepatic immunohistochemical analysis was used as control of the acute phase reaction status. In the liver, cell immunostaining revealed a perinuclear, cytoplasmic localization of Hp within hepatocytes, following mainly a periacinar pattern. Extrahepatic immunohistochemical analysis revealed positive cells in the glandular acini and duct epithelial cells of the salivary gland and intrasarcoplasmic immunolabelling of random diaphragmatic myofibers. A possible role of both salivary gland and diaphragmatic muscle on local Hp production could be postulated based on the present immunohistochemical study, which supports the concept that other cells besides hepatocytes may have the potential to produce Hp in the pig.

**Key words:** Haptoglobin, Pig, Immunohistochemistry, Monoclonal antibody

*Offprint requests to:* Jose Joaquin Cerón Madrigal, Department of Animal Medicine and Surgery, University of Murcia, 30100, Espinardo, Murcia, Spain. e-mail: jjceron@um.es

## Introduction

Acute phase proteins (APP) are serum proteins which are up- and down-regulated following homeostasis disturbance such as infection, inflammation, tissue injury or neoplasia (Heegard et al., 1998). The synthesis of acute phase proteins (e.g. Haptoglobin (Hp), serum amyloid A and C-reactive protein) is mainly produced in the liver and is induced by proinflammatory cytokines such as interleukin 6 and tumor necrosis factor (Hiss et al., 2008).

In pigs, studies about extrahepatic production of APP have been focused on the respiratory tract. An extrahepatic production of C-reactive protein has been reported in vascular smooth muscle cells (Kuji et al., 2007), pulmonary fibroblast and endothelial cells in the lung (Päiväniemi et al., 2009). Moreover, Hp has been located in airway epithelial cells and immigrated leucocytes (Hiss et al., 2008) and in intestinal lymph nodes and peripheral lymphoid tissues of pigs (Skovgaard et al., 2009).

Serum haptoglobin has been used as an inflammatory marker to assess animal health (Eurell et al., 1992), monitor antibiotic therapy (Hultén et al., 2003), and as a tool for veterinary inspection at slaughter (Petersen et al., 2002). Measurements of Hp in saliva or meat juice samples have been proposed as suitable alternatives to serum (Gutiérrez et al., 2009a) since they have several analytical advantages. Saliva is obtained with a simple non-invasive and minimally stressful methodology that could be performed by personnel with minimal training. On the other hand, meat juice is easy to obtain by cutting a diaphragmatic muscle piece at post-mortem examination of animals, either at slaughterhouse without reducing the speed of the

slaughter line or at farm. In fact, meat juice samples are being used for monitoring specific pathogens such as *Salmonella* (Nielsen et al., 1998), Aujezsky's disease virus (Le Potier et al., 1998), *Trichinella* (Kapel et al., 1998), *Actinobacillus pleuropneumoniae* (Wallgren et al., 2000), or Porcine Reproductive and Respiratory Syndrome virus (PRRSv) (Gutiérrez et al., 2009b; Gómez-Laguna et al., 2010).

However, the mechanism by which APPs appear in saliva and meat juice has not been revealed. In order to elucidate this topic, we aimed to investigate the possible extrahepatic localization of Hp in both tissues salivary gland and diaphragmatic muscle. For this purpose we produced a specific mAb against porcine-Hp which recognized native Hp by ELISA, Western Blot and immunohistochemistry.

## Materials and methods

### *Purification of porcine Hp*

Hp was purified from porcine serum, with high levels of Hp, as described before (Fuentes et al., 2011). Briefly, serum was fractionated with ammonium sulphate at a final concentration of 50%, to separate albumin and immunoglobulins, followed by a chromatographic purification using Superdex TM 200 10/300 GL column (Amersham Biosciences, Uppsala, Sweden) and Fast performance liquid chromatographic equipment (FPLC) (Amersham Biosciences, Uppsala, Sweden). Fractions corresponding to pure porcine Hp were pooled and evaluated by SDS-PAGE in 12% poly-acrylamide gels. Electrophoresis was performed for one hour at 180V in a vertical electrophoresis chamber (MINI-Protean Electrophoresis System, Bio-Rad Laboratories, California, USA).

Gel was digitalized and Image analysis software (ImageQuant™ TL, GE Healthcare Life Sciences, Munich, Germany) was used to calculate the molecular weight of the unknown bands. The lanes containing molecular weight markers were identified as standard to calculate the bands of similar molecular weight across all the lanes within a tolerance of 4% of lane height. To verify the presence of porcine Hp in the obtained protein bands, the main band obtained in SDS-PAGE of reduced purified Hp was excised and sent to The Developing and Support Research Center (CAID) of the University of Murcia (Spain) for Mass spectrometry identification.

### *Monoclonal antibody production*

For immunization purposes, Balb/c mice were intraperitoneally injected fortnightly with 50$\mu$g of pure porcine Hp. The first dose of antigen was emulsified with Complete Freund's adjuvant (Sigma Aldrich, St Louise, USA) and the next two immunizations with Incomplete Freund's adjuvant (Sigma Aldrich, St Louise, USA). The final booster was done by intravenous injection of the antigen without adjuvant. The spleen was removed at day 3 and cells were fused with the non-Immunoglobulin-secreting myeloma X63-Ag8.653 using polyethylene glycol 1500 (Sigma Aldrich, St Louise, USA) as reported before (Köhler and Milstein, 1975).

