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Pig major acute-phase protein and haptoglobin serum concentrations correlate with PCV2 viremia and the clinical course of postweaning multisystemic wasting syndrome

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Abstract

The aim of the present longitudinal study was to assess the evolution of two acute phase proteins (APPs), pig-major acute phase protein (pig-MAP) and haptoglobin (HPT), in serum from pigs that developed postweaning multisystemic wasting syndrome (PMWS) in comparison to healthy and wasted non-PMWS affected pigs. In addition, evidence of infection with other pathogens and its relation with variations in APPs concentrations was also assessed. Fourteen independent batches of 100 to 154 pigs were monitored from birth to PMWS outbreak occurrence in 11 PMWS affected farms. Pigs displaying PMWS-like signs and age-matched healthy controls were euthanized during the clinical outbreak. PMWS was diagnosed according to internationally accepted criteria and pigs were classified as: i) PMWS cases, ii) wasted non-PMWS cases and iii) healthy pigs. At the moment of PMWS occurrence, pig-MAP and HPT concentration in PMWS affected pigs were higher than in healthy ones (p<0.0001). No differences in APPs serum concentrations between subclinically PCV2 infected pigs and healthy non-PCV2 infected pigs (based on quantitative PCR on serum results) were detected. Results showed a significant correlation between PCV2 loads and both pig-MAP (R=0.487 to 0.602, p<0.0001) and HPT (R=0.326 to 0.550, p<0.05 to 0.0001) concentrations in serum of PMWS affected pigs, indicating that the acute phase response in PMWS affected pigs occurred concomitantly to PCV2 viremia. No other pathogen, apart from PCV2, was consistently related with variations in APPs concentrations. A ROC analysis, made to determine the capacity of discrimination of both APPs between PMWS affected and non-affected pigs, showed higher sensitivity and specificity values using pig-MAP compared to HPT. These results suggest that pig-MAP might be a better indicator of PMWS status than HPT. Moreover, the fact that APR occurred some days
before the start of clinical signs suggests that APPs could provide valuable prognostic information for PMWS development.

**Keywords:** Haptoglobin; pig-major acute phase protein (pig-MAP); acute phase proteins (APPs); acute phase reaction (APR); porcine circovirus type 2 (PCV2); postweaning multisystemic wasting syndrome (PMWS).

1. Introduction

The acute phase response (APR) is an innate, non-specific immune response which occurs after many different stimuli such as infections, tissue damage, neoplastic growth or immunological disorders (Baumann and Gauldie, 1994; Gruys et al., 2005a). This complex reaction is mediated by pro-inflammatory cytokines and involves both local and systemic reactions, including fever, increase in muscle protein catabolism, alterations in sleep and appetite patterns, and changes in the concentration of a group of plasma proteins which are called acute phase proteins (APPs) (Gruys et al., 2005a). These proteins are mainly synthesised in the liver and are classified according to the direction of change (positive APPs if their concentration increases, negative if it decreases), and according to the extent to which their concentrations change (minor, intermediate or major) during the APR (Petersen et al., 2004; Gruys et al., 2005a).

APPs have been described as useful for assessing health in humans and animals (Petersen et al., 2004; Gruys et al., 2005b). In pigs, APPs concentrations have been demonstrated to be increased after experimental infections with several bacteria, viruses or parasites, as for instance *Streptococcus suis* (Sorensen et al., 2006), porcine reproductive and respiratory syndrome virus (PRRSV) (Asai et al., 1999) and
Toxoplasma gondii (Jungersen et al., 1999). Moreover, increases in APPs concentrations have also been detected in pigs affected by tail- and ear-biting or after stress induced by transport conditions or changes in the pattern of food administration (Parra et al., 2006; Piñeiro et al., 2007; Salamano et al., 2008). Specifically, haptoglobin (HPT) and pig-major acute phase protein (pig-MAP) have been suggested as the most robust APPs as indicators of infection in pigs (Sorensen et al., 2006).

Porcine circovirus type 2 (PCV2) is considered the essential infectious agent for postweaning multisystemic wasting syndrome (PMWS) development, but it is also known that other triggering factors are necessary for the full expression of the clinical disease (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés et al., 2005). The internationally accepted criteria for diagnosis of PMWS include the presence of compatible clinical signs (mainly wasting and respiratory distress), moderate to severe lymphocyte depletion with granulomatous inflammation in lymphoid tissues, and detection of moderate to high amounts of PCV2 within these lesions (Sorden, 2000; Segalés et al., 2005). Although it is known that APPs serum concentrations are increased in PMWS affected pigs (Segalés et al., 2004; Parra et al., 2006), few field data are available in the literature. Moreover, the longitudinal evolution of APPs in animals that finally develop the disease has not been assessed.

