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**Kinetics of the response of four positive acute phase proteins
in pigs experimentally infected with toxigenic *Pasteurella multocida***

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Abstract

In the present study acute phase proteins (APP) responses in pigs after infection with toxigenic strain of *Pasteurella multocida* (Pm) were evaluated. Twelve piglets from a herd free from toxigenic Pm were used. Six of them were infected intranasally with Pm. CRP, Hp, SAA and PigMAP concentrations were measured using commercial ELISA tests. CRP and Hp were significantly induced from 2 days post inoculation (dpi). The concentration of Hp in inoculated pigs remained elevated until the end of the study. The concentrations of SAA and Pig-MAP increased significantly from 3 dpi, and remained elevated to 5 or 7 dpi, respectively. Strong correlations were observed between concentration of Hp or SAA and changes in the lungs. No correlations were found between levels of APP in serum and changes observed in the turbinates. On the basis of our investigation, we cannot state that evaluation of APP concentrations in serum may provide useful information about severity of atrophic rhinitis. However, the concentration of all investigated APP increased after inoculation. Thus, APP-measurements may reveal ongoing infection. Monitoring of APP concentrations in the pig herds may help to pinpoint infected animals even before clinical signs are present. If found, these “APP-high” animals may be chosen next for pathogen-specific diagnostics. Early diagnosis and therapy may prevent the infection from spreading in the herd. Moreover, monitoring of APP concentrations in serum may be useful for selecting clinically healthy pigs

before integration into an uninfected herd. Future studies should focus on the possibility of distinguishing infected and non-infected pigs under field conditions.

Key words: pigs, acute phase protein, *Pasteurella multocida*, early diagnostics

1. Introduction

The acute phase response is an innate, nonspecific early response of the organism caused by various stimuli (Heegaard 1998, Carroll et al., 2004, Grau-Roma, 2009). This reaction is mediated by pro-inflammatory cytokines and involves local and systemic reactions, including changes in the concentrations of acute phase proteins (APP) (Murata et al., 2004, Grau-Roma, 2009). The occurrence and progress monitoring of infection by APP measurements in blood samples is used extensively in human patients (Tsiakalos et al., 2009, Chai et al., 2010). Similar diagnostic value of APP has been proposed in veterinary medicine (Carroll et al., 2004). Moreover APP are possible candidates to monitor the health status of pigs herds (Alava et al., 1997, Sorensen et al., 2006).

APP patterns differ among species. In swine, C-reactive protein (CRP), haptoglobin (Hp), serum amyloid A (SAA) and pig major acute phase protein (Pig-MAP) are the main positive APP (Lampreave et al., 1994, Heegaard et al., 1998, Carpintero et al., 2007). Negative APP in pigs include albumin, Apolipoprotein A-I (Apo) and transthyretin (Gruys et al., 1994, Lampreave et al., 1994, Carpintero et al., 2007). Serum concentrations of CRP and SAA in pigs increase early after an inflammatory stimulus, reach the maximum levels within 2-3 days followed by a rapid decline. Pig-MAP and Hp increases slightly later (reach a peak within 5 days) and remain elevated for longer period (up to 2 weeks) (Petersen et al., 2004, Sorensen et al., 2006).

Pasteurella multocida (Pm) is an aetiological agent of progressive atrophic rhinitis (PAR) and plays a role in multifactorial bronchopneumonia in pigs. PAR is caused by toxigenic Pm, with or without *Bordetella bronchiseptica* (Bbr). The nonprogressive form is caused by Bbr (de Jong, 2006). Both toxigenic and nontoxigenic strains of Pm have been associated with pneumonia (de Jong, 2006). Atrophic rhinitis (AR) is an infectious disease of pigs characterized by nasal discharge, shortening or twisting of the snout, atrophy of the

turbinate and reduced productivity (de Jong, 2006). It is generally accepted that a herd in which toxigenic Pm is present is defined as affected with PAR, whether or not clinical signs of the disease are evident (Pedersen et al., 1988).