Hybridomas were selected by growth in RPMI 1640 culture medium (Cambrex Bioscience, Verviers, Belgium) containing hypoxanthine, aminopterin, and thymidine (Sigma Aldrich, St Louise, USA) and supplemented with 12% Fetal bovine serum (Gibco, Invitrogen, Hilden, Germany), 2 mM glutamine (Gibco, Invitrogen, Hilden, Germany), 1 mM pyruvate (Sigma Aldrich, St Louise, USA), 100 U/mL of penicillin and 100 mg/mL of streptomycin (Sigma Aldrich, St Louise, USA). Hybridoma cell supernatants were analyzed by a direct ELISA using 96-well plates (Maxisorp surface, Nunc Brand, Roskilde, Denmark) coated with 250 ng/well of purified Hp. Briefly, 250 $\mu$l of PBS with 5% of semi-skimmed milk were added to each well and incubated for 1 hour at room temperature to block unbound sites in the plates. After four washes with 200 $\mu$l of phosphate buffer saline (PBS) with 1% of Tween 20, 100 $\mu$l of supernatant were added and incubated 1 hour. A volume of 100 $\mu$l of a 1:2500 dilution of secondary anti-mouse IgG with horse radish peroxidase (Pierce Biotechnology, Rockford, USA) was applied after an additional four washed and was followed by 1 hour incubation in darkness. A final wash cycle was performed and 100 $\mu$l of a solution of ABTS substrate was used to reveal the enzymatic signal, which was proportional to the amount of specific mouse anti-Hp porcine antibody in each sample. The absorbance of the signal was detected in a Spectrophotometer (Microplate spectrophotometer PowerWave 340, BioTek instruments, Winoosky, VT, USA). Specific anti-Hp hybridomas were subcloned twice by limiting dilution and four stable clones, which produced high titles of antibody, were obtained.

All procedures involving animals were approved by the Murcia University Ethics Committee in accordance with the University of Murcia Institutional Animal Care Guidelines.

### *Purification of mAbs by affinity chromatography column*

The mAbs were partially purified from cell culture supernatant using precipitation with ammonium sulphate at a final concentration of 60% (Ito, 2000). After centrifugation for 30 minutes at 3000xg, the pellet, which contains antibodies, were dialyzed against PBS buffer to remove sulphate ions. Antibodies in the dialyzed buffer were purified using haptoglobin-affinity column chromatography by using CNBr-activated Shepharose-4B (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's procedures.

To purify mAbs, previously precipitated with ammonium sulphate, antibody solution was loaded onto the affinity column at room temperature without incubation, followed by an extensive wash with 50 ml of

PBS. The antibody bound material was eluted with PBS pH 11 as previously described (Yueh et al., 2007). One ml of each fraction was collected in a tube containing 50 $\mu$l of 1 M Tris-HCl buffer pH 6.8, to immediately neutralize the pH value. Fractions absorbance were measured to show the elution profile, by using the spectrophotometer detailed above, and then fractions containing anti-Hp porcine antibodies were pooled and concentrated using Centricon centrifugal filters (Ultracel YM-10, Millipore Corporation, USA).

The purity of the mAb suspension obtained after purification was assessed by SDS-PAGE using a 12% poly-acrylamide gel.

### *Animals and samples*

Ten conventional pigs (five healthy and five diseased) from a finishing unit of 1,800 animals were used in the present study. The farm was seropositive to PRRSv, porcine circovirus type 2 (PCV2), *Mycoplasma hyopneumoniae and A. pleuropneumoniae*.

Samples from healthy animals were collected at abattoir. These animals were transported for no longer than 1.5 h and were rested for 4 h before slaughter. They were killed by cutting the throat after being stunned in a chamber containing carbon dioxide, and were bled out with a Rotastick pump (Anitec, Malmö, Sweden). Blood samples were collected during the slaughter process by using vacutainer tubes and were allowed to clot for 1 h at room temperature. Serum was separated by centrifugation (2000xg for 15 min). Carcasses and organs were carefully examined and samples of liver, parotid salivary gland and diaphragmatic muscle were collected in 10% neutral buffered formalin.

Diseased animals were randomly chosen among pigs of the same finishing unit with clinical signs of disease and poor body condition that were not suitable to be sent to the slaughterhouse. Afterwards, the animals were killed by an intravenous overdose of sodium pentobarbital (Dolethal, Vétoquinol, Lure cedex, France) and complete postmortem examinations were performed. Blood samples were collected during the slaughter process as reported with healthy animals. Samples from the same tissues as in healthy animals were collected in 10% neutral buffered formalin. All sick pigs presented gross and microscopic lesions compatible with porcine respiratory disease complex.

Hp serum levels of all ten animals were quantified using a previously validated time-resolved immuno-fluorometric assay (Gutiérrez et al., 2009a).

### *Western blot*

Western blot analysis was performed to demonstrate the specificity of the mAb produced. A total of 2.5 $\mu$g of purified porcine Hp and 5 $\mu$g of porcine serum proteins, from a healthy and a diseased pig (see section 2.4), under reduced and non-reduced conditions, were loaded on 12% SDS-PAGE following the procedure described before. Following electrophoresis separated proteins were electro-blotted onto PVDF membranes (Hybond-P, Amersham, UK) by using a Semi-Dry trans-blot (Bio-Rad, Hercules, CA) for 1 hour at 25 mA. The membranes were then blocked overnight with RotiBlock (Carl Roth, Karlsruhe GmbH) and incubated for 1 hour with 15 mL of 1:2500 dilution in blocking solution of the anti-Hp porcine antibody labelled to horseradish peroxidase (EZ-Link Plus Activated Peroxidase, Pierce Biotechnology, Roclford, USA) at 0.9 mg/mL. After three washes with TPBS (0.05% Tween 20 Phosphate buffered saline buffer 7.5 pH) and an additional wash with PBS the reaction was developed using a chromogen (4-chloro-1-naphthol) (Miller and Gemeiner, 1992).