The aim of this study was to assess the evolution of two APPs, pig-MAP and HPT, in serum throughout the productive life of pigs that developed PMWS in comparison to healthy pigs and pigs that developed wasting without fulfilling PMWS diagnosis. Moreover, the infection dynamics of PCV2 and several other pathogens was monitored by quantitative PCR, nested PCR and/or serological techniques to determine potential
associations between studied APPs concentrations and the moment of infection for each pathogen.

2. Materials and methods

2.1. Study design

Two longitudinal case-control studies in PMWS affected farms, one in Spain and one in Denmark, were performed as previously described (Grau-Roma et al., 2009). Briefly, both studies were carried out using similar designs, and were performed on 13 independent farm batches (6 in Spain and 7 in Denmark) of 100 to 154 animals per batch, coming from 10 different farms (3 from Spain and 7 from Denmark). Studied piglets were ear-tagged at 1 week of age and monitored until the occurrence of the PMWS outbreak. Nasal and rectal swabs as well as blood samples were serially collected from those piglets at established weeks of age (1, 3, 7 and 11 in Spain, and 1, 4, 6 and 9 in Denmark) and at the time of PMWS outbreak occurrence (necropsy). When the PMWS compatible clinical picture (Segalés, 2002) appeared at the studied farms, pigs displaying PMWS-like signs and healthy age-matched pigs were selected, euthanized and necropsied. At necropsy, sections of lymphoid tissues were collected and fixed by immersion in neutral-buffered 10% formalin to assess the pathological status of both clinically healthy and diseased animals.

All treatments, housing, husbandry and slaughtering conditions followed the European Union Guidelines and Good Clinical Practices.

2.2. Histopathology
Formalin fixed tissues were dehydrated and embedded in paraffin blocks. Two consecutive 4 µm thick sections containing collected lymphoid tissues from each pig were cut from each block. One section was processed for haematoxylin and eosin stain, while the other was used for PCV2 nucleic acid detection by in situ hybridization (ISH) (Rosell et al., 1999) for Spanish cases, and for immunohistochemistry (IHC) for PCV2 antigen detection (Jensen et al., 2006) for Danish cases. Pathological evaluation and PMWS diagnosis was carried out using a previously described scoring system evaluating the PCV2 amount and the intensity of lymphoid depletion and granulomatous infiltration present in lymphoid tissues (Grau-Roma et al., 2009). Three different categories of pigs were established: i) PMWS cases: corresponding to pigs showing clinical wasting and with moderate to severe lymphoid lesions and moderate to high amount of PCV2 antigen/nucleic acid; ii) wasted non-PMWS cases: corresponding to pigs showing clinical wasting but without or with slight PMWS characteristic histopathological lesions and no or low amount of PCV2 antigen/nucleic acid within lymphoid tissues; iii) Healthy pigs: corresponding to pigs showing good clinical condition, without or with slight PMWS characteristic histopathological lesions and no or low amount of PCV2 antigen/nucleic acid within lymphoid tissues.

2.3. Acute phase proteins determination in serum

Two APPs, HPT and Pig-MAP, were measured in serum of studied pigs. Both APPs were determined in all longitudinally collected sera from Spanish necropsied pigs (107 pigs coming from 3 different farms). For the Danish pigs, serum samples collected at 1, 6 or 9 weeks of age and at necropsy from only those pigs with available serum at the moment of necropsy were analyzed. (53 pigs coming from 7 different batches).
Pig-MAP concentration was determined by a sandwich enzyme-linked immunosorbent assay (ELISA), using a commercial kit based on two anti-Pig-MAP specific monoclonal antibodies (PigCHAMP Pro Europa S.A.), according to the manufacturers’ instructions. This kit was based on an ELISA previously developed and validated (Piñeiro et al., 2009b). HPT concentration was determined by a sandwich ELISA as previously described (Sorensen et al., 2006). Pig-MAP and HPT results were expressed as mg per millilitre of serum (mg/ml).