To our knowledge, apart from the study of Francisco et al. (1996) who dealt with Hp only, there has been no reports regarding APP responses after infection with toxigenic Pm. Therefore, the aims of the present study were: 1) to evaluate APP response after the pigs had been infected with the toxigenic strain of Pm and 2) to evaluate the correlation between levels of investigated APP in serum and severity of infection caused by toxigenic Pm and/or 3) to establish the usefulness of CRP, Hp, SAA and/or Pig-MAP measurements in identification of infected animals

2. Materials and methods

2.1. Animals

The twelve piglets of Landrace/Pietrain/Duroc crosses, approximately 5-week-old, both sexes, from a herd with high health status, free from toxigenic Pm were used.

The herd was seronegative to porcine reproductive and respiratory syndrome virus and Aujeszky disease virus. No evidence of pleuropneumonia, streptococcosis and AR was recorded at any age group of pigs on the farm based on clinical, serological and pathological examinations. Pigs were not vaccinated against atrophic rhinitis.

During the experiment, piglets were housed in two independent, isolated units, one for the control pigs and one for the infected pigs. Feed (antibiotic free) and water were offered *ad libitum*.

2.2. Bacterial strain and culture conditions

A DNT producing Pm type D isolate, which originated from a pig with the clinical form of PAR, was used for the experimental infection. It was cultured on blood agar at 37 °C for 24 h. A suspension of this culture to 0.7 McFarland turbidity (which corresponds with

approximately 1.5×10^8 colony forming units (CFU)/ml) was prepared in PBS. A plate count was also performed to quantify the accurate number of viable bacteria (final result 1.6×10^8 CFU/ml).

2.3. Experimental design

Prior to inoculation, animals were tested for serum antibodies against dermonecrotic toxin (anti-DNT Ab) produced by Pm. Additionally, the nasal and tonsil swabs from all piglets were evaluated for presence of toxigenic Pm according to procedures described below.

On day 0, six piglets (n°1-6) were inoculated intranasally with toxigenic Pm (3.2×10^8 CFU/pig). Six non-inoculated pigs served as control pigs (n° 7-12).

Ten days post inoculation (dpi) the pigs were euthanized. Necropsy was performed immediately after the animals were sacrificed.

All procedures were approved by the Local Ethical Commission.

2.4. Clinical and pathological examination

Rectal temperatures were assessed daily and clinical signs of disease were recorded. Fever was defined as body temperature of $\geq 40^\circ\text{C}$.

Blood samples were collected on days -7, 0 (challenge), 1, 2, 3, 5, 7 and 10 dpi. Serum was harvested after centrifugation and stored at -20°C (not longer than three weeks) for further analyses. Nasal and tonsil swabs were taken from each pig at 10 dpi. Macroscopic lesions in the respiratory tract were recorded during necropsy. Samples of lungs and bronchoalveolar lavage fluids (BALF) were collected aseptically for further analyses..

2.4.1. Turbinate score

On day 10 pigs were euthanized and snouts were sectioned at the upper first premolar tooth. The lesions in the left and right turbinates and septum were scored as 0, 1, 2 and 3 as was described previously (Gatlin et al , 1996). Normal turbinates were graded as 0. Slight but obvious atrophy was graded as 1. Moderate atrophy of not less than half of the turbinates was

graded at 2. Severe atrophy of the dorsal and ventral scrolls was graded as 3. The three scores (from left and right turbinates and septum) were then added together and divided by 3, to determine final visual turbinate scores (TS) for each pig, ranging from 0 to 3.

2.4.2. Lung Score

Lungs were assessed according to the scheme described by Christensen et al. (1999). If no changes were found in the lobe, it was scored as 0%. Changes in the right lung were scored as follows: apical lobe 10%, cardiac lobe 10%, diaphragmatic lobe 35% (all together 55%). Changes observed in the left lung were scored as: apical lobe 5%, cardiac lobe 5%, diaphragmatic lobe 30%. Changes in intermediate lobe was scored as 5%. All recorded scores were then added together, to determine final visual lung score for each pig, ranging from 0 to 100%.

2.5. Laboratory examination

2.5.1. Swabs and tissue samples

Nasal and tonsil swabs, lungs sample and BALF were tested for presence of Pm. The standard bacteriological methods were used for detection of Pm in the nasal and tonsil swabs, lung samples and BALF, as described previously (Markowska-Daniel et al. 2010). For Pm isolation collected samples were streaked onto agar containing 5% horse blood and incubated for 24h at 37°C in 7.5% CO₂ atmosphere. Strains with characteristic colony morphology were identified by Api 32E tests (BioMerieux, France). Additionally, for detection of genes encoding DNT, the PCR test was performed according to the previously described procedure (Register et al., 2006).