The affinity and specificity of the mAb to Hp was compared with a commercially available affinity purified rabbit polyclonal anti-Hp porcine (Immunology Consultants Laboratory, Inc., Newberg, USA) (15 mL of 1:2000 dilution in blocking solution of 1 mg/mL) by western blot, according to the protocol described above, using both isolated Hp protein and porcine serum proteins from a healthy and a diseased pig. Membranes were digitalized and the molecular weights of the developed positive bands were calculated using specific software as reported before to gel electrophoresis analysis.

### *Immunohistochemical study*

All tissues, liver, salivary gland and diaphragmatic muscle, from a healthy and a diseased pigs (see section 2.4), were processed for immunohistochemical analysis using the same protocol based on the avidin-biotin-peroxidase complex technique (ABC) as previously described (Hsu and Raine, 1981). Briefly, tissue sections were dewaxed and dehydrated through graded ethanol and the endogenous peroxidase activity was quenched in $H_2O_2$ 3% in methanol for 30 min. The sections were washed with PBS and incubated for 30 min at room temperature with 100 $\mu$l per slide of blocking solution (10 % normal goat serum, Sigma Chemical Company, St. Louise, MO, USA) in a humid chamber. For antigen retrieval, tissues were placed in citrate buffer 10 mM pH=6 and heated for 5 min at 450W followed by 6 min at 150W in a microwave. Anti-porcine Hp mAb (100 $\mu$L of 1:50 dilution from an aliquot of 0.6 mg/mL) was incubated with tissue sections overnight at 4°C in a humid chamber followed by 30 minutes incubation at room temperature with the secondary antibody (biotinylated polyclonal goat anti-mouse immunoglobulins, Dako Denmark A/S, Glostrup, Denmark) (100 $\mu$L of 1:50 dilution from an aliquot of 0.8 mg/mL). An avidin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) was applied for 1 h at room temperature. Labelling of porcine Hp in tissue sections was visualized or revealed by using a specific substrate according to the manufacturer's instructions (NovaRED™ substrate kit, Vector Laboratories, Burlingame, CA, USA). Finally, sections were

counterstained with Mayer's haematoxylin, dehydrated and mounted. For negative controls, tissue sections were analyzed as reported above, but replacing the primary antibody with blocking solution (primary antibody-omitted negative control).

## Results

### *Preparation of the immunogenic protein*

Hp was purified from serum samples and the chromatogram obtained is shown in figure 1A. According to studies reported before (Fuentes et al., 2011), Hp would be presented in peak 2, so the fractions corresponding to this peak were pooled and evaluated by SDS-PAGE under denaturing reducing and non-reducing conditions. Two bands of 42.87 kDa and 13.47 kDa were obtained under reducing conditions (Fig. 1B lane 1) and a band of around 133.20 kDa under non-reduced conditions (Fig. 1B lane 4). Approximate molecular weights of each band were calculated by using the specific software reported above.

Mass spectrometric analysis verified that porcine Hp was purified since the highest molecular weight band, of around 42 kDa in the gel, was identified as haptoglobin precursor from sus scrofa (NCBInr Database, Reference Sequence: NP_999165.1) by using an electrospray ion source mass spectrophotometer (Trap XCT Plus de Agilent Technologies, Santa Clara, USA).

### *Production and purification of monoclonal antibody*

The supernatant of multiple clones were initially screened by ELISA and most of them were found to be weakly secreting mAbs against Hp in the process of subcloning and were consequently discarded. Four clones producing monoclonal antibodies against Hp, which showed a good secretion of mAbs, were characterized, in which the clone named 4E4A6C2 showed the highest reactivity with Hp by ELISA (data not shown) and was chosen for further analysis.

The selected clone was purified by precipitation with ammonium sulphate followed by affinity chromatography. Fig. 2A shows a typical chromatographic profile for mAbs affinity purified against porcine Hp.

The purity of the monoclonal antibody analyzed on

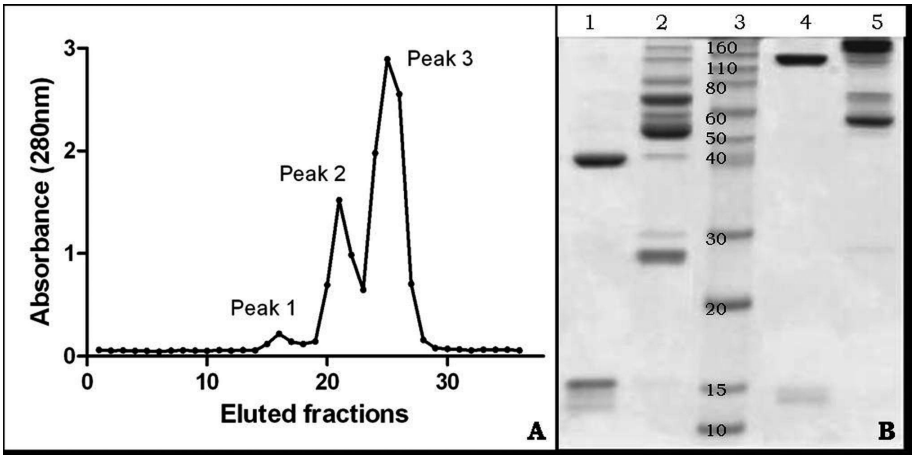


**Fig. 1. A.** Chromatogram of porcine Hp purification by fast performance liquid chromatography after ammonium sulphate immuno-precipitation. Peak 2 represent Hp fractions. Y axis: absorbance at 280nm wavelength. X axis: eluted fractions following purification. **B.** SDS-PAGE of purified porcine Hp using 12% SDS-PAGE under reduced and non-reduced condition with DTT. Lane 1: 2.5 $\mu$g of reduced Hp pool fraction from peak 2 chromatogram. Lane 2: 5$\mu$g of reduced porcine serum proteins. Lane 3: Apparent molecular weight of protein standards (kDa) (Novex® Sharp Protein Standards, Invitrogen, Hilden, Germany). Lane 4: 2.5 $\mu$g of non-reduced Hp pool fraction from peak 2 chromatogram. Lane 5: 5 $\mu$g of non-reduced porcine serum proteins.