2.4. Quantitative PCV2 PCR

PCV2 real time quantitative PCR (qPCR) was performed on all longitudinally collected serum samples. DNA was extracted from 200 µl of serum from Spanish (Nucleospin® Blood, Macherey-Nagel, GmbH & Co KG, Düren, Germany) and Danish samples (QIAamp DNA Mini Kit, Qiagen® GmbH, Germany), according to the manufacturers’ instructions. DNA was eluted in 100 µl and 200 µl of elution buffer in Spain and Denmark, respectively.

PCV2 DNA was quantified using two previously described qPCR techniques on Spanish (Olvera et al., 2004) and Danish (Hjulsager et al., 2009) samples, respectively. The performance of both qPCRs was compared in a previous study based on field samples (Hjulsager et al., 2009). This latter work showed a highly significant linear association between results of the two qPCR assays. However, the method performed by the Danish laboratory had higher sensitivity and yielded systematically higher PCV2 load values than the one used in the Spanish laboratory. All PCV2 qPCR results were expressed as log$_{10}$ (number of PCV2 copies per ml of sera).
2.5. Serology

Serological analyses were performed on all longitudinally collected serum samples. In Spain, ELISAs were used to detect antibodies against porcine reproductive and respiratory syndrome virus (PRRSV) (HerdCheck PRRS virus antibody test, IDEXX, Inc., USA), Aujeszky’s disease virus (HerdCheck anti-PRV gpI, IDEXX, Inc., USA), porcine parvovirus (PPV) (Ingezim PPV, Ingenasa, Spain), swine influenza virus (SIV) (Civtest Suis Influenza, Laboratorios Hipra, Spain), *Mycoplasma hyopneumoniae* (Civtest Suis *Mycoplasma Hyopneumoniae*, Laboratorios Hipra, Spain), and *Salmonella* spp. (Salmonella covalent mix-ELISA, Svanovir™, Svanova, Sweden). In Denmark, assays used were previously described immunoperoxidase monolayer assays (IPMAs) to detect antibodies against European and American PRRSV strains (Sorensen et al., 1998), ELISAs to detect antibodies against PPV (Madsen et al., 1997) and *Lawsonia intracellularis* (Boesen et al., 2005), and haemagglutination inhibition (HI) test to detect antibodies against H1N1 and H3N2 subtypes of SIV, which was essentially carried out according to the OIE manual (OIE manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008). A seroconversion against studied pathogens was considered to be present when an increase in the antibody titres in two consecutive samples or when a positive result preceded by a previous negative result was detected.

2.6 *Mycoplasma hyopneumoniae* nested PCR

It is known that the appearance of antibodies to *M. hyopneumoniae* can be delayed as much as 8 weeks after infection (Sitjar et al., 1996). Thus, in order to avoid underestimating its potential prevalence in studied animals, a nested PCR (nPCR) assay to detect *M. hyopneumoniae* was performed in the Spanish nasal swabs collected at the
moment of necropsy (Calsamiglia et al., 1999). For 1 out of 107 Spanish pigs the nasal swab sample was lacking.

2.6. Statistical analyses

For statistical evaluation, the SAS 9.1.3 software was used (SAS institute Inc., Cary, North Carolina, USA). A repeated measurement analysis of variance according to the general linear model procedure (PROC MIXED) and LSMEANS follow-up test was used to evaluate APPs mean concentrations differences between: i) PMWS cases, wasted non-PMWS and healthy pigs at each sampling time and within them at different samplings points; ii) healthy PCV2 subclinically infected pigs (PCV2 qPCR positive) and healthy non-infected pigs (PCV2 qPCR negative) at different sampling points; iii) seroconverted and non-seroconverted pigs against studied pathogens at each sampling; and iv) *M. hyopneumoniae* infected (nPCR positive) and non-infected (nPCR negative) at necropsy. Constant correlation was assumed between repeated measurements. Post-hoc multiple comparisons were addressed using the Tukey test. PCV2 qPCR data were log10 transformed prior to graphic representation, and negative results were coded as 0. A Pearson’s chi-square test was used to compare the proportions of seroconverted animals to each studied pathogen at the moment of necropsy between the three categories of pigs studied; same comparison was also established using *M. hyopneumoniae* nPCR results. The level of significance for all analyses (α) was set to p<0.05.