2.5.2. Serum analyses

For all analyses serum samples were tested in duplicate, according to the manufacturer's recommendations.

Anti-DNT Ab were measured using a commercial ELISA test (PMT ELISA, Oxoid, Hampshire, UK) according to the manufacturer's specification.

For determination of APP commercial tests were used according to the manufacturer's recommendation (PigMAP KIT ELISA from PigCHAMP Pro Europa S.A, Spain; Phase C-Reactive Protein CPR-Porcine, Phase Serum Amyloid A Assay and Haptoglobin Kit from Tridelata Development Ltd, County Kildare, Ireland). Prior to analyses serum samples were diluted as follows: 1:2000 for CRP, 1:4 for Hp, 1:500 for SAA and 1:1000 for Pig-MAP.

2.6. Statistical analysis

The obtained data were subjected to the W. Shapiro-Wilk's test of normality and the Levene's test of equal variances. A nonparametric Kruskal-Wallis test with post hoc multiple comparisons for comparison of all pairs was used for comparison of mean APP concentrations and levels of anti-DNT antibodies. For analysis of correlation between measured parameters the Spearman-Rang correlation (nonparametric) were used. Differences with $\alpha < 0.05$ were considered as significant. All calculations were performed with the Statistica 8.0 (Statsoft, Poland) computer program.

3. Results

3.1. Clinical signs

The infection method used in our study resulted in clinical signs, including sneezing and coughing. In half of the infected pigs, accelerated respiratory rates and dyspnoea were also observed. Inoculation of pigs with toxigenic Pm did not produce a remarkable febrile response. Rectal temperature was elevated over 40°C in three inoculated pigs, however these elevations were of short duration. In the control pigs no clinical signs of any disease were seen.

3.2. Serological response against *Pasteurella multocida* DNT

Infection with Pm resulted in an increase of anti-DNT Ab in the serum. Unlike the control pigs, the mean level of anti-DNT Ab started to increase significantly from 5 dpi, and tended to increase till the end of study (10dpi).

3.3. Microbiological examination

In the nasal and tonsil swabs taken before inoculation, no DNT producing Pm were found (bacteriological examination and PCR test). The results of the bacterial isolation, as well as the identification of Pm genes encoding DNT with the use of the PCR technique, in samples taken from infected pigs at 10 dpi are given in Table 1. Reisolation of Pm from nasal swabs was successful only in 2 out of 6 pigs (2/6), while from BALF in 5/6 pigs. The use of the PCR technique improves detection of Pm from nasal swabs in 4/6 pigs and has no influence on Pm detection in BALF (5/6 pigs). For detection of Pm, tonsils swabs seem to be more useful clinical samples than nasal swabs. Reisolation of Pm and identification with the use of PCR was successful in 4/6 and 5/6, respectively.

3.4. Pathological examination

Postmortem examination revealed typical lesions deriving from toxigenic Pm infection in pigs. In 5/6 inoculated pigs atrophy of turbinates was observed (mean TS = 0.76 ± 0.41 , range 0–1.33). The most severe atrophy observed in the present study was scored as 1.33 (Fig. 1).

Lesions typical of pneumonic pasteurellosis were found in the lungs of 3/6 inoculated pigs. In all cases, the toxigenic Pm were reisolated from the lung samples and BALF in a pure culture. The mean lung score was $15\% \pm 16.61\%$ (range 0–40%).

3.5. Acute phase proteins

All investigated APP were strongly induced after infection, with mean maximum levels from day 2 to 3. The time course of mean APP concentration (\pm SD) in infected pigs

revealed significant changes during the study period (Fig. 2). In the control pigs levels of investigated APP remained relatively constant.

C-reactive protein. Prior to inoculation, pigs had CRP-values below 14.5 µg/mL (mean 10.21 ± 4.78), while 24 h after infection the mean CRP level was higher in comparison to day 0 ($p=0.0662$). A significant increase of mean CRP concentration was observed at 48 h after infection ($p<0.001$), reaching a mean level of 83.29 ± 53.81 mg/ml (8-fold increase). The maximum individual CRP levels were observed between 1 to 3 days after infection (mean 2 ± 0.70). Starting from 72h after inoculation, the mean concentrations of CRP did not differ significantly from that observed on day 0, as well that observed in the control pigs ($p \geq 0.05$).