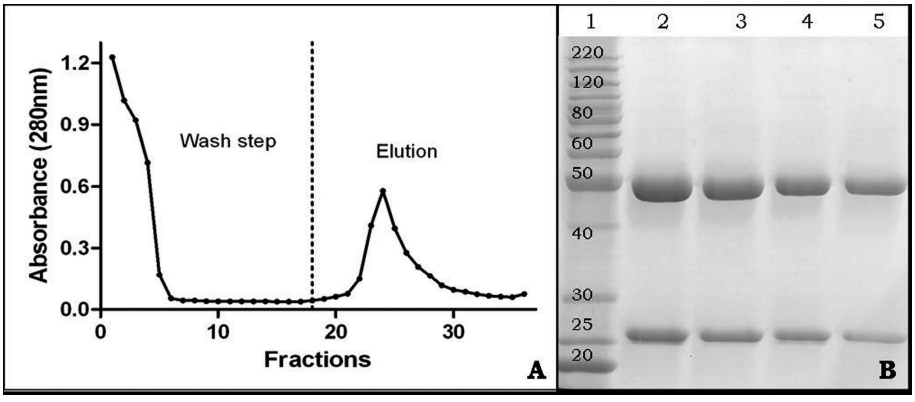


**Fig. 2. A.** Typical purification profile of monoclonal antibody against porcine Hp on affinity-column. Vertical discontinuous line: end of washing and start of elution step of specific antibodies. **B.** Analysis of purified monoclonal antibody using 12% SDS-PAGE under reduced condition with DTT. Lane 1: Apparent molecular weight of protein standards (kDa) (BenchMark Protein Ladder, Invitrogen, Hilden, Germany). Lanes 2-3: 5 $\mu$g of purified mAbs. Lane 4-5: 2.5 $\mu$g of purified mAbs.

SDS-PAGE showed 2 bands of 50kDa and 25kDa respectively (Fig. 2B). The first band corresponded to the heavy chain of immunoglobulins meanwhile the second band showed the light chain of immunoglobulins.

### *Animal sampling*

The levels of Hp obtained in serum of healthy and diseased animals are shown in table 1. In the group of healthy animals a median Hp level of 0.47 mg/mL was observed, while higher concentrations appeared in diseased pigs with a median of 6.50 mg/mL. As animals in each group had similar concentrations of Hp in serum, since the 25$^{th}$ and 75$^{th}$ percentiles obtained for healthy and diseased animals were small, pigs from each group were treated as having similar acute phase reactions. A representative animal of each group was randomly selected and used to show the results of both western blot and immunohistochemistry analysis. These animals corresponded to pig number 3 and 5 from healthy and diseased groups, respectively.

### *Western blot analysis*

Western blot analyses were performed by using both the in-house produced mAb and the commercial rabbit polyclonal antibody against porcine Hp (Immunology Consultants Laboratory, Inc., Newberg, USA), and provided a strong positive signal with a molecular weight which corresponds to the molecule of porcine haptoglobin when the purified protein and serum samples were used (Fig. 3).

When the commercial antibody was applied as positive control (Fig. 3A), one band of around 42 kDa was observed in reduced pure haptoglobin and serum from a diseased pig (lane 2 and 4). Moreover, a band between 110 and 160kDa, of approximately 133 kDa, was obtained for non-reduced haptoglobin and serum from a diseased pig (lane 5 and 7 respectively), similar to those observed with the mAb. Neither in reduced nor in non-reduced conditions were positive bands observed when serum from a healthy pig was applied (lane 3 and 6).

**Table 1.** Concentrations of haptoglobin in serum samples of the healthy and diseased pigs included in the study measured by time-resolved immunofluorometry. SD: standard deviation.

| Number of animals | Serum Hp levels (mg/mL) | |
|---|---|---|
| | Healthy pigs | Diseased pigs |
| 1 | 0.56 | 7.57 |
| 2 | 0.71 | 6.50 |
| 3 | 0.30 | 6.63 |
| 4 | 0.47 | 4.82 |
| 5 | 0.42 | 4.68 |
| Median | 0.47 | 6.50 |
| 25th percentile | 0.36 | 4.75 |
| 75th percentile | 0.63 | 7.10 |

By using the in-house produced mAb (Fig. 3B), a band between 110 and 160 kDa, approximately 133 kDa, was observed in lane 5 and 7, which corresponds to non-reduced Hp from purified protein and serum from a diseased pig (theorical molecular weight 120 kDa), meanwhile non-positive reactive bands were observed under reduced conditions when the mAb were used in both purified protein and serum (lane 2-4). In addition,