To determine the capacity of discrimination of both Pig-MAP and HPT between PMWS affected and healthy pigs, a Receiver Operating Characteristic (ROC) analysis from samples collected at necropsy was carried out using Statsdirect (version 2.6.6,
CamCode, Ashwell, UK). Moreover, ROC analysis was also performed to determine the
diagnostic performance of both APPs on clinically affected animals. For this later
analysis, only wasted pigs were considered, considering PMWS as “diseased” and
wasted non-PMWS as “non-diseased” animals. Taking into account that APPs
congcentration was determined by the same laboratorial tests in both studies, all samples
collected at necropsy coming from Spain and Denmark were analysed together.

3. Results

3.1 Studied animals, histopathology and acute phase proteins

The number of pigs from which APPs concentrations were analysed and their
pathological classification (PMWS, healthy and wasted non-PMWS) is detailed in Table
1. Mean±SD of weeks of age at necropsy for PMWS, healthy and wasted non-PMWS
pigs were 14.0±2.1, 14.7±2.5, 15.2±2.7, respectively in Spain, and 11.2±1.5, 10.0±3.5,
10.9±1.9, respectively, in Denmark.

The longitudinal evolution of pig-MAP serum concentrations is displayed in figure 1. Pig-MAP concentration in PMWS affected pigs was higher than in non-PMWS affected
ones (healthy and wasted non-PMWS) either in Spain (p<0.0001) and Denmark
(p<0.001) at the moment of necropsy. Although no statistically significant differences
were found between healthy and wasted non-PMWS pigs, the mean pig-MAP serum
concentration at the moment of necropsy in wasted non-PMWS was higher than in
healthy ones (Spain: p=0.0715; Denmark: p=0.1472). Moreover, the Pig-MAP
concentration in PMWS pigs at the sampling prior to necropsy in Spain was higher than
its concentration in non-PMWS affected pigs (healthy and wasted non-PMWS)
(p<0.01). Accordingly, in the Spanish pigs, the increase of pig-MAP concentration in
PMWS affected pigs was already significant at the sampling prior to necropsy, compared to basal pig-MAP concentrations detected at weeks 1 to 7. In Denmark, a significant increase in Pig-MAP concentration was only detected at necropsy in PMWS pigs. No statistical significant changes in pig-MAP concentration were found among all samplings in healthy and in wasted non-PMWS pigs from both countries.

The longitudinal evolution of HPT serum concentrations is displayed in figure 2. HPT concentration at the moment of necropsy was higher in PMWS than in healthy pigs either in Spain (p<0.0001) and Denmark (p<0.01). On the other hand, wasted non-PMWS could not be distinguished from the other two groups in Denmark, but had significantly lower HPT concentration than PMWS (p<0.001) in Spain. Regarding the longitudinal evolution of HPT concentration within each group, HPT increased progressively in both PMWS and wasted non-PMWS pigs in Spain from 11 weeks of age (p<0.01) to the necropsy moment (p<0.0001). In Denmark, only PMWS affected pigs gave significant increase in HPT concentrations with respect to the basal concentrations detected at the 1st week of age (p<0.01). No statistically significant variation was found among the HPT concentrations of healthy pigs neither in Spain nor Denmark.

Pig-MAP concentrations obtained at the moment of necropsy were statistically correlated with the global average scoring (histopathology plus PCV2 detection in lymphoid tissues) in Spain (R=0.622; p<0.0001) and also in Denmark (R=0.524; p<0.0001). On the other hand, significant correlation between HPT concentration at the moment of necropsy and the global average scoring was detected in Spain (R=0.443; p<0.0001), but only a tendency was observed in Denmark (R=0.254; p=0.066).
Moreover, pig-MAP and HPT concentrations were significantly correlated (Spain: \(R=0.462, p<0.0001\); Denmark: \(R=0.576, p<0.0001\)).

### 3.2 PCV2 qPCR

PCV2 loads in sera are schematically displayed together with APPs results in figures 1 and 2. PCV2 loads increased progressively from the 1st week of age until necropsy in Denmark and during the last two samplings in Spain. Maximum PCV2 loads were observed at the moment of disease outbreak (necropsy) in both countries.

