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Correlation between serum acute phase proteins, lung pathology, and disease severity in pigs experimentally co-infected with H3N2 swine influenza virus and *Bordetella bronchiseptica*

Małgorzata Pomorska-Mól, Iwona Markowska-Daniel, Krzysztof Kwit, Kinga Urbaniak, Zygmunt Pejsak

Department of Swine Diseases,
National Veterinary Research Institute, 24-100 Pulawy, Poland,
mpomorska@piwet.pulawy.pl

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Abstract

The kinetics of C-reactive protein (CRP), haptoglobin (Hp), serum amyloid A (SAA), and pig major acute protein (Pig-MAP) response in pigs co-infected with H3N2 swine influenza virus (SwH3N2) and *Bordetella bronchiseptica* (Bbr) was studied, with assessment of potential correlations between the concentration of acute phase proteins (APPs) in serum samples, lung lesions, and the clinical course of the disease in co-infected pigs. The standard bacteriological methods for detection of Bbr and PCR technique for identification of Bbr and SwH3N2 were used. The serum concentrations of APPs were measured using ELISA. The concentration of CRP, SAA, and Pig-MAP was significantly higher from 2 to 4 or 5 dpi. The concentration of Hp was elevated until the end of the study. Significant correlations were found between the serum concentration of SAA and Pig-MAP and clinical score, and between the concentration of SAA and lung score. Apart from their potential as biological markers for co-infections, SAA and Pig-MAP levels have additive value since they are related to the severity of infection. The results indicate that measurement of APP (*i.e* SAA) may prove valuable in assessing the severity of respiratory infection in pigs, and may be of supportive value in the clinical evaluation of animals and in the selection of more appropriate treatment.

Keywords: swine, acute phase proteins, swine influenza virus, *Bordetella bronchiseptica*, diagnostics markers.

Introduction

Acute phase reaction is attributed to a group of changes that occur in response to physiological changes, such as infection, physical trauma or malignancy, leading to tissue damage (3, 23). The most important component of the response comprises the acute phase proteins (APP). Acute phase proteins reflect the defence and adaptation mechanisms, which take place in the organism before an immunological response (16). The clinical utility of APP measurements (*e.g*. evaluation of treatment efficacy, discrimination between bacterial and viral infections, evaluation of disease severity) has been studied both in humans and animals (1, 8, 10, 13, 19). A significant correlation between APP level in serum and the severity of the disease has been previously reported for some infections in humans (10, 17), bovines (11), and horses (14). The relationship between serum APP concentration and the extent of pathological lesions was also studied in pigs (26, 29, 30); however, the knowledge about the kinetics of APP response and the correlation between serum APP, lung pathology, and the course of disease in pigs co-infected with H3N2 swine influenza virus (SIV) and *Bordetella bronchiseptica* (Bbr) is restricted.

Co-infection with two or more pathogens commonly occurs in pig respiratory diseases, thus they are generally considered a multifactorial problem (25). The list of pathogens that are responsible for respiratory infections in pigs is extensive and includes both viral and bacterial agents. Swine influenza virus is

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one of the most important disease-causing agents in pigs, both as a primary pathogen and as an agent predisposing to secondary bacterial infection. Swine influenza (SI) in general is a self-limited infection characterised by high morbidity and low mortality; however, secondary infection, especially bacterial, may significantly exacerbate the SIV-associated illness and increase the risk of death (23). Bacterial infections can occur as concurrent infections (*i.e*. co-infections) with influenza, or develop after influenza infection. It has been shown that the bacteria belonging to the genus *Bordetella* can be responsible for a potentially severe complication of influenza that can progress rapidly to a serious infection with severe pneumonia (2, 20). In pigs, Bbr is involved in the aetiology of atrophic rhinitis, bronchopneumonia, and has been shown to contribute to porcine respiratory disease complex (PRDC) (7). As it has been previously shown, SIV may enhance Bbr colonisation in the respiratory tract (20). Additionally, prior infection with Bbr has been shown to have led to an increased colonisation with other bacteria, and to a more severe course of disease (5, 6).

The objective of the present study was to determine the kinetics of APP response in pigs experimentally co-infected with H3N2 SIV and Bbr, and to assess whether the correlation between serum concentrations of APP, lung lesions, and the clinical course of the co-infection exists.

Material and Methods

Animals. A total of 16 6-week-old piglets (gilts and boars) from a high health status herd were used. During the study the piglets were housed in isolated units, one for the control and one for the infected pigs. Feed and water were offered *ad libitum*.