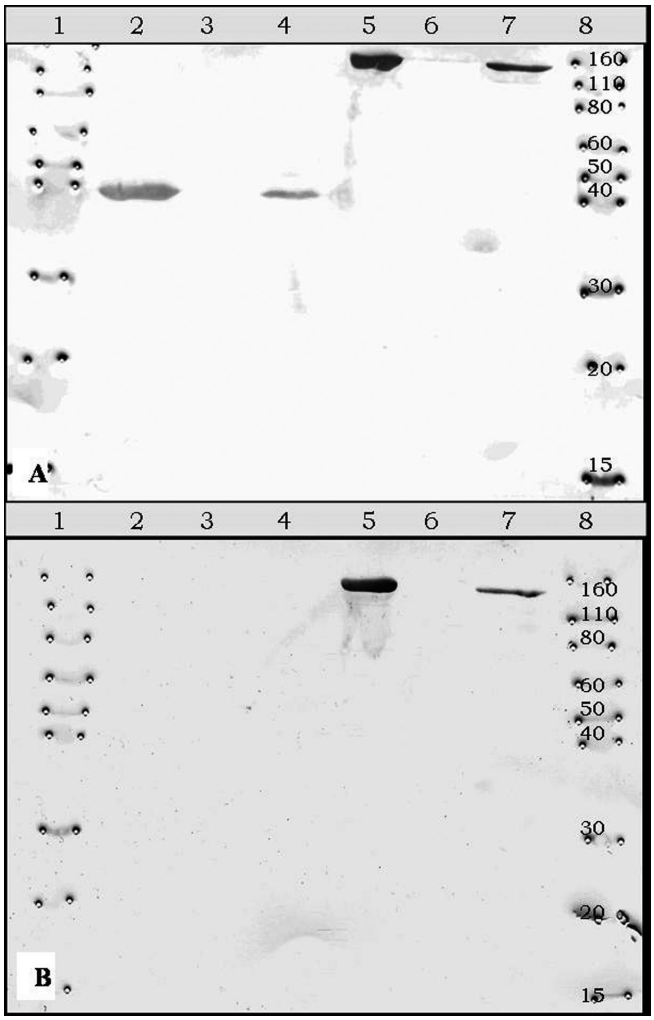


**Fig. 3.** Immunoblot of the anti-porcine Hp commercial polyclonal antibody **(A)** and mAb **(B)** in swine haptoglobin and serum proteins. Lane 1: Apparent molecular weight of protein standards (kDa) (Novex® Sharp Protein Standards, Invitrogen, Hilden, Germany). Lane 2: positive bands against 2.5 $\mu$g of purified reduced porcine Hp pool fraction from peak 2 chromatogram. Lane 3: Positive bands against 5 $\mu$g of reduced healthy porcine serum proteins. Lane 4: Positive bands against 5 $\mu$g of reduced diseased porcine serum proteins. Lane 5: positive bands against 2.5 $\mu$g of purified non-reduced porcine Hp pool fraction from peak 2 chromatogram. Lane 6: Positive bands against 5 $\mu$g of non-reduced healthy porcine serum proteins. Lane 7: Positive bands against 5 $\mu$g of non-reduced diseased porcine serum proteins. Lane 8: Apparent molecular weight of protein standards (kDa) (Novex® Sharp Protein Standards, Invitrogen, Hilden, Germany).

non-positive reaction band was observed when a healthy serum (lane 6) was applied under non-reduced conditions.

### *Immunohistochemical analysis*

The presence of Hp in the liver of the diseased animal was prominent and evidenced as perinuclear, small to medium sized globules (1 to 5 $\mu$m in diameter) in the cytoplasm of the hepatocytes (Fig. 4A). The labelling was homogeneously distributed in the hepatic acini, although it was more intense in the periacinar area (Fig. 4A). Free globules were also randomly observed within the lumen of blood vessels of portal triads and central hepatic vein. Occasional intracytoplasmic immunolabelled globules were observed in the cytoplasm of some Kupffer cells. In contrast, immunohistochemistry demonstrated a mild to poor immunolabelling of Hp in the liver of the healthy pigs (Fig. 4B). No presence of Hp at all was observed when the primary antibody was omitted (Fig. 4C).

In diseased pigs immunolabelling of scattered glandular epithelial cells in haphazardly located glandular acini of the parotid salivary gland was observed, coinciding with a more intense hepatic presence of Hp. These glandular epithelial cells showed a diffuse, cytoplasmic immunostaining (Fig. 5A, arrows). In addition, the presence of Hp was also observed within the cytoplasm of epithelial cells in the ducts of the salivary gland showing two patterns of immunolabelling: (1) multiple, immunolabelled globules occupying the whole cytoplasm (Fig. 5B); (2) diffuse, cytoplasmic immunolabelling, more intense at the apical border of the cell (Fig. 5C, arrow). A few positive large, polygonal to spindle, resembling macrophage-like or myofibroblastic cells showed immunhistochemical staining in the interstitium (Fig. 5A, arrowhead). Moreover, a diffuse, intracytoplasmic expression of Hp was observed in isolated postganglionar vegetative fibres of the salivary gland of the sick animal (Fig. 5D, arrows). In the salivary gland from healthy animals, a mild immunolabelling was observed within the cytoplasm of duct epithelial cells (Fig. 5E, arrows), whereas no glandular epithelial cell was immunolabelled. In addition, no staining was observed in the primary antibody-omitted negative control (Fig. 5F).

All diseased animals showed a multifocal immunostaining of myofibers of skeletal muscle. Immunolabelled myofibers were usually in groups of two or three, although scattered immunolabelled myofibers were also observed (Fig. 6A, inset). The immunostaining of positive myofibers consisted of a faint diffuse sarcoplasmic immunolabelling, together with multifocal, variable diameter, intrasarcoplasmic granules (Fig. 6A, arrows and arrowheads, respectively). In addition, intracytoplasmic immunolabelling of large elongated to spindle cells, resembling histiocytic- or fibroblast-like cells was detected. A moderate to marked background was observed within the lumen of blood vessels in the interstitium of the skeletal muscle. The fibres of diaphragmatic muscle displayed a negative labelling against Hp in healthy animals (Fig. 6B), and also when the primary antibody was omitted (Fig. 6C).

## Discussion

In the present study we have produced a monoclonal antibody against porcine Hp which showed reactivity with this protein both in Western Blot and immunohistochemical analysis. By using this antibody,

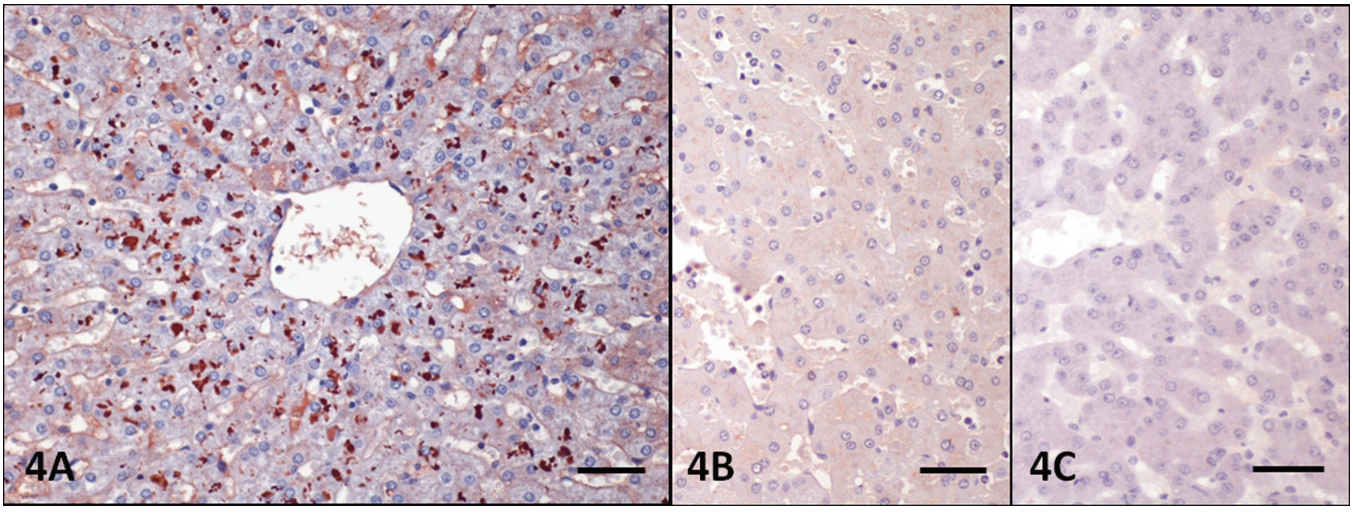


**Fig. 4. A.** Intracytoplasmic globules immunolabelled with the porcine-specific mAb Hp in hepatocytes from the periacinar (centrolobulillar) area of a hepatic lobuli from a sick animal. IHC. **B.** Mild and faint immunostaining against porcine-Hp in a healthy animal. IHC. **C.** No labelling observed in the omit-negative control from a sick animal. IHC. Bars: 50 $\mu$m.

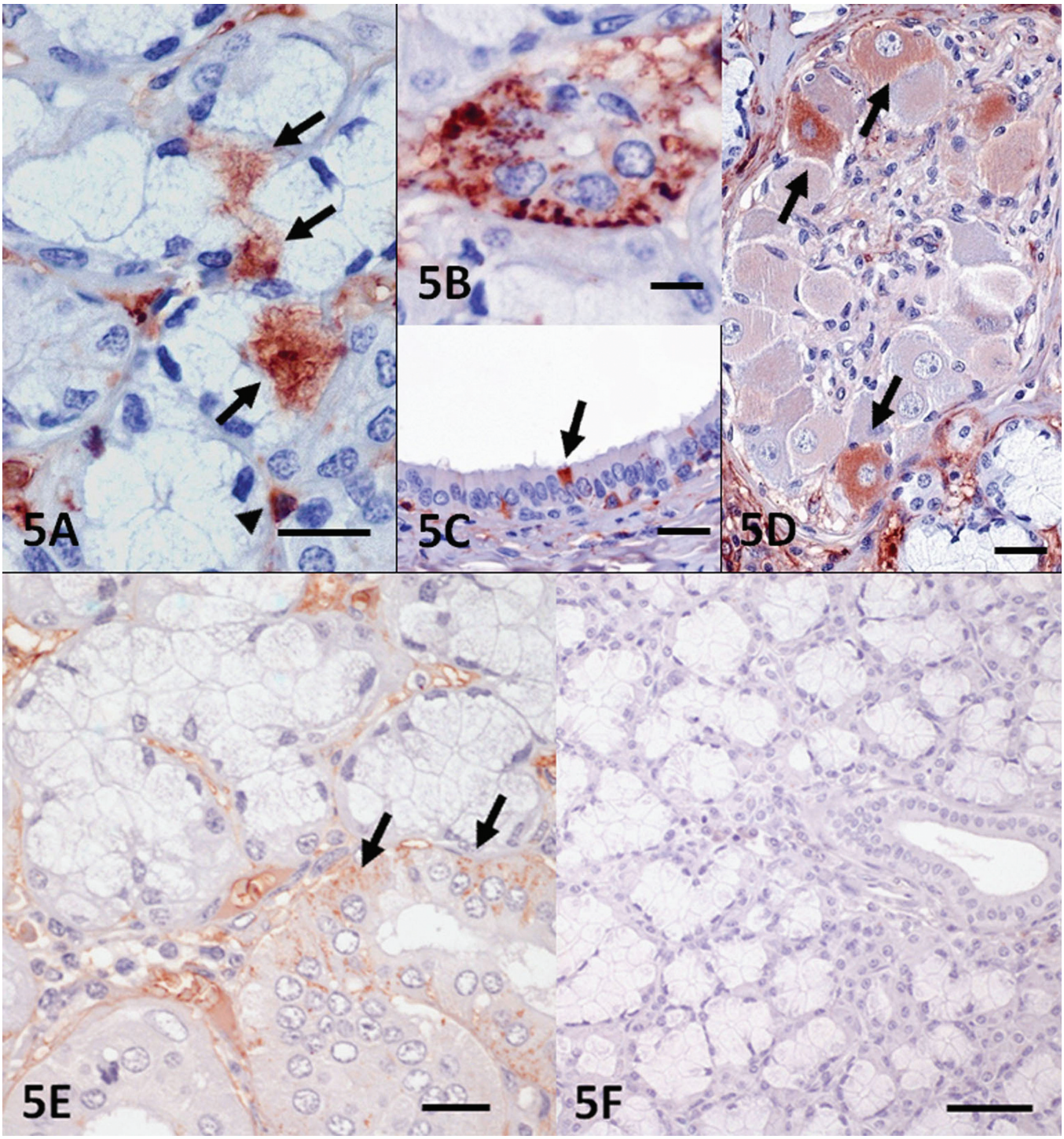


**Fig. 5. A.** Intracytoplasmic immunolabelling of epithelial glandular cells (arrows) and an interstitial cell (arrowhead) in the parotid salivary gland of a sick animal. IHC. **B.** Numerous intracytoplasmic immunostained globules in the cytoplasm of duct epithelial cells of the salivary gland in a sick animal. IHC. **C.** An epithelial cell from a duct of the salivary gland of a sick animal displaying a diffuse, apical, cytoplasmic expression of Hp (arrow). IHC. **D.** Expression of Hp in the cytoplasm of postganglionar vegetative fibres (arrows) of the parotid salivary gland in a sick animal. IHC. **E.** Mild immunolabelling within the cytoplasm of few duct epithelial cells (arrows), but no immunolabelling of glandular epithelial cells, in the parotid salivary gland from a healthy animal. IHC. **F.** Lack of immunostaining in the omit-negative control from a sick animal. IHC. Bars: A, E, 20 $\mu$m; B, 15 $\mu$m; C, D, 30 $\mu$m; F, 70 $\mu$m.

we have evaluated the possible local presence of Hp in salivary gland and diaphragmatic muscle tissues of healthy pigs in order to gain knowledge about the source of the haptoglobin that is measured in saliva and meat juice samples as a health and welfare biomarker.

At the first stage, to isolate specific mAbs against porcine Hp, pure protein purified from serum samples of pigs was used as immunogen. The purified protein had a complete molecular weight of approximately 133 kDa, as previously described (Yang and Mao, 1999), which in reduced conditions showed two subunits, one of approximately 42k Da (1ß chain) and another of 12 kDa (1$\alpha$ chains) which corresponded to porcine haptoglobin, as has been reported before (Hiss et al., 2003; Fuentes et al., 2011) and in agreement with mass spectrometric identification.

By using the purified protein as immunogen, monoclonal antibodies were specifically produced against porcine Hp using Balb/c mice and resulting in the establishment of at least four clones with high affinity. The protocol used to purify mAb was similar to that previously reported for human anti-Hp mAb purification (Yueh et al., 2007). The purity of the mAb was high, according to our electrophoretic results, since only two bands of 50 and 25kDa were observed in SDS-PAGE, which corresponded to the high and light chains of immunoglobulins, respectively (Miller and Goldfarb, 2006).

The mAbs produced did not react with any other component of porcine serum under reduced and non-reduced conditions, as evaluated by Western Blot, when a serum sample from a diseased animal was applied, so it could be usefully applied in our immunohistochemical study with high specificity. Western Blot analysis, by using the mAb produced, revealed only one positive band that corresponded to purified non-reduced Hp. According to those results, our clone $4E_4A_6C_2$ specifically reacts with the native molecule and thus the epitope of the mAb was accessible under native conditions. To verify the affinity of the antibody against porcine Hp by western blot, a commercial specific polyclonal antibody was used as control by analyzing pure Hp, serum from a healthy pig and serum from a diseased pig, obtaining similar results under non-reduced conditions. Neither the in-house produced mAb nor the commercial polyclonal antibody revealed positive bands when serum from a healthy animal was applied. This lack of positive response could be due to the low concentration of Hp presented in serum of healthy animals.

When the effective production of Hp was investigated by immunohistochemistry in liver tissue section, immunostaining was observed in the cytoplasm of hepatocytes. Extracellular staining was also observed in lumina of blood vessels, showing, possibly, the active synthesis and secretion of Hp in liver as has been