Haptoglobin. Preinoculation individual levels of Hp were found to be below 0.74 mg/ml, and the highest individual induced level reached above 6.15 mg/ml (at 3 dpi). Changes in serum Hp concentrations were observed in all Pm infected pigs. The concentration of Hp had increased by 48h after inoculation, by which time the mean concentration of Hp was significantly higher than in the control pigs ($p<0.01$). The highest concentrations of Hp were observed between the second and third day (2.6 ± 0.54). The mean peaked level was around 5-fold higher compared to the mean preinoculation concentration. The levels of Hp remain elevated to the 10 dpi, however, a significant differences between concentration of Hp in the control animals and animals infected were observed only up to 7 dpi ($p<0.05$).

Serum amyloid A. The mean concentration of SAA did not increase significantly during the first 48 h after inoculation. A statistically significant increase of mean SAA concentration, as compared to the control pigs, was observed on days 3 and 5 post inoculation ($p<0.01$). The highest concentrations of SAA in particular animals were seen on 3.6 ± 1.34 dpi (between 2 and 5 dpi). Mean peak level reached 125.01 ± 81.53 µg/ml, this was over 30-fold higher compared to day 0-level. From 5 dpi the SAA concentrations had decreased and

reached preinoculated levels around 7 dpi. Starting from 7 dpi the mean level of SAA did not differ significantly between the control and the infected pigs.

Pig Major Acute Protein. Preinoculation levels of Pig-MAP were found to be below 0.95 mg/ml (mean 0.83 mg/ml \pm 0.12). Concentration of Pig-MAP significantly increased 72 h after challenge ($p < 0.001$) and remained elevated until about 96 h. The highest Pig-MAP concentrations in particular pigs were detected between 2 to 5 dpi (mean 3.6 ± 1.34). The maximum mean level of Pig-MAP, observed at 3 dpi, was around 4 times higher compared to day 0-level. The mean concentration of Pig-MAP returned to pre-inoculation value at 10 dpi.

Strong, significant correlations were found between concentrations of Hp and SAA, as well as Hp and Pig-MAP ($r = 0.78$ and 0.96 , respectively), and between Pig-MAP and SAA ($r = 0.82$) in inoculated pigs. Additionally, significant correlations were observed between concentration of Hp and SAA at the day of necropsy and changes in the lungs (respectively $r = 0.81$ and $r = 0.94$, $p < 0.05$). Surprisingly, no significant correlations were found between levels of investigated APP in serum and TS.

4. Discussion

Measurements of APP have extensively been used for: monitoring of health status, evaluation of treatment efficacy or immunization protocols and in monitoring the progression of an infectious disease (Carroll et al., 2004, Sorensen et al., 2006, Petersen et al., 2004, Carpintero et al., 2007, de la Fuente, 2008). Previous studies in pigs have described the kinetics of APP response in the course of various diseases (Hall et al., 1992, Heegaard et al., 1998, Sorensen et al., 2006). Until now, though, there have been no reports published on the development over time of acute-phase response characterized by more than one APP after infection with toxigenic Pm. Francisco et al. (1996), observed a slight correlation between Hp level in serum and extent of turbinate atrophy after infection with Pm. However in Francisco's

et al. (1996) report, there was no data about kinetics of Hp concentrations in serum during the early stage of the infection.

Therefore, in our study, the early response of Hp, CRP, SAA and Pig-MAP induced by infection with toxigenic Pm was characterized, during the first 10 dpi. In addition, the relationship between APP concentrations and pathological changes were analyzed.

Almost a 5-fold increase in Hp, after inoculation with Pm, places pig Hp into the category of moderate APP (Murata et al., 2004). Similar induction levels and relative increases in pig Hp were observed by others (Hall et al., 1992, Sorensen et al., 2006), while (Heegaard et al., 1998) found a much stronger reaction (over a 25-fold increase). In the study by Sorensen et al. (2006), Hp showed a 10-fold increase and stayed elevated until the end of the study (14 dpi). Similar prolonged Hp responses were also found in the present study. However, in the previously mentioned experiment by Sorensen et al. (2006), the Hp responded later compared to our results (peak levels were seen around 5 dpi). In the study by Heegaard et al., (1998), the kinetics of the Hp response to *A. pleuropneumoniae* were similar to the response seen in our study.