Influenza virus and *Bordetella bronchiseptica* inocula. Swine influenza virus Sw/Ghent/172/2008, subtype H3N2 (SwH3N2), provided through the courtesy of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, was used for the experimental infection. The virus stocks used for inoculation represented the third passage in embrionated chicken eggs. The concentration of SIV was evaluated in Madin-Darby canine kidney (MDCK) cells. The inoculations of $10^{7.7}$ $TCID_{50}$ of the virus in 1.5 mL of PBS were given.

Twenty-four-hour culture of Bbr field strain, isolated from a pig with clinical form of non-progressive atrophic rhinitis (NPAR), suspended in a phosphate-buffered saline (PBS) to a final concentration of 1.5×10^8 cfu mL^{-1}, was used for inoculation (2 mL/pig).

Experimental design. On the day of the inoculation (day 0), 11 piglets were intranasally inoculated with SwH3N2 and Bbr. The inocula were delivered separately to both nostrils. The pigs from the control group (n = 5) were mock-inoculated (PBS).

Blood was sampled on -7, 0 (inoculation), 1, 2, 3, 5, 7, and 10 dpi. Serum samples were stored at -20ºC prior to analysis (not longer than 3 weeks). Nasal swabs were collected at 2, 3, 4, 5, 7, and 10 dpi.

Complete necropsy procedures were performed on all the pigs, immediately after euthanasia. The pigs were euthanized by an intravenous injection of pentobarbital (Morbital, Biowet Pulawy, Poland). A total of six co-infected piglets were euthanized on days 3 (three animals) and 5 (three animals) after the inoculation. Additional five co-infected pigs were euthanized at 10 dpi. The piglets from the control groups (n = 5) were euthanized at 10 dpi. The samples from the trachea and lungs were collected aseptically for further analysis (bacteriological, PCR).

Clinical and pathological examination. The rectal temperature and clinical score were examined daily. The pigs were scored for the respiratory signs as follows: respiratory rate: 0 - normal (≤40), 1 - slightly elevated (41-45), 2 - moderately elevated, slight abdominal breathing (46-50), 3 - clearly elevated, dyspnoea (>50); nasal discharge: 0 - absent, 1 - present; coughing: 0 - absent, 1 - present; sneezing: 0 - absent, 1 - present. All scores per item were added up and a clinical score (CS) of each individual pig (0-6) was calculated. The temperatures of 40°C or over were recognised as fever. Lung lesions were scored using the method described previously (27). The final lung score (LS) calculated for each individual pig ranged from 0 to 28.

Laboratory examinations

Swabs and lung samples. Nasal swabs and lung samples were tested for the presence of Bbr using standard bacteriological methods and PCR technique as described previously (22). SIV genetic material was detected in the nasal swabs and lungs with the use of the real time reverse transcription (RRT)-polymerase chain reaction (PCR) method, as described before (31). Real-time PCR results are given as ++ (Ct value <30; positive), + (Ct value 30-35; weak positive), - (Ct value >35, negative).

Measurement of acute phase proteins. The following tests for the determination of serum concentrations of C-reactive protein (CRP), haptoglobin (Hp), serum amyloid A (SAA), and pig major acute protein (Pig-MAP) were used: Pig C-reactive protein (Life Diagnostics Inc., USA) for CRP, Pig haptoglobin (Life Diagnostics Inc., USA) for Hp, PigMAP KIT ELISA (PigCHAMP Pro Europa S.A, Spain) for Pig-MAP, and Phase Serum Amyloid A Assay (Tridelta Development Ltd, Ireland) for SAA. All tests were used according to the manufacturer's recommendations. Before analysis, serum samples were appropriately diluted (1:2000 for CRP, 1:35 000 for Hp, 1:500 for SAA and 1:1000 for Pig-MAP).

Statistical analysis. Statistical analyses of various parameters were performed using Statistica 8.0 (Statsoft) software. Comparisons between groups at each time point were assessed using the Mann-

Whitney U test. The differences between the lungs and the clinical scores were analysed with a Kruskal-Wallis test. The correlation between the APP concentrations and the clinical or pathological characteristics was determined by Spearman's rank correlation coefficient (nonparametric). The differences with $\alpha < 0.05$ were considered as significant.

Results

Clinical signs. In all co-infected pigs the clinical signs, including sneezing, coughing, nasal discharge, and fever were observed. The rectal temperatures of infected pigs are presented in Fig. 1.

