

## Significance of different types and levels of antigen-specific immunity to *Actinobacillus pleuropneumoniae* infection in piglets

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**ABSTRACT:** The aim of the study was to verify under experimental conditions the hypothesis that a reliable protection of piglets against *Actinobacillus pleuropneumoniae* (App) infection can be obtained by colostrum-derived antibodies and/or a low-dose infection from sows naturally infected with App. Twenty-eight piglets were allocated into four groups. Piglets of groups 1A and 1B were the progeny of App-seronegative sows, piglets of groups 2A and 2B originated from App-seropositive sows. At the age of 4 weeks, piglets in groups 1A and 2A were exposed to a low infection dose with the pathogenic strain of App serotype 9 ( $4 \times 10^4$  CFU/ml). At the age of 8 weeks, all four groups of piglets were exposed to the App serotype 9 challenge ( $2.5 \times 10^7$  CFU/ml). We compared the differences in clinical, radiological, pathological changes and cytological findings in blood and bronchoalveolar lavage samples between groups with different levels of antigen-specific immunity after challenge. Piglets that obtained colostrum-derived antibodies and experienced a low-dose App infection were best protected against App challenge. More apparent clinical and pathological changes were observed in groups protected with either only colostrum-derived antibodies or active immunity induced by a preceding low-dose infection. However, none of the types of protection prevented developing of the disease and characteristic changes in the lungs. Cell counts changes in blood and lavage gave evidence of only bacterial infection in progress; however, between-group differences were not marked. Antibodies (IgG, IgA) present in the respiratory tract at the time of challenge played a major role in protection against App infection.

**Keywords:** bacterial infection; bronchoalveolar lavage; mucosal immunity; pneumonia; porcine; protection; respiratory tract

Porcine pleuropneumonia is a contagious disease of the respiratory tract of pigs that is worldwide considered as a disease causing great direct and indirect economic losses within swine herds in the past 20–25 years (Sebunya and Saunders, 1983; Fenwick and Henry, 1994; Satran and Nedbalcova, 2002; Dreyfus et al., 2004). The aetiological agent of the infection, *Actinobacillus pleuropneumoniae* (App), causes severe and fatal fibrinous hemorrhagic necrotizing pneumonia especially in growing pigs (Sebunya and Saunders, 1983). Except for

the acute form of App with typical clinical signs, various forms of chronic disease and asymptomatic carriers can be found (Fenwick and Henry, 1994; Tascon et al., 1996; Bosse et al., 2002).

Pathogenesis of App is a multifactorial process and not all aspects have yet been recognized (Tascon et al., 1996; Haesebrouck et al., 1997; Bosse et al., 2002). None of the prophylactic measures adopted in herds so far provides absolute protection. Current vaccines may reduce losses caused by the disease, reduce mortality rates, minimize

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the extent of lung damage, but do not prevent the development of the carrier state, and subsequent spread of the infection (Fenwick and Henry, 1994; Tascon et al., 1996; Satran et al., 2003). No effective therapeutic options to eliminate the bacteria exist (Wallgren et al., 1999a,b; Stipkovits et al., 2001; Tanigawa and Sawada, 2003).

It is widely believed that the key role in protection of the immune system in porcine pleuropneumonia is played by specific IgG antibodies circulating in blood (Bosse et al., 1992; Hensel et al., 1994). In the initial phases, they diffuse through the hemato-alveolar barrier of the lung parenchyma disturbed by inflammation. Some data exists on the presence of specific antibodies in bronchoalveolar lavage fluids (Bosse et al., 1992; Hensel et al., 1994; Loftager et al., 1995; Pabst et al., 1995); however, it is mostly related to the experiments aimed at other problems and their results only partially contribute to the explanation of immune mechanisms of App protection. Many authors refer to the fact that the mere presence of antibodies on surface of mucosa or in serum does not provide complete protection against App (Fenwick and Henry, 1994; Hensel et al., 1995; Furesz et al., 1997; Haesebrouck et al., 1997). These antibodies in association with the other humoral and cellular components of the inflammatory exudates may prevent severe forms of the disease (Bertram, 1985; Cruijisen et al., 1992).

After survival the natural infection, pigs develop protective immunity against further challenge infection (Nielsen and Mandrup, 1977; Nielsen, 1984; Inzana, 1991). The observations demonstrated that a natural low-dose infection also confers complete protection to subsequent challenge (Furesz et al., 1997). Cruijisen et al. (1995) found in their experiments that convalescent pigs were really solidly protected only against homologous strain reinfection. After heterologous strain reinfection the degree of protection varied. Nielsen (1975), Nicolet (1992) and Vigre et al. (2003) presumed that colostrum-derived antibodies also provide protection of piglets under natural conditions. Natural low-dose infection from the dam (Fenwick and Henry, 1994) also provides partial or total protection of piglets under natural conditions. Mechanisms and prospective constituents of the immune system in these models of protection have not been investigated yet.

The purpose of the present study was to verify these hypotheses and to deepen the knowledge of mechanisms that participate in the protection against actinobacillary pleuropneumonia.

## MATERIAL AND METHODS

The animal care protocol for this experiment followed the Czech guidelines for animal experimentation and was approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic (No. 59-2000, registration No. 369).

All experiments were conducted in accredited barrier-type stables (accreditation certificate No. 1020/212/A/98).

### Animals and experimental design

Twenty-eight piglets of Large White breed, 8 weeks old, with average body weight of 25 to 30 kg were used in the experiment. The piglets originated from two farms. Piglets of groups 1A and 1B were the progeny of 2 sows that were purchased from the farm without occurrence of clinical form of App infection (beneath designated as non-immune sows). The sows were not vaccinated and antibodies against lipopolysaccharidic (LPS) antigen App9 have not been detected by serological analyses of their blood. Piglets of groups 2A and 2B originated from 2 sows that were purchased from the farm with occurrence of clinical form of App (beneath designated as immune sows). Antibodies were transferred from the sows with high titres of serum antibodies against LPS App9 detected by serological analyses to the piglets through colostrum; that was confirmed by the detection of high levels of serum antibodies against LPS App9 in 7-day old piglets. All piglets were born in experimental barrier-type stables. They were weaned after colostrum intake, fed with artificial diet and kept separately.

At the age of 4 weeks, piglets in groups 1A (piglets with antibody-free colostrum intake) and 2A (piglets with the intake of colostrum containing antibodies), were exposed to a low infection dose with the pathogenic strain of App (low-dose infection). Piglets were infected intranasally in the period of inspirium and infection dose was administered to the second third of each nasal cavity. Clinical signs after this low-dose infection were moderate. Present App infection was confirmed by changes in absolute and differential cell counts in blood collected in regular intervals. Moderate inflammation of the respiratory tract was also confirmed by differential cell counts in BALF samples two weeks after low-dose infection.

Table 1. Characteristics of the groups

	Group 1A	Group 1B	Group 2A	Group 2B
Numbers of piglets in groups ( <i>n</i> )	7	7	7	7
Intake of antibodies against App9 by colostrum	no	no	yes	yes
App9 infection at the age of 4 weeks (low-dose infection)	yes	no	yes	no
App9 infection at the age of 8 weeks (challenge)	yes	yes	yes	yes

At the age of 8 weeks, all 4 groups of piglets (Table 1) were exposed to the infection with a high infection dose of App (challenge). Piglets were again infected intranasally in the period of inspirium and infection dose was administered to the second third of each nasal cavity. Piglets that did not die during the experiment were euthanized at the age of 13 weeks and the experiment was completed.

### Bacterial strain

Field strain of *Actinobacillus pleuropneumoniae* (App) biotype 1, serotype 9 (field isolate from the department of bacteriology of our Institute, KL2-2000) in the 4th passage was used for the infection. The strain was cultured at 37°C in BHI (Brain Heart Infusion Broth, HiMedia, India) supplemented with 10 µg/ml NAD (Nicotinamide Adenine Dinucleotide trihydrate, AppliChem GmbH, Germany) for 6 hours. Concentration of the grown culture was measured photometrically and adjusted with sterile PBS to optic density OD<sub>550</sub> = 1, which corresponded to the concentration  $5 \times 10^9$  CFU/ml App and subsequently was diluted with PBS to the same concentration as that used for experimental infections. Pigs were infected intranasally; total volume of each infection dose was 4 ml (2 ml per nostril). The final concentration of low and high infection doses were  $4 \times 10^4$  CFU/ml and  $2.5 \times 10^7$  CFU/ml, respectively.

### Clinical monitoring

Monitoring of the health status of the animals began immediately after birth. Clinical signs of the disease were recorded.

One day before the challenge and during whole the challenge period, body (rectal) temperature and clinical signs of App infection were recorded (increased respiratory rate, dyspnoea, coughing, anorexia and lethargy).

### Radiological examination of thorax (X-rays)

Two weeks after the challenge, thoracic radiographs (dorsoventral and laterolateral projections) were obtained using a portable X-ray machine (Gierth 80HE, output of 15 mA at 80 kV). These radiographs were reviewed for the evidence of pulmonary or bronchial disease as indicated by bronchial, alveolar or interstitial patterns. Pleural or mediastinal abnormalities were also recorded. Each animal was assigned an average score. The lesions were assessed as 0 (normal), 1 (alveolar pattern), 2 (alveolar and slight interstitial pattern), and 3 (severe interstitial pattern).

### Pathological examination

Piglets were euthanized by *i.v.* administration of a barbiturate on day 35 after the challenge. Pigs that died before the end of the experiment were immediately submitted to necropsy. All the pigs were examined for pathological changes, especially in the lungs, according to the method by Hannan et al. (1982). The extent of the observed pulmonary lesions was very accurately drawn in a lung diagram. The extent of pulmonary lesions was assessed for each lobe in the range from 0 (without changes) to 5 (solely lesions). The maximum total score possible for each complete lung (7 lobes) was 35.

### Blood sampling

Blood samples for haematological examination were taken up from the piglets from the age of 2 weeks in week-intervals and one day before the challenge and the first three days after challenge in 6-hour intervals. Blood for serological analyses was taken up one day before challenge. Blood was collected by jugular venipuncture (a) into heparin tubes (30 I.U./ml) for haematological examination

and (b) into serum tubes or vacutainers for isotype-specific antibodies determination. Heparinized blood samples were used immediately. Clotted blood was centrifuged at 5 000 G for 20 minutes and the separated serum was stored at  $-20^{\circ}\text{C}$  prior to serological analyses.

### Lavage fluid sampling

Intravital lavages were performed in only 4 piglets from the group 1A and in 4 piglets from the group 2A one day before the low-dose infection (as the young age categories of piglets do not well tolerate anaesthesia via injection), and in all piglets of these groups two weeks after the low-dose infection. Lavages were also conducted in all piglets one day before the challenge and 2 weeks after the challenge. In case death occurred or after euthanasia when the experiment was completed, postmortem lung lavage was performed.

Lavages were performed in piglets fasting for 20 hours, under general anaesthesia with combination of tiletamin-zolazepam+xylazin+ketamin. One ml of this mixture contained 250 mg of each substance. The mixture was injected intramuscularly in the dose of 1 ml/10 kg of body weight. All anaesthetized animals were premedicated with a parasympatolytic preparation atropine (0.05 to 0.1 mg/kg, *i.m.*). The lavages were performed as follows:

(a) Bronchoalveolar lavage without the use of an endoscope: a modification of the method by van Leengoed and Kamp (1989a). The pig was fixed in the sternal position. An endotracheal tube was inserted into the trachea and 1 to 2 ml/kg of sterile PBS (pH 7.2) was slowly infused via a polyvinyl tube with the inner diameter of 2 mm placed into the distal parts of the airways, towards the bronchus. About 60 percent of the infused saline was recovered as aspirate (bronchoalveolar lavage fluid, BALF) and was filtered and centrifuged for 15 minutes at 200 G. Supernatant was stored at  $-20^{\circ}\text{C}$  prior to serological analyses. The sediment was diluted in 1 ml of PBS and used for BALF examination.

(b) Postmortem lung lavage: 200 to 250 ml sterile PBS was administered to the lungs (i.e. up to maximum filling of the lungs) through excised extrathoracic part of trachea. Subsequently, about 60 percent of the infused saline was recovered as aspirate and processed in the same way as BALF.

### Haematological and BALF examination

White blood cell (WBC) counts both in blood and lavages, erythrocyte counts and haemoglobin (Hb) concentrations were determined using electronic cell counter (Digicell 500, Contraves AG, Switzerland). Differential WBC counts in blood samples were determined using Giemsa-stained blood smears and cells were classified as neutrophils, lymphocytes and monocytes, with at least 200 cells being differentiated. Cells from the BALF sediment were classified on Giemsa stained slides as macrophages, neutrophils and lymphocytes, with at least 200 cells being differentiated.

### Serological analyses (ELISA)

The levels of serum immunoglobulin IgM, IgG and lavage immunoglobulin IgG, IgA antibodies specific for lipopolysaccharidic (LPS) antigen App9 were determined by isotype-specific indirect enzyme linked immunosorbent assays (ELISA) with purified LPS App9 antigens. Concentrations of each of the reagents used in ELISA were determined by checkerboard titrations. Crude LPS was extracted by the method of Darveau and Hancock (1983). The antigen in carbonate-bicarbonate buffer, pH 8.6 was pipetted into wells (0.3  $\mu\text{g}$  in 100  $\mu\text{g}$  per well) of 96-well polystyrene microtitration plates (Gama Dalečín, Czech Republic). The plates were incubated in a cool place for 18 hours and subsequently washed three times with PBS, pH 7.2, containing 0.05% Tween 20. Then 100  $\mu\text{l}$  of diluent (PBS, pH 7.2, containing 0.05% Tween 20 and 0.5% casein hydrolysate) was pipetted into each well. The wells of the first row were compared with 50  $\mu\text{l}$  of blood serum samples pre-diluted 1 : 33, or undiluted BALF samples and threefold dilution series were prepared transferring 50  $\mu\text{l}$  of the mixture into 100  $\mu\text{l}$  of diluent. After 60 minutes of incubation in a wet chamber at  $37^{\circ}\text{C}$ , the wells were washed three times and 100  $\mu\text{l}$  of Goat anti-pig Ig horse radish peroxidase conjugate (Goat anti-pig IgM-mu chain specific HRP or Goat anti-pig IgG-Fc fragment HRP; Bethyl, USA) was added into each well. After 1-hour incubation at  $37^{\circ}\text{C}$ , the plates were washed four times and 100  $\mu\text{l}$  of TMB substrate solution (Test-line, Czech Republic) was added into each well. The reaction was stopped after 15 minutes incubation by adding 50  $\mu\text{l}$  of 2 M sulphuric acid and absorbances

were read at 450 nm using the multichannel spectrometer iEMS Reader (LabSystems iEMS). Control sera were included in all tests. Positive control serum was collected from conventional pigs with clinical form of pleuropneumonia caused by App9, negative control serum originated from a non-vaccinated App-free SPF pig. The result of titration is an optical density red at dilutions 1 : 1 000 for IgM and 1 : 3 000 for IgG in serum samples and at dilution 1 : 1 for IgA and IgG in BALF samples.

## Data analysis

Statistical and graphical software STAT Plus (Matoušková et al., 1992) was used for statistical evaluation of data. Significance of between-group changes detected by clinical monitoring following App challenge was assessed with Fisher's test; changes in radiological and pathological examinations were assessed with one-tail Mann-Whitney nonparametric test. For statistical evaluation of the between-group differences in haematological and BALF analysis results, Bartlett's test of homogeneity of variance, analysis of variance and subsequently Scheffé test of contrasts were used. Box-Cox transformations were used for obtaining correctness of the results before own data testing because normality of basic distributions was not achieved.

Regression analysis (polynomial regression type of second order) was used for determination of correlation between the results of radiological examination (radiological score) and gross examination (lung score).

Significance of between-group changes detected by serological analyses before App challenge was assessed with one-tail Mann-Whitney nonparametric test.

## RESULTS

### Characterization of the animals before challenge

One day before the beginning of the challenge, no piglets showed any clinical sign of the disease, body temperature was within physiological range (38.5 to 39.8°C) as well as the monitored haematological parameters (erythrocytes 5 to  $8 \times 10^{12}$  cells/l, Hb 85 to 125 g/l, WBC 5 to  $15 \times 10^9$  cells/l, including differential WBC counts – neutrophils 30 to 50%;

lymphocytes 45 to 60%; monocytes 5 to 10%). The counts of nucleated cells in BALF ranged between 0.8 and  $5 \times 10^9$  cells/l, and in the differential count of BALF cells, macrophages prevailed (85 to 98%) and low counts of neutrophils (0 to 5%) and lymphocytes (5 to 10%) were present, only in group 1A due to the previous low-dose infection, the average counts of macrophages, neutrophils and lymphocytes were 52.5%, 27.5% and 20%, respectively.

### Levels of antigen-specific antibodies before challenge

Using serological analyses, the levels of serum immunoglobulin IgM, IgG and lavage immunoglobulin IgA, IgG antibodies specific for LPS antigen App9 were determined in serum and BALF samples collected from all piglets at the age of 8 weeks, one day before challenge (Figure 1).

The highest levels of IgM antibodies were found in sera from group 1A. These values were statistically significantly higher ( $P < 0.01$ ) than in other groups. Lower levels of antibodies of this isotype were determined in group 2A; despite that the values were statistically significantly higher ( $P < 0.01$ ) in comparison with groups 1B and 2B. In the two latter groups (1B and 2B), IgM levels were extremely low (Figure 1A).

The highest levels of IgG antibodies were recorded in sera from group 1A, however with relatively high between-animal variability. High levels of these antibodies were also detected in group 2A. In contrast, in 2B group animals, IgG levels were low and IgG levels in 1B group were statistically significantly lower ( $P < 0.01$ ) than in the other groups (Figure 1B).

In BALF samples, the highest levels of IgA antibodies were observed in group 1A; these values were statistically significantly higher ( $P < 0.01$ ) than in groups 1B and 2B. IgA levels in the other groups were low. IgA levels in animals of group 1B were statistically significantly lower ( $P < 0.01$ ) than in animals of groups 1A and 2B (Figure 1C).

The highest levels of IgG in BALF were assessed in animals of 2A and 2B groups; these values were statistically significantly higher ( $P < 0.01$ ) than in group 1B. High levels of antibodies of IgG isotype were also detected in group 1A. In contrast, very low IgG levels were detected in group 1B (Figure 1D).

In general, the highest levels of IgG and IgM in serum and IgA in BALF were found in the 1A group

in comparison with the other groups of animals; the former had also high levels of IgG in BALF. In contrast, the lowest levels of antibodies of all isotypes were detected in group 1B. The highest levels of IgG antibodies in BALF were observed in groups 2A and 2B; group 2A also exhibited high serum levels of IgM and IgG.

### Characterization of the animals after challenge

Challenge induced the outbreak of the disease in pigs of all groups but with different course (clinical and pathological signs) in respective groups of animals. Summarized results of clinical, radiological and pathological findings are shown in Table 2.

Pathological changes were studied in all experimental piglets after death or after euthanasia five weeks following challenge, and only the respiratory tract was found to be affected. Using regression analysis (polynomial regression type of second order), statistically highly significant correlation ( $P < 0.01$ ) between the results of radiological examination (radiological score) and gross examination (lung score) was detected.

Based on haematological examination after challenge, Hb concentrations and erythrocyte cell counts declined (70 to 110 g/l; 3 to  $6 \times 10^{12}$  cells/l) and total counts of leucocytes increased (25 to  $65 \times 10^9$  cells/l) in all piglets, mainly due to the increase in neutrophils counts. Changes in differential WBC counts were also recorded (lymphocyte counts decreased from 55% to 30% and neutrophil

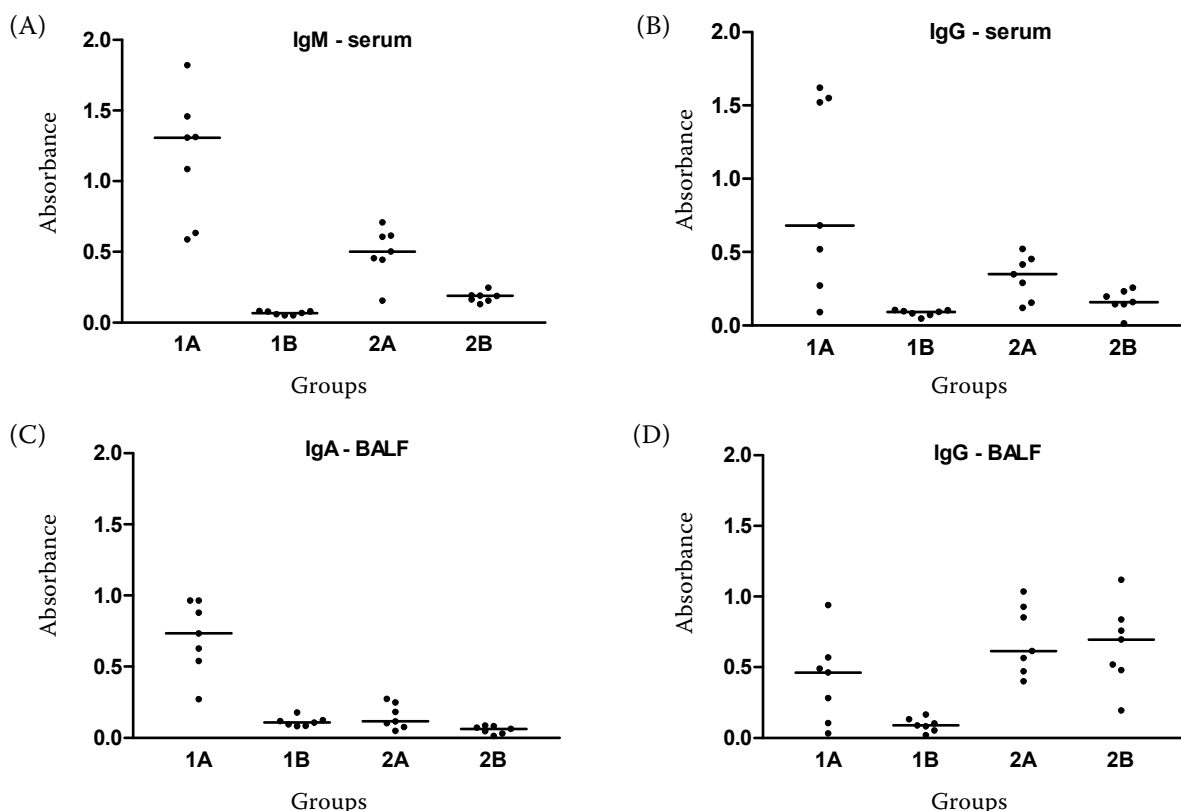


Figure 1. Isotype-specific antibody responses against lipopolysaccharidic antigen *A. pleuropneumoniae* serotype 9, determined by ELISA, in serum and bronchoalveolar lavage fluid (BALF) samples obtained one day before challenge with *A. pleuropneumoniae* serotype 9, each group is characterized as absorbance of individual animals and medians

(A) IgM antibody responses in serum; absorbance – optical density read at serum dilution 1 : 1 000

(B) IgG antibody responses in serum; absorbance – optical density read at serum dilution 1 : 3 000

(C) IgA antibody responses in BALF; absorbance – optical density read at BALF dilution 1 : 1 000

(D) IgG antibody responses in BALF; absorbance – optical density read at BALF dilution 1 : 3 000

Groups 1A, 1B, 2A, 2B – characteristics of the groups (see Table 1)

Table 2. Results of clinical observations, radiological and pathological findings following challenge with *A. pleuropneumoniae* serotype 9

		Group 1A (n = 7)*		Group 1B (n = 7)*		Group 2A (n = 7)*		Group 2B (n = 7)*	
Clinical monitoring**	Increase of temperature above 40°C <sup>y</sup>	2 <sup>a</sup>	(29)	7 <sup>a</sup>	(100)	6	(86)	5	(71)
	Increase of respiratory rate <sup>y</sup>	7 <sup>a</sup>	(100)	7 <sup>b</sup>	(100)	0 <sup>abc</sup>	(0)	7 <sup>c</sup>	(100)
	Vomiting <sup>y</sup>	0 <sup>a</sup>	(0)	7 <sup>abc</sup>	(100)	0 <sup>b</sup>	(0)	3 <sup>c</sup>	(43)
	Cough <sup>y</sup>	0 <sup>a</sup>	(0)	5 <sup>abc</sup>	(71)	0 <sup>b</sup>	(0)	0 <sup>c</sup>	(0)
	Death <sup>y</sup>	0	(0)	3	(43)	0	(0)	0	(0)
Radiological, pathological examinations***	Occurrence of pleuritis <sup>y</sup>	4	(57)	6	(86)	4	(57)	5	(71)
	Occurrence of abscesses <sup>y</sup>	4 <sup>ab</sup>	(57)	2	(29)	0 <sup>a</sup>	(0)	0 <sup>b</sup>	(0)
	Radiological score (mean) <sup>z</sup>	1.71 <sup>a</sup>		2.75		1.00 <sup>ab</sup>		1.29 <sup>c</sup>	
	Lung score (mean) <sup>z</sup>	5.0 <sup>a</sup>		16.1 <sup>abc</sup>		2.4 <sup>b</sup>		3.0 <sup>c</sup>	

Groups 1A, 1B, 2A, 2B – characteristics of the groups (see Table 1)

<sup>a, b, c</sup> represent the pairs of parameters in the lines between which the significant differences ( $P < 0.05$ ) were found (Fisher's test, Mann-Whitney test)

\*number of piglets in the group

\*\*results of clinical monitoring during the first day following challenge

\*\*\*results of radiological examination two weeks following challenge, and pathological examination after death or after euthanasia five weeks following challenge

<sup>y</sup>number of affected piglets (percentages of affected piglets)

<sup>z</sup>mean of scores of individual animals (see Material and Methods)

counts increased from 40% to 75%) in all groups. The highest differences were found between the values detected in animals before the beginning of the experiment and in experimental animals 24 hours post challenge. The counts of neutrophils were significantly higher ( $P < 0.01$ ) and the counts of lymphocytes were significantly lower ( $P < 0.01$ ) on days 1, 7, 14 and 21 after challenge compared to the counts detected before challenge. No significant differences between respective experimental groups were detected either.

By intravital BALF collection, 2 weeks after challenge,  $0.8$  to  $5 \times 10^9$  cells/l were recovered. Changes in differential BALF cell counts were recorded in all groups of animals. Decline in macrophage counts (from 92% to 51%, occasionally less than 30%) and increased counts of lymphocytes (from 4% to 16%) and neutrophils (from 4% to 25%, occasionally more than 55%) were registered. Intensity of changes varied between respective piglets, but no significant between-group differences were detected in experimental groups. However, statistically significantly higher percentage of neutrophils and statistically sig-

nificantly lower percentage of macrophages in BALF compared with the values detected before challenge was recorded in group 1B. The differential cell counts gradually returned to physiological values from week 2 to 5 post challenge and no changes in cell counts were recorded in groups 1A, 2A and 2B five weeks post challenge, in postmortem BALF samples. Marked changes in cell counts persisted in only 3 piglets from group 1B within five weeks post infection.

### Group 1A

Increased respiratory rate and moderate dyspnoea occurred 5 to 10 hours post infection in all piglets of group 1A; increased body temperature was detected in 2 piglets (40.4 and 40.6°C). Clinical signs disappeared within three days.

Moderate or intermediate changes were detected by radiological examination; only one animal was seriously affected (interstitial lung pattern).

Pulmonary lesions of chronic character, necrotizing-fibrinous pneumonia with/without pleuritis

and pericarditis were detected in the piglets sacrificed 5 weeks after the challenge. The changes often only persisted as encapsulated abscesses or the affected lung tissue regenerated by scarring.

### **Group 1B**

All piglets of group 1B vomited and their respiratory rate increased within 5 to 10 hours; cough was recorded in 5 piglets within 14 hours. Increased body temperature was detected in all piglets (40.2 to 41.0°C). In 3 piglets from this group, a sharp decrease in body temperature (35.8 to 37.0°C) followed its peak immediately post infection, additional impairment of the health status was registered on day 1; these piglets died (within 24 to 36 hours post infection). In the other piglets clinical signs disappeared within 7 days after challenge.

Serious radiological findings were recorded in piglets, except for 1 piglet.

In the animals from group 1B (in subjects found dead), acute pleuropneumonia of acute-necrotic type with fibrinous pleuritis and pericarditis was detected. A low amount of exudate was found in the thoracic cavity. Bronchial lymph nodes were hyperemic and enlarged. Pulmonary lesions of chronic character, necrotizing-fibrinous pneumonia with/without pleuritis and pericarditis were detected in the other piglets (sacrificed 5 weeks after the challenge). The changes often persisted only as focal sequestra and encapsulated abscesses.

### **Group 2A**

Increased body temperature appeared in most piglets of group 2A (40.1 to 41.0°C). Within 10 hours they reached their maximum and then returned to physiological values. Piglets did not show any other sign of the disease.

Only moderate radiological changes were observed in this group.

Pulmonary lesions of chronic character, necrotizing-fibrinous pneumonia with/without pleuritis and pericarditis were detected in the piglets euthanized 5 weeks after the challenge. The changes often persisted only as focal sequestra or the affected lung tissue was regenerated by scarring. The lesions of diaphragmatic lobes prevailed.

### **Group 2B**

In group 2B, the body temperature increased in 5 piglets (40.1 to 40.8°C), respiratory frequency increased in all the animals within 10 hours with concurrent vomiting in 3 piglets. The day after infection, all the signs disappeared.

Only moderate and intermediate changes were observed in the group. Moderate changes were detected in 5 piglets and intermediate changes were detected in 2 piglets.

Pulmonary lesions of chronic character, necrotizing-fibrinous pneumonia with/without pleuritis and pericarditis were detected in the piglets (sacrificed 5 weeks after the challenge). The changes often persisted only as focal sequestra or the affected lung tissue was regenerated by scarring. The lesions of diaphragmatic lobes prevailed.

Based on the clinical, radiological and pathological changes it can be summarized that most marked changes were detected in group 1B, i.e. in piglets without colostrum-derived antibodies and without preceding low-dose infection. In contrast, minimum changes were registered in the group of piglets 2A, i.e. in piglets with colostrum-derived antibodies, which were administered a low dose of the infectious agent at the age of 4 weeks. No significant differences between groups with colostrum-derived antibodies only (2B) and with a low-dose infection only (1A) were detected, except for increased occurrence of lung abscesses in group 1A.

## **DISCUSSION**

The aim of the present study was to verify practical observations and the hypothesis that efficient protection against actinobacillary infection can be provided to piglets by colostrum-derived antibodies and/or direct infection from sows naturally infected with actinobacillus. This hypothesis was tested in experimental conditions based on the comparison of the course of infection after challenge with App serotype 9 in piglets with different levels of antigen-specific immunity. The challenge course and severity of the induced disease was evaluated on the basis of between-group differences in clinical symptoms, the extent of radiological and pathological findings after challenge as well as differences in haematological parameters of blood and lavages. The level of protection was compared with the lev-



els of total and local antigen-specific antibodies. The purpose of the present study was to investigate the levels of particular antibodies in serum and BALF immediately before the challenge and their potential effect and role in the protection against App infection.

Clinical signs after low-dose intranasal infection in 4-week old piglets were moderate as usually seen in field and experimental conditions (van Leengoed and Kamp, 1989b). Present bacterial infection indicated changes in blood and BALF cell counts in concord with van Leengoed and Kamp (1989b)

This preceding low-dose infection induced active immune response with antibody formation. Specific IgM and IgG antibodies in serum and IgA and IgG antibodies in BALF were detected in animals that did not passively obtain antibodies through colostrum, but experienced a low-dose infection (group 1A). The fact that they were post-infection antibodies can be demonstrated by the between-group differences of their levels in groups 1A and 1B (both the groups without colostrum-derived antibodies but group 1B without low-dose infection). Marked between-animal differences in Ig serum levels were detected in animals of group 1A; the animals however, did not markedly differ in clinical and pathological findings after challenge. It follows that significance of Ig in BALF samples is much higher in the protection against App infection. The lowest levels of all types of antibodies were detected in the animals of group 1B before challenge in comparison with the other monitored groups.

The immune response with production of IgM and IgG in serum and probably also IgA in BALF was also seen after low-dose infection in animals of group 2A with colostrum-derived antibodies (see differences of Ig levels between groups 2A and 2B). IgG antibodies may have partly originated from colostrum. However, the presence of these colostrum-derived antibodies partly inhibited the immune response after low-dose infection (see differences of Ig levels between groups 1A and 2A).

It has been long since Nielsen (1975) indicated that antibodies against App were transferred from immune sows via colostrum to the offspring, and titres in colostrum whey samples were not significantly different from serum titres of dams. Suckling piglet serum titres rose to the same level as the colostrum whey titre, within the first 24 hours of life (Nielsen, 1975). Levels of these antigen-specific antibodies in piglets gradually decrease along with growth (Vigre et al., 2003). None of the mentioned studies inves-

tigated the persistence of antibody isotype characteristic. The finding of the present experiment that colostrum-derived antibodies penetrate to respiratory tract is noteworthy. Charley and Corthier (1977) demonstrated that no immunoglobulin can penetrate through the bronchoalveolar barrier of normal lungs in growing and adult pigs, but they documented that the penetration of colostrum-derived antibodies in newborn piglets and lambs may occur, as also described by Bradley et al. (1976), Mensik et al. (1971a,b) and Smith et al. (1976). This can be caused by means of the so called neonatal Fc receptor (FcRn) that has been recently described in bovine lung by Mayer et al. (2004). FcRn encoding gene has been cloned and characterized in pigs (Schnulle and Hurley, 2003). These colostrum-derived antibodies in BALF likely allowed penetration of fewer bacteria to the lower parts of respiratory tract or reduced the effect of bacteria toxins as described by Kamp et al. (1991), and consequently the lung parenchyma was less affected as also confirmed by BALF examination before challenge. In the animals of group 2A (with colostrum-derived antibodies) despite previous low-dose infection the differential cell counts gradually returned to physiological values and no changes in cell counts were recorded before challenge, in contrast to group 1A (without colostrum-derived antibodies) where the changes in cell counts persisted.

The highest between-group differences in the course of challenge infection were evident in clinical symptoms, radiological and gross findings in lungs and pleura. In contrast, results of haematological analysis and changes in differential cell counts in BALF reflected only current bacterial infection, however without marked between-group differences and without correlation with the extent of affected respiratory tract. Most marked clinical changes were recorded after challenge in piglets that were not protected either by colostrum-derived antibodies or by active immunity induced by a preceding low-dose infection (group 1B). The challenge infection induced an acute course of the disease in this group, in concordance with Sebunya and Saunders (1983). It corresponded to our hypothesis; likewise correctness of intranasal administration and the right dose of infectious agent were confirmed. Piglets from the other groups with either of the mentioned forms of specific protection (groups 1A, 2A and 2B) showed a substantially weaker course of infection. Similar signs were observed by van Leengoed and Kamp

(1989b) after experimental infection with App. Van Leengoed and Kamp (1989b) suggested that early clinical signs, except for changes in body temperature, cannot be of a predictive value for the development and course of the disease. We reached the same conclusions: current infection was manifested by elevated body temperature; however, fatal course of infection could be only expected when the body temperature suddenly and sharply decreased. In 3 piglets from group 1B, a sharp decrease in body temperature followed its peak immediately post infection and the piglets died within 24 to 72 hours post infection.

Despite the above mentioned differences in clinical manifestation, inflammatory infection process was induced in all of the piglet groups in the present study. It is documented, except others, by changes in haematological parameters of blood and changes in cell counts in BALF. On the first day after the challenge infection, the decrease in erythrocyte counts as well as in haemoglobin contents was observed, in concord with the findings of van Leengoed and Kamp (1989b). It is also the consequence of one of the ways bacteria obtain iron as an essential nutrient for their growth in the host organism (Deneer and Potter, 1989; Bosse et al., 2002). The total leukocyte count in blood increased primarily due to the increase in neutrophil counts; these changes indicated an acute course of bacterial infection. Our findings are in concordance with those found by van Leengoed and Kamp (1989b) in blood picture.

It is necessary to considerately evaluate the cell count changes in the lower parts of the respiratory tract and to compare them with the findings by other authors, since many authors (Garcia et al., 1986; Gehrke and Pabst, 1990) based on their investigations, pointed out that the data and results may differ because of different lavage techniques used, different breed, age and health status. In our study, a slightly modified method of van Leengoed and Kamp (1989a) was used. Values of the total and differential cell counts in healthy pigs (before experimentation) described in our study corresponded to the reference values reported by other authors (Mayer and Lam, 1984; Zeidler and Kim, 1985; van Leengoed and Kamp, 1989b; Ganter and Hensel, 1997). Ganter and Hensel (1997) observed that increased cell counts in BALF often coincide with an increase in the number of bacteria and indicate an irritated lung in clinically healthy pigs. In contrast to this observation, the total number of nucleated

cells in BALF from our experimental piglets did not change significantly after infection. The differential count of BALF cells was altered and was followed by the pattern indicating bacterial infection. The proportion of granulocytes tended to increase and the number of macrophages decreased. These types of changes in BALF cells counts have been observed by other authors (Baarsch et al., 1995). Based on the results of the present study we can confirm that the number of granulocytes is the most reliable parameter to distinguish between healthy and diseased pigs (Ganter and Hensel, 1997).

Pathological findings demonstrated in our study were characteristic of App infection, i.e. acute pleuropneumonia in the piglets that died (from group 1B), and chronic fibrinous pneumonia with/without pleuritis and pericarditis in the other piglets; identical findings were described by Sebury et al. (1982), Bosse et al. (1992) and Hensel et al. (1995). Whereas the extent of gross changes correlated with the results of radiological examination, correlation of clinical course and subsequent pathological changes was seen only in the most affected group (1B) which did not have any protection (either active or passive). Blood and BALF picture changes did not correlate with the extent of postmortem pulmonary lesions, even in the most affected group (1B).

The present results confirmed our hypothesis that piglets without colostrum-derived antibodies and without preceding low-dose infection, i. e. piglets that had the lowest levels of both total and local antibodies, were least protected against challenge. On the other hand, piglets with the intake of colostrum-derived antibodies from the dam and concurrent experience of a low-dose App infection at the age of 4 weeks were best protected against App. However, none of the types of protection was able to prevent penetration of the infectious agent and the start of an inflammatory process. The present study demonstrated that the crucial role in the protection against APP is played by antibodies present in respiratory tract – colostrum-derived and/or actively formed IgG and IgA, most likely combined with other mechanisms that have not been investigated in the present study.

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## REFERENCES

- Baarsch M.J., Scamurra R.W., Burger K., Foss D.L., Maheswaran S.K., Murtaugh M.P. (1995): Inflammatory cytokine expression in swine experimentally infected with *Actinobacillus pleuropneumoniae*. *Infection and Immunity*, 63, 3587–3594.
- Bertram T.A. (1985): Quantitative morphology of peracute pulmonary lesions in swine induced by *Haemophilus pleuropneumoniae*. *Veterinary Pathology*, 22, 598–609.
- Bosse J.T., Johnson R.P., Nemec M., Rosendal S. (1992): Protective local and systemic antibody responses of swine exposed to an aerosol of *Actinobacillus pleuropneumoniae* serotype 1. *Infection and Immunity*, 60, 479–484.
- Bosse J.T., Janson H., Sheehan B.J., Beddek A.J., Rycroft A.N., Kroll J.S., Langford P.R. (2002): *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. *Microbes and Infection*, 4, 225–235.
- Bradley P.A., Bourne F.J., Brown P.J. (1976): The respiratory tract immune system in the pigs – I. The distribution of immunoglobulin containing cells in the respiratory tract mucosa. *Veterinary Pathology*, 13, 81–89.
- Charley B., Corthier G. (1977): Local immunity in the respiratory tract. II. – Relationship of serum and local antibodies. *Annales de Microbiologie*, 128B, 109–119.
- Cruijsen T.L., van Leengoed L.A., Dekker-Nooren T.C., Schoevers E.J., Verheijden J.H. (1992): Phagocytosis and killing of *Actinobacillus pleuropneumoniae* by alveolar macrophages and polymorphonuclear leukocytes isolated from pigs. *Infection and Immunity*, 60, 4867–4871.
- Cruijsen T., van Leengoed L.A., Ham-Hoffies M., Verheijden J.H. (1995): Convalescent pigs are protected completely against infection with a homologous *Actinobacillus pleuropneumoniae* strain but incompletely against a heterologous-serotype strain. *Infection and Immunity*, 63, 2341–2343.
- Darveau R.P., Hancock R.E. (1983): Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *Journal of Bacteriology*, 155, 831–838.
- Deneer H.G., Potter A.A. (1989): Effect of iron restriction on the outer membrane proteins of *Actinobacillus (Haemophilus) pleuropneumoniae*. *Infection and Immunity*, 57, 798–804.
- Dreyfus A., Schaller A., Nivollet S., Segers R.P., Kobisch M., Mieli L., Soerensen V., Hussy D., Miserez R., Zimmermann W., Inderbitzin F., Frey J. (2004): Use of recombinant ApxIV in serodiagnosis of *Actinobacillus pleuropneumoniae* infections, development and prevalidation of the ApxIV ELISA. *Veterinary Microbiology*, 99, 227–238.
- Fenwick B., Henry S. (1994): Porcine pleuropneumonia. *Journal of the American Veterinary Medical Association*, 204, 1334–1340.
- Furesz S.E., Mallard B.A., Bosse J.T., Rosendal S., Wilkie B.N., MacInnes J.I. (1997): Antibody- and cell-mediated immune responses of *Actinobacillus pleuropneumoniae*-infected and bacterin-vaccinated pigs. *Infection and Immunity*, 65, 358–365.
- Ganter M., Hensel A. (1997): Cellular variables in bronchoalveolar lavage fluids (BALF) in selected healthy pigs. *Research in Veterinary Science*, 63, 215–217.
- Garcia J.G.N., Wolven R.G., Garcia P.L., Keogh B.A. (1986): Assessment of interlobar variation of bronchoalveolar lavage cellular differentials in interstitial lung disease. *American Review of Respiratory Disease*, 133, 444–449.
- Gehrke I., Pabst R. (1990): Cell composition and lymphocyte subsets in the bronchoalveolar lavage of normal pigs of different ages in comparison with germfree and pneumonic pigs. *Lung*, 168, 79–92.
- Haesebrouck F., Chiers K., Van Overbeke I., Ducatelle R. (1997): *Actinobacillus pleuropneumoniae* infections in pigs: the role of virulence factors in pathogenesis and protection. *Veterinary Microbiology*, 58, 239–249.
- Hannan P.C.T., Bhogal B.S., Fish J.P. (1982): Tylosin tartrate and tiamutilin effects on experimental piglet pneumonia induced with pneumonic pig lung homogenate containing mycoplasmas, bacteria and viruses. *Research in Veterinary Science*, 33, 76–88.
- Hensel A., Pabst R., Bunka S., Petyold K. (1994): Oral and aerosol immunization with viable or inactivated *Actinobacillus pleuropneumoniae* bacteria: antibody response to capsular polysaccharides in bronchoalveolar lavage fluids (BALF) and sera of pigs. *Clinical and Experimental Immunology*, 96, 91–97.
- Hensel A., Stockhofe-Zurwieden N., Petzoldt K., Lubitz W. (1995): Oral immunization of pigs with viable or inactivated *Actinobacillus pleuropneumoniae* serotype 9 induces pulmonary and systemic antibodies and

- protects against homologous aerosol challenge. *Infection and Immunity*, 63, 3048–3053.
- Inzana T.J. (1991): Virulence properties of *Actinobacillus pleuropneumoniae*. *Microbial Pathogenesis*, 11, 17–23.
- Kamp E.M., Popma J.K., Anakotta J., Smits M.A. (1991): Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. *Infection and Immunity*, 59, 3079–3085.
- Loftager M.K., Eriksen L., Nielsen R. (1995): Mucosal and serum IgA antibodies in pigs following infection with *Actinobacillus pleuropneumoniae*. *Advances in Experimental Medicine and Biology*, 371B, 935–938.
- Matoušková O., Chalupa J., Cígler M., Hruška K. (1992): STAT Plus – Manual (in Czech). Veterinary Research Institute, Brno. 168 pp.
- Mayer P., Lam C. (1984): Porcine alveolar macrophages, isolation, morphological and functional characteristics. *Journal of Veterinary Medicine, Series A – Animal Physiology Pathology and Clinical Veterinary Medicine*, 31, 59–71.
- Mayer B., Kis Z., Kajan G., Frenyo L.V., Hammarstrom L., Kacskovics I. (2004): The neonatal Fc receptor (FcRn) is expressed in the bovine lung. *Veterinary Immunology and Immunopathology*, 98, 85–89.
- Mensik J., Franz J., Pospisil J., Krejci J. (1971a): The local transport of antibodies in the protection of calves and piglets against viral respiratory infection. *Acta Veterinaria Brno*, 40 (Suppl. 2), 75–81.
- Mensik J., Pospisil J., Franz J., Dreslerova J. (1971b): Local effect of passively acquired colostral antibody on the development of experimental swine influenza infection in suckling pigs. *Zentralblatt Veterinarmedizin, Reihe B*, 18, 804–818.
- Nicolet J. (1992): *Actinobacillus pleuropneumoniae*. In: Leman A.D., Straw B., Mengeling W.L., D'Allaire S., Taylor D.J. (eds.): *Diseases of Swine*. Iowa State University Press, Ames. 401–408.
- Nielsen R. (1975): Colostral transfer of immunity to *Haemophilus parahaemolyticus* in pigs. *Nordisk Veterinær Medicin*, 27, 319–328.
- Nielsen R. (1984): *Haemophilus pleuropneumoniae* serotypes – cross protection experiments. *Nordisk Veterinær Medicin*, 36, 221–234.
- Nielsen R., Mandrup M. (1977): Pleuropneumonia in swine caused by *Haemophilus parahaemolyticus*. A study of the epidemiology of the infection. *Nordisk Veterinær Medicin*, 29, 465–473.
- Pabst R., Delventhal S., Hensel A., Petzoldt K. (1995): Immunization by the enteral and respiratory route with viable and inactivated bacteria results in a differential increase in lymphocyte subsets in the bronchoalveolar space. *Advances in Experimental Medicine and Biology*, 371B, 1459–1461.
- Satran P., Nedbalcova K. (2002): Prevalence of serotypes, production of Apx toxins, and antibiotic resistance in strains of *Actinobacillus pleuropneumoniae* isolated in the Czech Republic. *Veterinari Medicina*, 47, 92–98.
- Satran P., Nedbalcova K., Kucerova Z. (2003): Comparison of protection efficacy of toxoid and whole-cell vaccines against porcine pleuropneumonia caused by endotracheal infection with *Actinobacillus pleuropneumoniae*. *Acta Veterinaria Brno*, 72, 213–219.
- Schnulle P.M., Hurley W.L. (2003): Sequence and expression of the FcRn in the porcine mammary gland. *Veterinary Immunology and Immunopathology*, 91, 227–231.
- Sebunya T.N.K., Saunders D.V.M., Osborne A.D. (1982): A model aerosol exposure system for induction of porcine *Haemophilus pleuropneumoniae*. *Canadian Journal of Comparative Medicine*, 47, 48–53.
- Sebunya T.N.K., Saunders D.V.M. (1983): *Haemophilus pleuropneumoniae* infection in swine. *Journal of the American Veterinary Medical Association*, 182, 1331–1337.
- Smith W.D., