PORCINE ACUTE PHASE PROTEIN RESPONSE TO EXPERIMENTAL INFECTION WITH *BORDETELLA BRONCHISEPTICA*

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Abstract

The response of five positive acute phase proteins (APP) was evaluated in pigs after infection with *Bordetella bronchiseptica* (Bbr). Twelve piglets from a herd with high health status were used. Six of them were infected intranasally with Bbr (3.4 x 10^6 cfu). The standard bacteriological methods and PCR were used for detection of Bbr in nasal swabs, lungs, and bronchoalveolar fluids. Serum APP concentrations were measured using commercial tests. Various kinetics of response was identified within the APP tested. C-reactive protein and serum amyloid A was characterised as a very fast, transient responder, while haptoglobin as a fast and very prolonged responder. Pig major acute phase protein was characterised as a fast, protracted responder. Alfa-1-acid-glycoprotein did not respond significantly after inoculation. No correlations were found between levels of APP in serum and changes in the turbinates. On the basis of the investigation we cannot state that evaluation of APP concentrations in serum may provide useful information about severity of atrophic rhinitis. However, the concentration of most investigated APP increased after inoculation. Thus, APP measurement could be the primary screening test prior to specific disease diagnosis. “APP-high” animals may be chosen next for pathogen-specific diagnostics. Moreover, monitoring of serum APP concentrations may be useful for selecting clinically healthy pigs before integration into an uninfected herd.

Key words: pigs, *Bordetella bronchiseptica*, acute phase proteins, early diagnosis.

The acute phase protein (APP) response to infection, inflammation, or trauma has been identified in a number of species and consists of alterations to the serum concentrations of several proteins, including APP (9, 12, 16). Occurrence and monitoring progression of infection by APP measurements in blood samples is used extensively in human patients (5, 20). Similar diagnostic value of APP has been proposed in veterinary medicine. Moreover, APP are possible candidates to monitor the health status of pig herds (1, 18).

It is known that the profile of APP response to stimulation differs between species. Although differences between species exist, in most animals serum concentrations of C-reactive protein (CRP) and serum amyloid A (SAA) increase early after an inflammatory stimulus, reaching the maximum levels within 2-3 d and then showing a rapid decline (3, 10, 12, 14). Alfa-1-acid-glycoprotein (AGP), pig major acute phase protein (pig-MAP), and haptoglobin (Hp), increase slightly later, reaching a peak within 3-5 d, and remain elevated for a longer period (17, 18).

*Bordetella bronchiseptica* (Bbr) is an aetiological agent of non-progressive atrophic rhinitis (NPAR). In combination with *Pasteurella multocida* this pathogen may be responsible for progressive form of the disease (PAR) (4, 8, 13).

To our knowledge, apart from the study of Francisco et al. (7), who dealt with Hp only, there were no reports regarding APP response to Bbr in pigs. Therefore, the aim of the presented study was to investigate the kinetics of five APP responses in pigs inoculated intranasally with Bbr.

Material and Methods

Animals. Twelve piglets, approximately 6 weeks of age, both sexes, from a herd with very good health status were used. Not every pig on the farm was vaccinated against atrophic rhinitis (AR). The herd was seronegative to porcine reproductive and respiratory syndrome virus and Aujeszky’s disease virus. On the grounds of clinical, serological, and pathological examinations no evidence of pleuropneumonia, streptococcosis, or AR was recorded at any age group of pigs on the farm. During the experiment, the piglets were housed in isolated unit. Feed (antibiotic free) and water were offered ad libitum.

Bacterial strain and culture conditions. 24-h culture of Bbr field strain, isolated from a pig with clinical form of NPAR, suspended in a physiological saline to a final concentration of 1.7 x 10⁶ cfu/mL, was...
used for intranasal inoculation. For reisolation of Bbr strains, tested samples (lungs, nasal swabs, bronchoalveolar lavage fluids - BALF) were simultaneously streaked onto agar containing 5% horse blood and selective G20G medium supplemented with gentamycin (10 mg/mL), penicillin (10 mg/mL), nystatin (10 mg/mL), and nitrofurantoin (10 mg/mL). Blood agar plates were incubated for 24 h at 37 °C in 8% CO₂ atmosphere, whereas G20G medium plates aerobically for 48-72 h at 37 °C. Strains with characteristic colony morphology were identified by standard methods (Gram-staining, tests for oxidase and catalase) (15).

**Experimental design.** On day 0, six piglets were inoculated intranasally with Bbr strain (2 ml of medium containing 1.7 x 10⁸ cfu/mL). Six control piglets received PBS as placebo. Rectal temperature and clinical signs of diseases were recorded daily. Fever was defined as body temperature of >40 °C. Blood samples were collected on days -7, 0 (inoculation), 1, 2, 3, 5, 7, and 10 post inoculation. Serum was stored at -20 °C. Nasal swabs were taken before inoculation and at the day of necropsy. Ten days post inoculation, the piglets were euthanized. Samples of the lungs and BALF were collected aseptically for further analyses. All procedures were approved by the Local Ethical Commission.