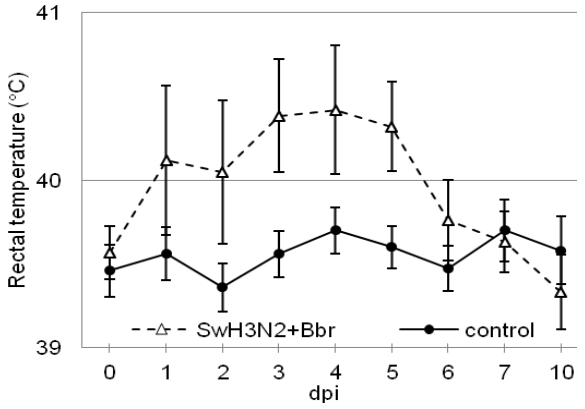


Fig. 1. Mean rectal temperature (±SD) of control pigs and pigs co-infected with swine influenza virus H3N2 (SwH3N2) and *Bordetella bronchiseptica* (Bbr)

In 4 out of 11 piglets accelerated respiratory rates and dyspnoea were also observed. The mean clinical score was 3.27 ± 1.19 (range 1-5). In the pigs euthanized at 3 dpi, the maximal CS ranged from 1 to 4, in the pigs euthanized at 5 dpi from 3 to 4, while in the pigs euthanised at 10 dpi from 2 to 5. In the control piglets, no clinical symptoms of any disease were seen. A significant correlation was found between the clinical and lung scores (R-Spearman = 0.83, $P < 0.05$).

Microbiological and pathological examination. No Bbr was found in the nasal swabs in the control pigs and the pigs before inoculation. The reisolation of Bbr from nasal swabs was only successful in 6 out of 11 inoculated piglets. The presence of genetic material of Bbr was confirmed in nasal swabs taken from all co-inoculated animals. The reisolation of Bbr from the lung tissues was possible in 5 out of 11 pigs. The presence of genetic material of Bbr was confirmed in 8 out of 11 pigs.

The presence of SIV genetic material in nasal swabs was confirmed in all co-inoculated pigs between 2 and 5 dpi. At 7 dpi only 2 out of 5 co-inoculated pigs shed a virus. The SIV genetic material was detected in the trachea and the lungs at 3 and 5 dpi in all co-inoculated pigs. No SIV RNA was found in the nasal swabs taken from the animals before inoculation and from control pigs, or in those taken at 10 dpi.

Gross purple-red consolidated pulmonary lesions were found in all co-inoculated pigs (Table 1, Fig. 2.). The mean lung score was 8.62 ± 4.31 (range 2 to 13). The mean lung score at 3, 5 and 10 dpi equalled 6.67 ± 5.51, 12.00 ± 1.73 and 5.00 ± 2.45 respectively, and did not differ significantly between the days ($P > 0.05$).

Table. 1. Lung score observed in co-infected and control pigs

	Time point	Lung score
Co-infected pigs	3 dpi	3
		4
		13
	5 dpi	13
		10
		10
	10 dpi	8
		7
		4
		2
		4
Control pigs (mean, n = 5)	10 dpi	0 ± 0.00

Acute phase proteins response. In general, all tested proteins were the markers of co-infection. The serum concentrations of CRP, Hp, SAA, and Pig-MAP were significantly higher after co-inoculation as compared to levels observed in the control pigs (Fig. 3.). In the control pigs, the concentration of all APPs did not change significantly during the study period.

The mean CRP serum level in pigs before inoculation was 13.77 μg mL^{-1} ± 8.36. A significant increase in CRP serum level in the inoculated pigs was observed from 2 to 5 dpi ($P < 0.05$) as compared to the control animals. The peak concentration observed at 3 dpi (114μg mL^{-1} ± 38) was over eightfold higher when compared to pre-inoculation level.

The serum concentration of Hp before the inoculation did not exceed 1.00 mg mL^{-1} (range 0.33 to 1.00 mg mL^{-1}). The kinetics of Hp response was different from the response of CRP. A significant increase in Hp concentration was observed in all co-inoculated pigs from 2 dpi until the end of the study (10 dpi) ($P < 0.05$). The mean peaked level (4.31 ± 1.59 mg/mL) was over sevenfold higher compared to day 0-level.

The kinetics of the SAA response was generally similar to that of CRP; however, an increase in the SAA concentration was most spectacular (30-fold increase compared to pre-inoculation value, 139.89 ± 66.55 μg mL^{-1}). A statistically significant increase in the mean SAA concentration, as compared to the controls, was observed from 2 to 4 dpi ($P < 0.05$).