CRP was induced from a level of around 10 µg/ml to around 83 µg/ml. This placed CRP in the category of having moderate reacting APP (Murata et al., 2004). Analogic increases were found by Sorensen et al. (2006) after *S. suis* inoculation and by Lampreave et al. (1994) upon turpentine induced inflammation. The very fast, non-protracted response of CRP was in accordance with results of previous experiments (Heegaard et al., 1998, Sorensen et al., 2006).

Comparable increases in Pig-MAP (about 4-fold) with similar absolute levels were found by Grau-Roma et al. (2009) in pigs with postweaning multisystemic wasting syndrome. On the other hand, Heegaard et al. (1998) and de la Fuente et al. (2008) found over a 13-fold increase in Pig-MAP after experimental infection with *A. pleuropneumoniae* or *Haemophilus*

parasuis, respectively. The kinetics of Pig-MAP response after inoculation with *S. suis* (Sorensen et al., 2006) was generally similar to, but more protracted than, the response seen in the present study. In the present study, the level of Pig-MAP increases later than the levels of Hp and CRP. This observation was similar to that reported by Heegaard et al. (1998).

In agreement with previous reports (Heegaard et al., 1998, Sorensen et al., 2006), yet in contrast to another report (Gruys et al., 1994) SAA was clearly found to be an APP in pig. The mean induction level was over 30-fold higher than before inoculation. A similar strong induction of SAA was observed after *S. suis* infection (Sorensen et al., 2006). However, in the present study, the response of SAA was observed later (mean peak day 3.6) and was more protracted (to 7 dpi).

After infection of pigs with toxigenic Pm various kinetics of responses could be identified within the APP tested. CRP and Hp were significantly induced from 2 dpi. The concentration of Hp in inoculated pigs remained elevated to the end of the study. The concentrations of SAA and Pig-MAP increased significantly from 3 dpi, and remained elevated to 5 or 7 dpi, respectively.

In the study by Francisco et al. (1996), a slight correlation between Hp concentration in serum and extent of turbinate atrophy was found after infection with Pm. Our results did not confirm those findings. In the present investigation, there was no correlation between levels of APP and degree of turbinate atrophy. Also, the recovery of Pm from nasal swabs did not correlate with the degree of turbinate atrophy, which is in agreement with previous reports (Sakano et al. 1992, Francisco et al. 1996). In the present study, correlations between TS and APP levels were evaluated according to (1) maximal concentrations of particular proteins, (2) concentrations at the day of necropsy and (3) geometric mean value (0-10dpi) calculated for each animal. The timing of sampling must be taken into consideration as it may be critical in showing a more precise correlation of the clinical signs or disease progression, as was

previously reported also by Francisco et al. (1996). In our investigation samples were collected more frequently and earlier, compared to another investigation (Francisco et al. 1996). The highest concentration variations of Hp, and the other investigated APP were between 0 and 7 dpi.

5. Conclusions

It is known that APP are markers of inflammation and are not specific of any disease (Petersen et al., 2004, Grau-Roma et al., 2009). The previously described potential use of APP (Heegaard et al., 1998, Petersen et al., 2004, Sorensen et al., 2006, de la Fuente, 2008, Carpintero et al., 2007) however, prompted us to look at the course of the main APP serum concentrations after infection of pigs with Pm – very important pathogen of swine. On the basis of our investigation we cannot state that evaluation of CRP, Hp, SAA or PigMAP concentrations in pig serum may provide useful information about severity of AR. Despite the fact that no relationship was observed between APP concentrations in serum and severity of AR, data presented in our paper provide clear evidence that all investigated APP strongly reacted after inoculation. Thus, APP-measurements may reveal ongoing infection.

With the above information in mind, monitoring of APP concentrations in the pig herds may help to pinpoint infected animals even before clinical signs are present. If found, these “APP-high” animals may be chosen next for pathogen-specific diagnostics. Early diagnosis and therapy may prevent the infection from spreading in the herd. Moreover, monitoring of APP concentrations in serum may be useful in selecting clinically healthy pigs before integration into an uninfected herd. Future studies should focus on the possibility of distinguishing infected and non-infected pigs under field conditions. The use of antibiotics in the herd would be then rationalized, improving the health of the pig herd and ultimately improving the economic results.

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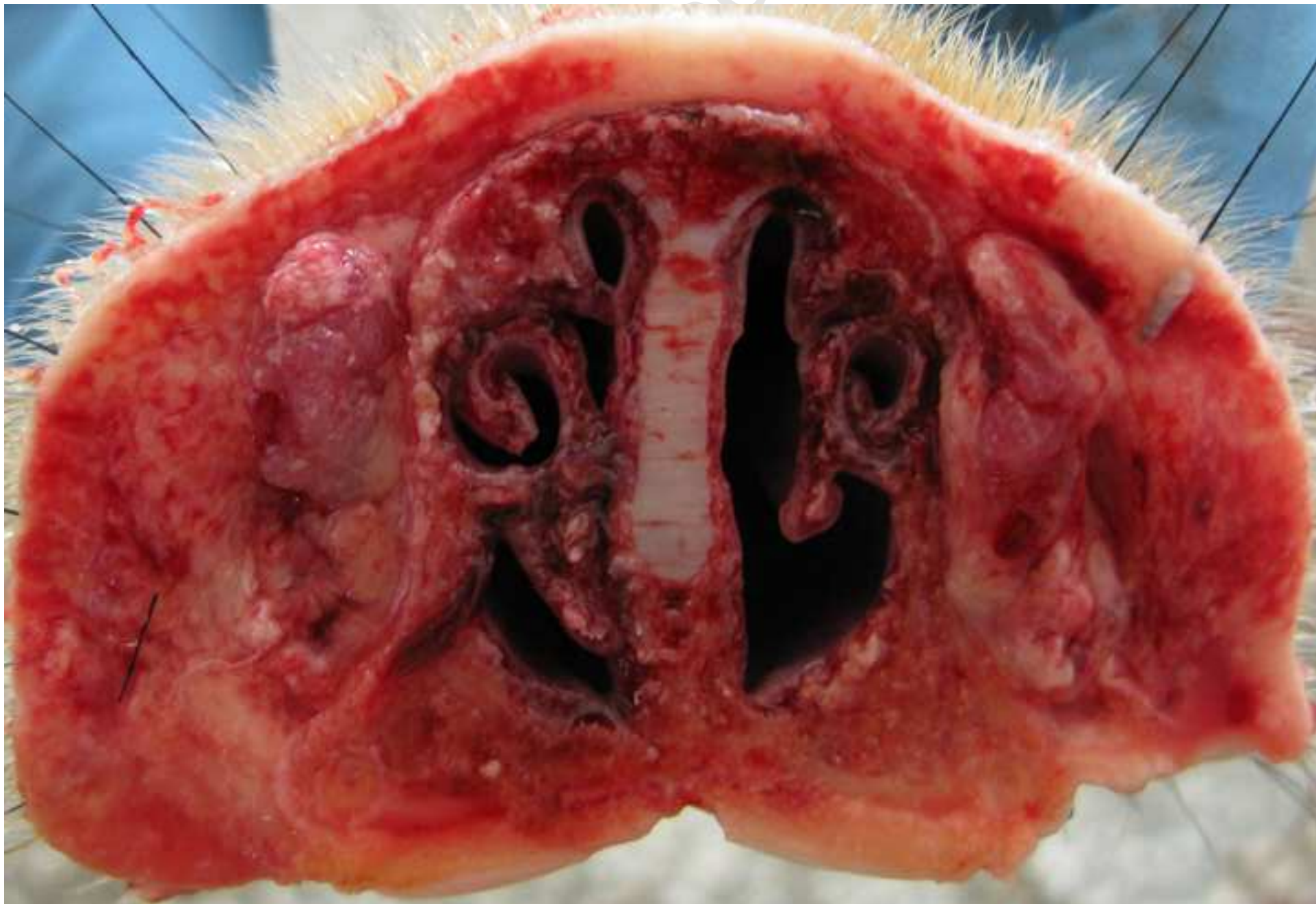
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Tab.1. Number of infected pigs from which the toxigenic *Pasteurella multocida* (Pm) were reisolated and/or identified with the use of PCR ten days after infection with Pm.