**Turbinate score.** The snouts were sectioned at the upper first premolar tooth during necropsy. The lesions in the left and right turbinates and septum were scored as 0, 1, 2, and 3, as was described previously (8). 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 as 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 piglet, ranging from 0 to 3.

**Examination of swabs and tissue samples.** Nasal swabs, lung samples, and BALF were tested for the presence of Bbr using standard bacteriological methods, as previously described (15). Additionally, the PCR technique was performed for both clinical samples and cultures. Strains of Bbr were identified by amplification of the dermonecrotxin (DNT) gene according to procedure described by Stepniwska and Markowska-Daniel (19).

**Measurement of acute phase proteins (APP).** For determination of APP, the commercial tests were used according to 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 Tridelta Development Ltd, Ireland, Porcine α1AG from Metabolic Ecosystem Co. Ltd., Japan). Prior to analyses, serum samples were diluted as follows: 1:2,000 for CRP, 1:4 for Hp, 1:500 for SAA, 1:1,000 for Pig-MAP, and 1:2 for AGP.

**Statistical analysis.** A Kruskal-Wallis test with *post hoc* multiple comparisons for comparison of all pairs was used for comparison of mean APP concentrations. For analysis of correlation between measured parameters, the Spearman-Rang correlation was used. Differences with α <0.05 were considered as significant. All calculations were performed with the Statistica 8.0 (Statsoft, Poland) computer programme.

**Results**

**Clinical signs.** The infection method used in the study induced clinical signs, including sneezing and coughing. In four out of six piglets also accelerated respiratory rates and dyspnoea were observed. In the control piglets no clinical sings of any disease were seen. The body temperature of five inoculated pigs increased over 40°C, 24 h post inoculation.

**Microbiological and pathological examination.** No Bbr were found (bacteriological examination and PCR test) in the nasal swabs taken before inoculation. The results of bacterial reisolation and identification of Bbr genes encoding DNT with the use of PCR technique in samples taken from infected piglets at the day of necropsy are given in Table 1. Reisolation of Bbr from nasal swabs was successful only in three, while from BALF in four inoculated piglets. The use of PCR technique improved detection of Bbr from nasal swabs (6/6) and from lungs (5/6).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Number of piglets from which <em>Bordatella bronchiseptica</em> (Bbr) was reisolated and/or identified with the use of PCR at the day of necropsy</th>
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<tr>
<td>Nasal swabs</td>
<td>Lung</td>
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<tr>
<td>Reisolation</td>
<td>3</td>
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<tr>
<td>PCR</td>
<td>6</td>
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Postmortem examination revealed lesions typical for AR. The atrophy of turbinates was observed in five inoculated piglets (mean TS = 0.71 ± 0.44).

**Acute phase proteins.** Most APP were strongly induced after inoculation. The concentrations of APP in infected piglets revealed significant changes during the study period (Fig. 1). In the control piglets levels of all investigated APP remained relatively constant.

Prior to inoculation all piglets had CRP-values below 21.55 μg/mL (mean 16.60 ± 3.27). Twenty four hours after inoculation, the mean CRP level was significantly higher (almost 8-fold increase) in comparison to day 0, (P<0.05). The maximum individual CRP levels were observed between 1 and 2 d post inoculation. Starting from 72 h after inoculation, the mean concentration of CRP did not differ significantly from that observed on day 0, and from the values detected in the control piglets (P≥0.05).

Preinoculation serum levels of Hp were found to be below 0.80 mg/mL. Changes in Hp concentrations were observed in all Bbr infected piglets. The concentration of Hp increased 48 h after inoculation.
Fig. 1. Concentrations of CRP, Hp, SAA, Pig-MAP, and AGP in serum of pigs before and on various time-points after intranasal inoculation with *Bordetella bronchiseptica*. Data are geometric means ± SD; dpi – days post inoculation.

* P<0.05 as compared to the control animals.
The mean peaked level was over 5-fold higher compared to mean preinoculation concentration. The highest individual concentrations of Hp were observed between the 2nd and 3rd d post inoculation. The levels of Hp remained significantly elevated until day 7 post inoculation (P<0.05).

The mean concentration of SAA significantly increased 24 h post inoculation. The statistically significant increase in mean SAA concentration, as compared to the control piglets, were observed on days 1, 2, and 3 after infection (P<0.05). The highest concentrations of SAA in particular animals were seen between the 2nd and 3rd d after inoculation. Mean peak level reached 80.50±46.03 μg/mL and was over 40-fold higher compared to day 0-level. From day 5, the SAA concentration reached the preinoculated level (P≥0.05) and did not differ significantly between the control and infected piglets.