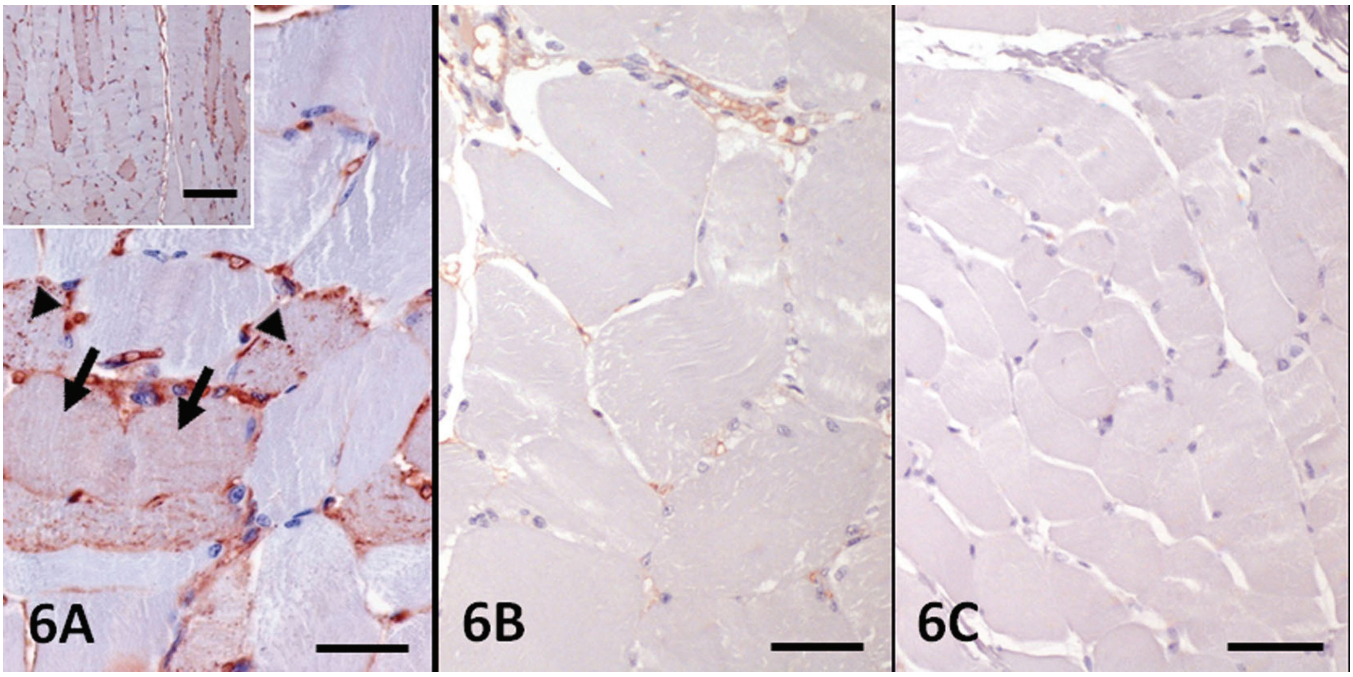


**Fig. 6. A.** Myofibers from diaphragmatic skeletal muscle showing a faint, diffuse sarcoplasmic immunolabelling against Hp (arrows) together with multifocal, intrasarcoplasmic granules (arrowheads). IHC. Inset. Detail of the distribution of the immunolabelled myofibers in groups of two or three with scattered immunolabelled myofibers. IHC. **B.** Mild immunostaining observed in the plasma of interstitial blood vessels from a healthy animal with no labelling of myofibers. IHC. **C.** No expression of Hp in the skeletal myofibers from an omit-negative control of a sick animal. IHC. Bars: A, B, 30 $\mu$m; Insert A, 130 $\mu$m; C, 45 $\mu$m.

reported elsewhere (Heegard et al, 1998). Previous studies have detected Hp mRNA in liver tissues, being higher in diseased animals (Skovgaard et al, 2009). However, to the author's knowledge, no immunohistochemical analysis has been performed until now in porcine liver sections. The results of the present study may verify that the acute phase response is produced in the liver involving the hepatic synthesis of Hp, since this protein was observed in the cytoplasm of hepatocytes. In addition, the positive immunostaining of hepatocytes against Hp served as a positive control of Hp production in the animals included in the study.

Our results also revealed information about the extrahepatic source of the Hp in saliva and meat juice samples. The presence of Hp in salivary gland and diaphragmatic muscle was demonstrated in the present study, an intense production of Hp in sick animals being observed compared with the lack of a marked immunolabelling in control animals. The localization of Hp observed in salivary gland may be related to an extrahepatic synthesis of this APP in this tissue, which may be supported by the immunostaining of both glandular and ductal epithelial cells of the parotid salivary gland. However, further studies, including analysis of mRNA, would be necessary to confirm this hypothesis since several mechanisms of transport of proteins from serum into salivary gland ducts have been reported (Wong, 2006), which could be responsible for the localization of specific proteins in duct cells. Those studies could provide new insights on the importance of Hp production in local tissues and its implication and role in cases of a systemic disease.

Skeletal muscle of diseased animals showed an immunohistochemical labelling in all sampled animals. The intrasarcoplasmic immunolabelling of myofibers points to a possible role of these cells in the production of Hp. Furthermore, interstitial cells and the plasma of blood vessels were also immunolabelled. Previous studies have defined meat juice samples as a mixture of serum, intracellular and lymphatic liquid (Nielsen et al., 1998). So, as a first approach and also in concordance to our results, it seems that Hp in meat juice and diaphragmatic tissue could be due to both *in situ* production by skeletal muscle myofibers and blood extravasations.

In our study, intracytoplasmic localization of Hp was also observed in postganglionar vegetative fibres of the salivary gland of a sick animal. Previous studies have reported the expression of Hp in the brain of mice, which was related to the defence of neurons against haemolytic products after an intracerebral haemorrhage (Zhao et al., 2009). The physio-pathological mechanism involved in this finding should be elucidated in the future.

In conclusion, our study has revealed, by immunohistochemical analysis using a specific mAb against porcine Hp, an extrahepatic presence of Hp in both salivary gland and diaphragmatic muscle tissues of pigs. This extrahepatic localization, in addition to an increase of the hepatic systemic production of Hp, would explain the increase of Hp levels found in saliva and meat juice samples in inflammatory conditions.

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