The concentration of Pig-MAP also increased after co-infection. Parallel to CRP, a significantly higher level was observed from 2 to 5 dpi ($P < 0.05$). However, the mean peak level of Pig-MAP was only three times higher as compared to day 0 level.

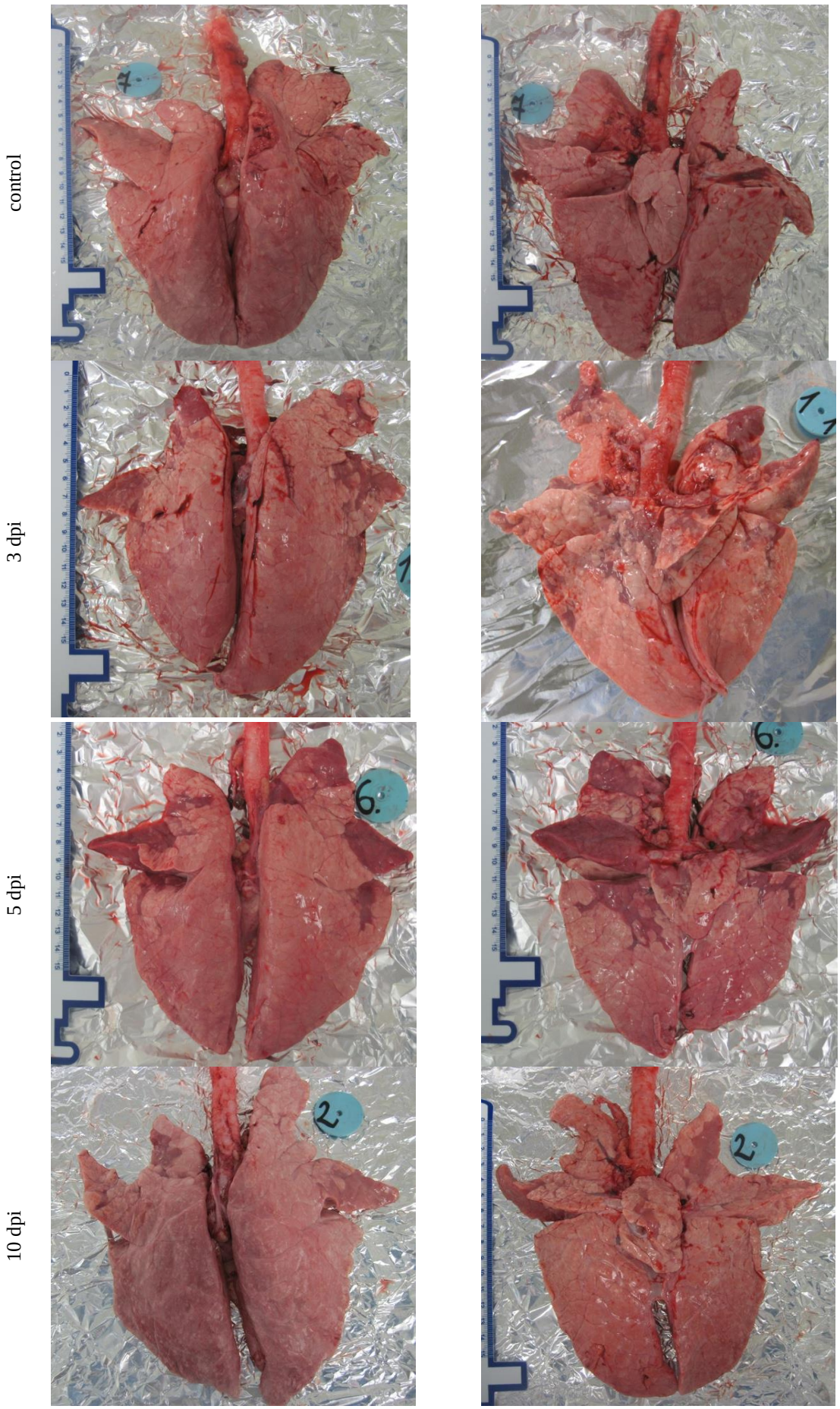


Fig. 2. Changes in the lungs of pigs co-infected with swine influenza virus (H3N2) and *Bordetella bronchiseptica*

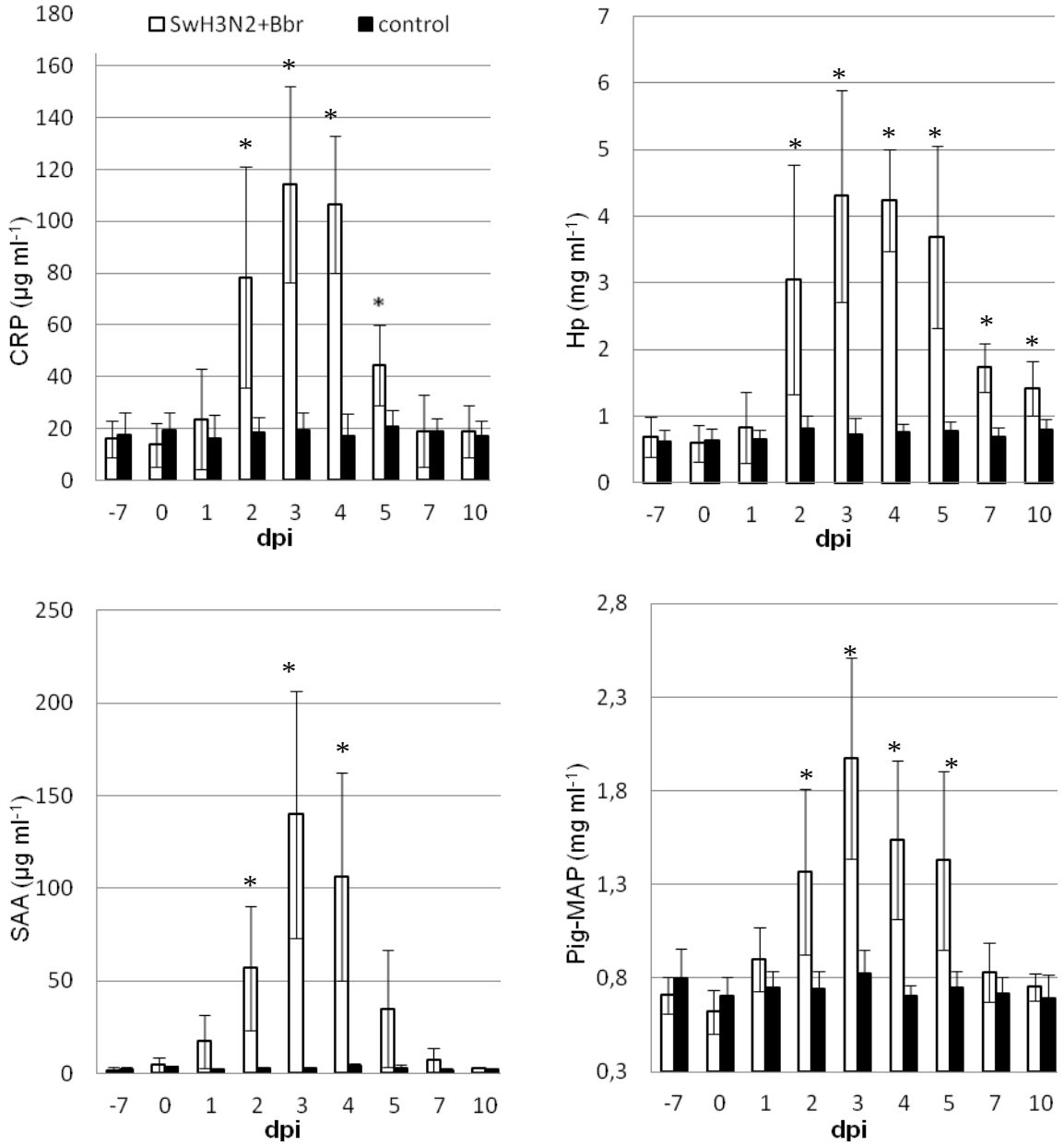


Fig. 3. The time course of mean APP concentration (±SD) in inoculated and control pigs during the study period;
* - $P < 0.05$,
SwH3N2+Bbr – pigs intranasally infected with swine influenza virus H3N2 and *Bordetella bronchiseptica*

Strong positive correlations were found between the concentrations of Pig-MAP and SAA in serum and the changes in the lungs (lung score) (R-Spearman = 0.73 and 0.64 respectively, $P < 0.05$), and between the concentration of clinical score and SAA (R-Spearman = 0.78, $P < 0.05$).

Discussion

In the present study, the CRP, Hp, SAA, and Pig-MAP responses during the first 10 dpi after intranasal co-inoculation of pigs with SwH3N2 and Bbr were determined. The second aim was to investigate the correlation between serum concentrations of CRP, Hp, SAA, Pig-MAP, and lung pathology and clinical course of the disease to evaluate their ability to predict the disease severity in pigs.

Respiratory disease is an important problem in swine industry and synergistic effects of viral and bacterial infections have been demonstrated in pigs (5, 6, 20, 29). SIV, porcine respiratory and reproductive syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), Bbr, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, and *Mycoplasma hyopneumoniae* are the most common agents associated with respiratory tract infections in pigs (25).

Influenza is typically a self-limited infection in pigs, but it could be complicated by bacteria.

Co-infections with bacteria often lead to bacterial pneumonia, and Bbr is one of the bacteria which may complicate the course of SI (20). Polymicrobial infections of the respiratory tract are infections of which the severity may vary from subclinical to severe. Therefore, a considerable interest has grown in the development of reliable markers that reflect the severity of the disease and may help to predict survival and the course of the disease in pigs. According to the earlier data (10, 11, 14, 17, 26, 29), it seems that APPs may be potentially useful as early markers of infection in pigs. However, the knowledge about the correlation between the response of various APPs and the disease severity in pigs is still insufficient. An improved outcome in the severe form of the disease is based on the early identification of disease severity and the implementation of proper treatment. The latter is important not only from the ethical, but also economical point of view. On the other hand, the overtreatment (especially with the use of antibiotics) should be avoided as it generates high costs and antibiotic resistance in animals. Currently, an approximate assessment of the severity of infection is available through a combination of clinical symptoms (fever, coughing, dyspnea, appetite). This method is often unsatisfactory and does not allow veterinarians to predict the risk of development of severe disease, requiring antibiotic treatment.

In the present study, similar kinetics, but not the magnitude, of CRP, SAA, and Pig-MAP responses was observed in co-infected pigs, while Hp revealed more protracted response. The mean concentration of Hp did not return to pre-inoculation level until the end of the study. A similar increase in the CRP concentration was found by Sorensen *et al.* (32) after *Streptococcus suis* inoculation, and by Lampreave *et al.* (18) upon turpentine induced inflammation. An increase in the Hp concentration was also in agreement with data reported by Hall *et al.* (12) and Sorensen *et al.* (32) during monoaetiological bacterial infections. A significant increase in the level of CRP, Hp, SAA, and Pig-MAP has also been observed in pigs single-inoculated with Bbr (the same strain and a similar dose of Bbr was used) (30). However, the kinetics and the magnitude of APP response was different during Bbr mono-infection. In a previous experiment, the concentration of CRP was significantly higher only from 1 to 2 dpi, but the mean maximum concentration was similar to that in the present study. In contrast, the mean peak level of Hp and SAA was higher in co-infected pigs than in mono-infected ones. The mean maximum concentration of Hp and SAA in Bbr-infected pigs reached 3.8 mg mL^{-1} and 80 μg mL^{-1} respectively. The response of Pig-MAP after the co-inoculation was the most similar to that observed previously in Bbr-infected pigs (30). However, during the co-infection with SwH3N2, the protraction of Hp response was observed. The mean maximal Hp concentrations were comparable in both experiments.

Besides their potential as biological markers of co-infections, SAA and Pig-MAP levels have additive value since they are related to the severity of infection. Significant correlations were found between the maximal concentrations of SAA and Pig-MAP in serum and clinical score, and between the maximal concentration of SAA and lung score. These results indicate that the measurement of APP such as SAA may prove valuable in assessing the severity of respiratory infection in pigs, and may be of supportive value in the clinical evaluation of animals and in the selection of more appropriate treatment. In agreement with the present results, the positive relationship between SAA and disease severity was also reported previously in pigs, humans, and other species (4, 9, 15, 28, 29).

The study was restricted due to a relatively small number of animals available for research. There is a need for additional research on the relationship between serum SAA concentration and the severity of infection, involving more animals and more pathogens. Studies on the utility of SAA measurement in evaluating treatment efficacy may also be of a great importance. More extensive research is necessary to assess the value of this marker as part of a clinical decision rule under field condition. Further studies should also focus on the determination of the cut-off levels of SAA to predict mild or severe course of infection.

Conflict of Interests Statement: The authors have no conflict of interest to declare.

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Animal Rights Statement: The experiment was approved by Local Ethics Commission (University of Life Sciences in Lublin, Poland).

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