Preinoculation levels of Pig-MAP were found to be below 0.78 mg/mL (mean 0.72±0.06 mg/mL). Concentration of Pig-MAP significantly increased 48 h after challenge (P<0.05) and remained elevated for about 24 h. The highest Pig-MAP concentrations in particular piglets were detected between the 2nd and 3rd d post inoculation. The maximum mean level of Pig-MAP, observed on day 3, was almost 3 times higher in comparison to day 0. The mean concentration of Pig-MAP returned to preinoculation value on day 5 post inoculation.

Serum AGP in Bbr-infected piglets did not change significantly, and the AGP concentrations ranged from 881.15 to 1,295.64 μg/mL. In the control piglets the concentration of AGP ranged from 909.14 to 1,063.2 μg/mL.

Strong correlations were found between concentrations of Hp and SAA, as well as Hp and Pig-MAP (r=0.71 and 0.79, respectively), and between Pig-MAP and CRP (r=0.75) in inoculated piglets. All correlations were significant (P<0.05). No significant correlations were found between levels of serum APP and changes observed in the turbinates.

**Discussion**

Measurements of APP have been extensively used for monitoring the health status and progression of infectious diseases (3, 17, 18). Previous studies in pigs have described the kinetics of APP response in the course of various diseases (11, 12, 18). Up till now, however, there have not been any reports published on the dynamics of the development of acute-phase response characterised by more than one APP after infection with Bbr. Therefore, in our study, an early response of CRP, Hp, SAA, Pig-MAP, and AGP induced by infection with Bbr was characterised during the first 10 d post inoculation, and the relationship between APP concentrations and pathological changes was analysed.

CRP was induced from a level of around 21 μg/mL to about 126 μg/mL. Similar increases were found by Sorensen et al. (18) after Streptococcus suis (S. suis) inoculation and by Lampreave et al. (14) upon turpentine induced inflammation. The very fast, non-protracted response of CRP in our study was in accordance with the results of previous experiments (12, 18).

Over a 5-fold increase in Hp, after inoculation with Bbr, places pig Hp into the category of moderate APP (16). Similar induction levels and relative increases in pig Hp were observed by others (11, 18). Heegaard et al. (12) found over a 25-fold increase in Hp after infection with A. pleuropneumoniae, however, the kinetics of the Hp response over time was similar to the response seen in our study.

Summarising, after infection of piglets with Bbr, various kinetics of responses could be identified within the APP tested. CRP could be characterised as a very fast, transient responder, while Hp as a fast and very prolonged responder. SAA could be described as a very fast and transient responder, while Pig-MAP as a fast, protracted responder.

Similar increase in Pig-MAP, with comparable absolute levels was found by Grau-Roma et al. (9) in pigs with post-weaning multisystemic wasting syndrome. On the other hand, Heegaard et al. (12) found over a 13-fold increase in Pig-MAP after experimental infection with A. pleuropneumoniae. The kinetics of Pig-MAP response after inoculation with S. suis (18) was generally similar, but more protracted than the response seen in the presented study. In accordance with data presented by Heegaard et al. (12), the level of Pig-MAP increased later than the levels of Hp and CRP.

In agreement with previous reports (12, 18), SAA was clearly found to be AAP in pig. Its mean induction level was over 40-fold higher than before inoculation. A similar strong induction of SAA was observed after S. suis infection (18).

Serum AGP in Bbr-infected pigs did not change significantly, which is consistent with observations reported previously by Asai et al. (2), who infected pigs with porcine reproductive and respiratory syndrome virus. Similar results were found also in other reports (6, 14). These results may suggest that AGP is not APP in pigs.

In the study by Francisco et al. (7), a slight correlation between Hp concentration in serum and extent of turbinate atrophy was found after infection with Bbr. Our results did not confirm that findings. In the presented investigation, there was no correlation between levels of APP and degree of turbinate atrophy. The correlations between TS and APP levels in our study were evaluated according to (1) maximal concentrations of particular proteins, (2) concentrations on the day of necropsy, and (3) geometric mean value (0-10 dpi) calculated for each animal. Timing of sampling must be taken into consideration as it may be critical for showing a more precise correlation of the clinical signs or disease progression, as was previously reported also by Francisco et al. (7). In our investigation, samples were collected more frequently and earlier, compared to the mentioned study (7). The
highest concentrations of Hp, and the other investigated APP were between 0 and 5 dpi.

The previously described potential use of APP (3, 12, 17, 18) prompted us to look at the course of the main APP serum concentrations after infection of pigs with Bbr – an important pathogen of swine. On the basis of our investigation we cannot state that evaluation of CRP, Hp, SAA, PigMAP, or APP concentrations in pig serum may provide a useful information about severity of AR. Though no relationship was observed between serum APP concentrations and severity of AR, the data presented in our paper provide clear evidence that all investigated APP strongly reacted after infection. Thus, APP measurement could be the primary screening test prior to specific disease diagnosis. The “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 determination of references values for various APP and possibility of distinguishing infected and non-infected pigs under field conditions.

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References