	Nasal swabs	Tonsil swabs	Lung (pure culture)	BALF (pure culture)
Reisolation	2/6	4/6	4/6	5/6
PCR	4/6	5/6	4/6	5/6



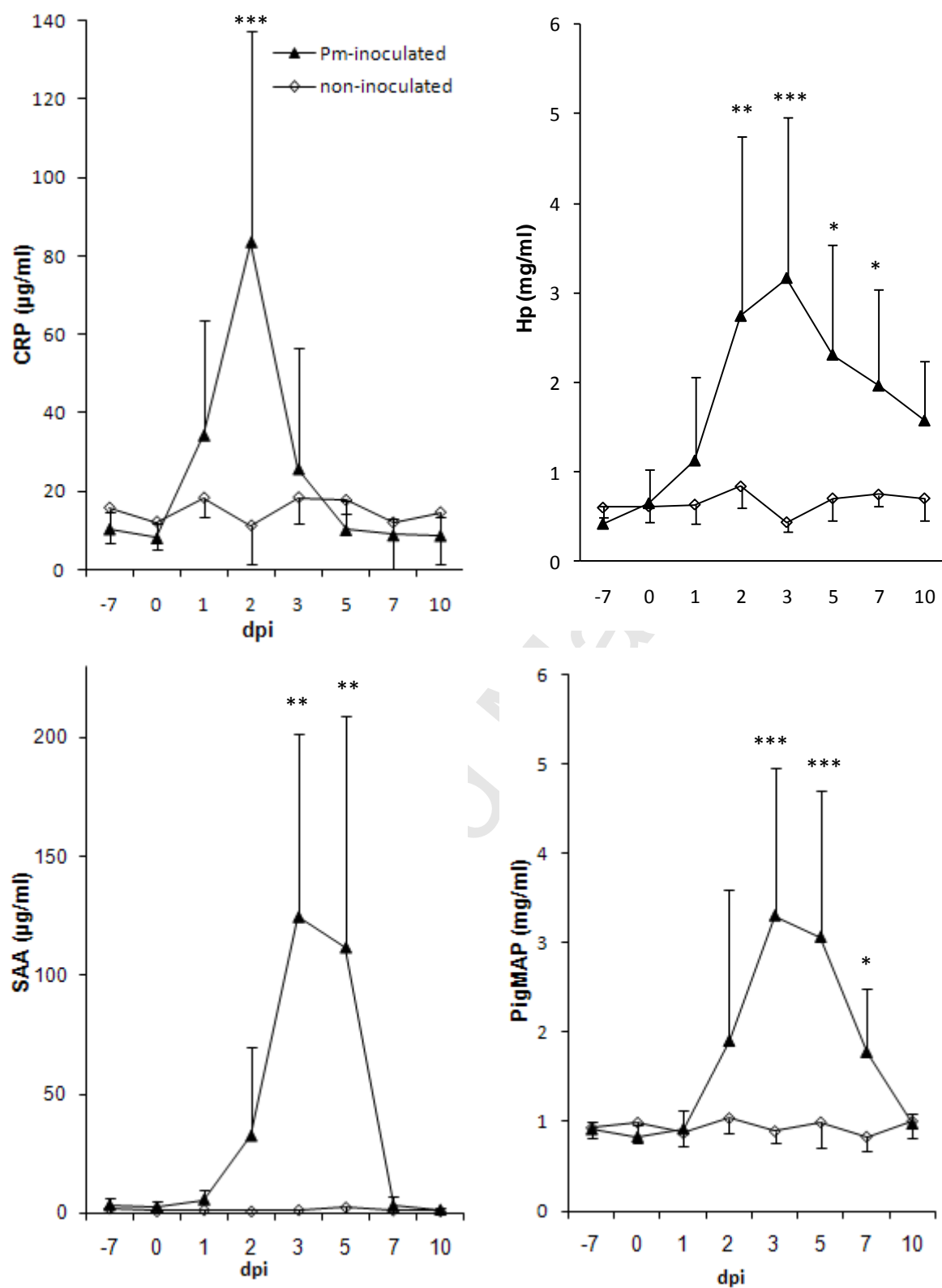


Fig.2. Concentrations of CRP, Hp, SAA and Pig-MAP in serum of pigs before and on various time-point after intranasal inoculation with toxigenic *Pasteurella multocida*. Data are geometric mean \pm SD.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ - significant increase as compared to control animals