Significant correlations were observed between pig-MAP serum concentrations and PCV2 loads in serum (Spain: \(R=0.278, p<0.0001\); Denmark: \(R=0.47, p<0.001\)). Considering the 3 groups of pigs separately (figure 3), PMWS affected pigs corresponded to the group with higher correlation between PCV2 load and pig-MAP (Spain: \(R=0.487, p<0.0001\); Denmark: \(R=0.602, p<0.0001\)), followed by the correlations observed in wasted non-PMWS pigs (Spain: \(R=0.218, p<0.01\); Denmark: \(R=0.437, p<0.01\)). On the contrary, no significant correlation was found between pig-MAP concentrations and PCV2 loads when only healthy pigs were considered (Spain: \(R=-0.152, p=0.07\); Denmark: \(R=0.2, p=0.194\) (figure 3).

Significant correlations were also found between HPT serum concentrations and PCV2 loads in serum (Spain: \(R=0.443, p<0.0001\); Denmark: \(R=0.326, p<0.001\)). Considering the 3 groups of pigs separately, the group of pigs with PMWS had again the highest correlation (Spain: \(R=0.550, p<0.0001\); Denmark: \(R=0.326, p<0.05\)), followed by the correlations observed in wasted non-PMWS pigs (Spain: \(R=0.437, p<0.0001\); Denmark: \(R=0.295, p=0.057\)). The lowest correlation between HPT concentration and PCV2 load...
was found in healthy pigs, being significant in Spain (R=0.186, p<0.05) but not in Denmark (R=0.273, p=0.73).

Taking into account that more than half of healthy Spanish pigs gave negative results at all samplings by PCV2 qPCR, mean APPs concentrations in healthy PCV2 infected and non-infected pigs were also compared. No statistical differences were seen between both groups of pigs neither in pig-MAP nor HPT concentrations. Such comparison was not done in any other group of pigs due to the low number of negative PCV2 qPCR results.

3.4 Serology and PCR against different swine pathogens

The number of pigs that had seroconverted against studied pathogens and PCV2 qPCR results at the moment of necropsy in both countries, and *M. hyopneumoniae* nPCR in Spain, are displayed in Table 2.

No serological evidence of infection to ADV was detected. Evidence of *M. hyopneumoniae*, PPV or PRRSV infections in Spain and SIV infection in Denmark were found in less than 10% of analysed pigs. Prevalence of SIV and *Salmonella spp.* in Spain and PPV, PRRSVe, PRRSVu and *L. intracellularis* in Denmark was detected in 25 to 55% of the studied pigs.

No differences in the proportions of infected and/or seroconverted animals to studied pathogens between PMWS, wasted non-PMWS and healthy pigs were observed. Moreover, no significant differences in APPs concentrations between seroconverted and non-seroconverted pigs to studied pathogens were detected. However, the 5 pigs that
gave positive nPCR results for *M. hyopneumoniae* in Spain had higher pig-MAP mean serum concentrations (mean±SD=4.47±4.49) than the 101 pigs that gave negative nPCR (2.35±2.13) (p<0.05).

### 3.5 ROC analysis

Optimal cut-off values for both studied APPs maximizing sensitivity (Sn) and specificity (Sp) to discriminate diseased and non-diseased pigs are displayed in Table 3. Sp and Sn values were higher when comparing PMWS versus healthy pigs than comparing PMWS versus wasted non-PMWS animals. Moreover, Sn and Sp values obtained using pig-MAP concentrations were always higher than when HPT was used. Further exploration of the data showed that, when comparing PMWS and healthy pigs, Sp values were slightly improved when the presence of PCV2 (detected by qPCR) was considered together with the APPs concentration. Thus, a potential pre-mortem diagnosis of PMWS could be established only when APP concentrations were above optimal threshold and qPCR gave a positive result. Following this condition, pigMAP and HPT maintained their Sn at 85.7% (73.8-93.6) and 67.9% (54.0-79.7), respectively, and Sp increased to 93.5% (82.1-98.6) in both cases.

### 4. Discussion

The present study represents the first longitudinal determination of pig-MAP and HPT concentrations in pigs that subsequently developed PMWS. Both APPs increased in parallel to the increase of PCV2 load in sera and reached the maximum values at the moment of disease manifestation. Pig-MAP and HPT concentrations were higher in PMWS affected pigs than in healthy ones, which is in agreement with previous reports.
This situation is probably a reflection of the systemic inflammatory status suffered by PMWS affected pigs (Rosell et al., 1999).

It is known that APPs serum concentrations might vary with the age (Piñeiro et al., 2009a). However, in the present study, samples from all pigs were collected at previously established weeks of age to accomplish a longitudinal study (Grau-Roma et al., 2009). Moreover, at the moment of necropsy, pigs displaying PMWS-like signs and healthy pigs were euthanized in an age-matched way. Therefore, the differences observed between the three studied groups of pigs should not be influenced by a potential age-effect.

The higher correlation between PCV2 load and both APPs detected in PMWS affected pigs, together with the absence or weak correlation detected in healthy pigs, indicates that the APR in PMWS affected pigs occurs concurrently with PCV2 infection, further supporting the idea that PCV2 is the essential infectious agent for PMWS development (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés et al., 2005). Based on qPCR PCV2 results, an important percentage of healthy and wasted non-PMWS pigs were also infected by PCV2. However, significant increase in APPs concentrations was only clearly observed in PMWS affected animals. It has been reported that infections with pathogens like *Streptococcus suis* produce an increase of APPs in both clinically and subclinically infected pigs (Sorensen et al., 2006). However, present data showed that, in accordance with previous results (Segalés et al., 2004), no differences between subclinically PCV2 infected pigs (healthy PCV2 qPCR positive pigs) and healthy non-PCV2 infected pigs (healthy PCV2 qPCR negative pigs) were detected. These findings suggest that sole infection with PCV2 does not imply a systemic inflammatory status
causing a detectable APR; only when the disease develops, HPT and pig-MAP concentrations increase significantly. Taking into account that PMWS affected pigs have higher serum viral loads than non-affected pigs (Brunborg et al., 2004; Olvera et al., 2004; Grau-Roma et al., 2009), a certain level of serum PCV2 load might be associated with the increase of HPT and pig-MAP serum concentrations. Therefore, it is likely that when the animal limits the PCV2 replication to certain levels and/or the virus is not able to overcome the defences of the host, the APR becomes much less evident or even non-detectable using HPT and pig-MAP determinations in sera.

It is difficult to collectively interpret the results of the pigs suffering wasting but without a PMWS diagnosis (wasted non-PMWS animals). This group of animals might include pigs suffering from wasting due to PMWS-unrelated causes, such as other infectious or non-infectious diseases and/or management conditions (Harding, 1997), but might also include PMWS convalescent pigs or pigs that were able to overcome or limit the disease expression (Segalés, 2002; Krakowka et al., 2005). The obtained pig-MAP concentration profile in wasted non-PMWS pigs was similar to the one obtained in healthy pigs. However, an increase in HPT concentration was detected at the two last samplings in Spain. Moreover, in Denmark, HPT concentrations at the moment of necropsy in wasted non-PMWS animals could not be distinguished from those in PMWS affected pigs. These results, together with the wider APPs concentration variation observed in wasted non-PMWS pigs compared to healthy ones, suggest that at least a proportion of wasted non-PMWS animals mounted an APR around the PMWS outbreak. On the other hand, this group of pigs had lower PCV2 serum loads than PMWS-affected ones and higher than healthy pigs during PMWS outbreak (Grau-Roma et al., 2009). Moreover, a correlation between PCV2 viral load and APPs concentration
was observed. Taken together, these findings suggest that the APR detected in the
groups of wasted non-PMWS affected pigs might also be associated with PCV2
infection.

The fact that an increase of both pig-MAP and HPT proteins was seen in Spanish pigs
prior to the PMWS outbreak suggests that the detectable APR occurred some days
before the start of clinical signs. Therefore, those APPs could provide valuable
prognostic information if proper timing of sampling is assured (Murata et al., 2004).
These increased values before the PMWS outbreak were not detected in the Danish
animals, probably due to the low number of samples analyzed at week 9 in each group.

Despite it is known that APPs are generic markers of inflammation and therefore are not
specific of any disease (Petersen et al., 2004; Gruys et al., 2005a), the previously
described potential use of APP as markers of health (Gruys et al., 2005b; Parra et al.,
2006) prompted us to evaluate their diagnostic performance for PMWS. Both HPT and
pig-MAP serum concentrations were significantly correlated. Nevertheless, pig-MAP
gave a better diagnostic value of PMWS, since this protein displayed higher Sp and Sn
values than HPT in the ROC analysis. Moreover, pig-MAP was able to differentiate
PMWS from non-PMWS affected pigs (comprising wasted non-PMWS and healthy
pigs) even at the sampling prior to PMWS development (at least in Spain). The
previously described individual higher variation in HPT concentrations (Piñeiro et al.,
2009a) could partially explain the lower diagnostic value of HPT. In any case, as
previously indicated, the APPs values of single reactants are not sensitive enough to
detect a special subject in a population livestock (Gruys et al., 2005a). It has been
suggested that the acute phase signal situation obtained for an individual animal can be
enhanced when the values of positive APPs are combined with negative APPs as an
index. Such an index could enhance Sn and Sp remarkably in comparison to single
APPs determination in the search for unhealthy subjects among a population of
apparently normal animals (Gruys et al., 2005a).

PMWS is considered a multifactorial disease in which the occurrence of PCV2 infection
is necessary but not sufficient (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés
et al., 2005). Present results showed that, among all studied pathogens, PCV2 was the
only one systematically present in PMWS affected farms and pigs. This fact suggests
that none of the other studied pathogens are necessary for the PMWS development
under field conditions. Moreover, no significant differences in APP serum
concentrations between those pigs showing evidences of infection for pathogens
different from PCV2 at the moment of clinical PMWS outbreak were detected, with the
only exception of \textit{M. hyopneumoniae} in Spain. Specifically, the 5 pigs that gave
positive PCR results for \textit{M. hyopneumoniae} had higher pig-MAP concentration than the
101 pigs with negative results. In fact, the two pigs with higher pig-MAP concentration
(10 and 9 mg/ml) corresponded to 2 out of the 5 \textit{M. hyopneumoniae} nPCR positive,
suggesting that PCV2 co-infection with other pathogens could increase the APR,
aggravating the severity of the disease (Ellis et al., 2004). The potential influence of
other not studied pathogens on the observed clinical signs and, therefore, on the APR of
the PMWS affected animals, can not be ruled out.

In conclusion, the present results indicate that the acute phase response in PMWS
affected pigs occurred concomitantly to PCV2 viremia. In addition, present work
supports the hypothesis that the increase of APPs levels depends on the development of
PMWS and not on infection with PCV2 alone, as previously suggested (Segalés et al., 2004).

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References


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Figure 1. Pig-MAP concentrations (mg/ml) (lines) and PCV2 loads (copies of PCV2/ml of sera) (columns) in PMWS (▲, ▲), healthy (○, ○) and wasted non-PMWS (■, ■) pigs at the different sampling points from Spanish (1A) and Danish (1B) farms. a,b,c indicate statistical significant differences in APP concentration between animal groups in a given sampling time. a’b’c’ indicate statistical significant differences in APP concentration within each animal group along the study period (p < 0.05). Error bars represents standard error of the mean.

1A.
1B.

<table>
<thead>
<tr>
<th>PMWS</th>
<th>$a'$</th>
<th>$a'$</th>
<th>$a'$</th>
<th>$b'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>$a'$</td>
<td>$a'$</td>
<td>$a'$</td>
<td>$a'$</td>
</tr>
<tr>
<td>Wasted non-PMWS</td>
<td>$a'$</td>
<td>$a'$</td>
<td>$a'$</td>
<td>$a'$</td>
</tr>
</tbody>
</table>
Figure 2. Haptoglobin concentrations (mg/ml) (lines) and PCV2 loads (copies of PCV2/ml of sera) (columns) in PMWS (▲), healthy (○), and wasted non-PMWS (-) pigs at the different samplings from Spanish (2A) and Danish (2B) farms. a,b,c indicate statistical significant differences in APP concentration between animal groups in a given sampling time. a’b’c’ indicate statistical significant differences in APPs concentration within each animal group along the studied period (p < 0.05). Error bars represents standard error of the mean.

2A.

<table>
<thead>
<tr>
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<th>PMWS</th>
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<th>Wasted non-PMWS</th>
</tr>
</thead>
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<td>weeks of age</td>
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</tr>
<tr>
<td>1</td>
<td>a’</td>
<td>a’</td>
<td>a’</td>
</tr>
<tr>
<td>3</td>
<td>a’</td>
<td>a’</td>
<td>a’</td>
</tr>
<tr>
<td>7</td>
<td>a’</td>
<td>a’</td>
<td>ab’</td>
</tr>
<tr>
<td>11</td>
<td>b’</td>
<td>a’</td>
<td>bc’</td>
</tr>
<tr>
<td>Necropsy</td>
<td>c’</td>
<td>a’</td>
<td>c’</td>
</tr>
</tbody>
</table>
2B.

<table>
<thead>
<tr>
<th></th>
<th>PMWS</th>
<th>Healthy</th>
<th>Wasted non-PMWS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td></td>
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<tr>
<td>2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
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</tbody>
</table>

Log10(copies PCV2/ml) vs. weeks of age.

Haptoglobin (mg/ml) vs. weeks of age.

Necropsy

PMWS: $a'$, $a'$, $a'$, $b'$
Healthy: $a'$, $a'$, $a'$, $a'$
Wasted non-PMWS: $a'$, $a'$, $a'$, $a'$
**Figure 3.** Correlation between pig-MAP concentration and PCV2 load in each studied category of pigs: PMWS (△, - - - -), wasted non-PMWS (□, - - - -) and Healthy (○, - - - -) in Spain (A) and Denmark (B).

3A.

![Graph showing correlation between pig-MAP concentration and PCV2 load in PMWS category in Spain.](image)

3B.

![Graph showing correlation between pig-MAP concentration and PCV2 load in wasted non-PMWS category in Spain.](image)
Table 1. Number of pigs within each pathological category from which haptoglobin and pig-MAP concentrations were determined in each country.

<table>
<thead>
<tr>
<th></th>
<th>PMWS</th>
<th>Healthy</th>
<th>Wasted non-PMWS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain</td>
<td>35</td>
<td>29</td>
<td>43</td>
<td>107</td>
</tr>
<tr>
<td>Denmark</td>
<td>21</td>
<td>17</td>
<td>15</td>
<td>53</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>46</td>
<td>58</td>
<td>160</td>
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</table>
Table 2. Number of seroconverted pigs against studied pathogens, and nPCR and/or qPCR results at the moment of necropsy in Spain (A) and Denmark (B). Results are indicated as the number of seropositive (serology) or positive (qPCR and nPCR) pigs at euthanasia during the PMWS outbreak respect the total of studied pigs. Percentage is given between brackets.

A.

<table>
<thead>
<tr>
<th></th>
<th>Serology</th>
<th>nPCR</th>
<th>qPCR</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PPV*</td>
<td>PRRSV</td>
<td>SIV</td>
</tr>
<tr>
<td>PMWS</td>
<td>2/35 (6)</td>
<td>1/35 (3)</td>
<td>16/35 (46)</td>
</tr>
<tr>
<td>Healthy</td>
<td>3/29 (10)</td>
<td>0/29 (0)</td>
<td>17/29 (59)</td>
</tr>
<tr>
<td>Wasted non-PMWS</td>
<td>2/43 (5)</td>
<td>1/43 (2)</td>
<td>26/43 (60)</td>
</tr>
<tr>
<td>Total</td>
<td>7/107 (7)</td>
<td>2/107 (2)</td>
<td>59/107 (55)</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>Serology</th>
<th>qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPV</td>
<td>PRRSV</td>
</tr>
<tr>
<td>PMWS</td>
<td>7/21 (33)</td>
<td>9/21 (43)</td>
</tr>
<tr>
<td>Healthy</td>
<td>11/17 (65)</td>
<td>7/17 (41)</td>
</tr>
<tr>
<td>Wasted non-PMWS</td>
<td>4/15 (27)</td>
<td>8/15 (53)</td>
</tr>
</tbody>
</table>

*Porcine parvovirus (PPV), porcine reproductive and respiratory virus (PRRSV), Aujeszky disease virus (ADV), swine influenza virus (SIV), Salmonella spp. (salm), Mycoplasma hyopneumoniae (Myco), porcine circovirus type 2 (PCV2), European and American PRRSV strains (PRRSVe, PRRSVu), Lawsonia intracellularis (law).
Table 3. Optimal cut-off, sensitivity and specificity values of ROC analyses for pig-MAP and Haptoglobin serum concentrations considering PMWS pigs as “diseased” and healthy or wasted non-PMWS as “non-diseased animals”.

<table>
<thead>
<tr>
<th>APPs</th>
<th>PMWS vs Healthy</th>
<th>PMWS vs Wasted non-PMWS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optimal cut-off</td>
<td>Sensitivity (CI95%)</td>
</tr>
<tr>
<td>Pig-MAP</td>
<td>≥1,3</td>
<td>85,7 (73,8-93,6)</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>≥2,7</td>
<td>67,9 (54,0-79,7)</td>
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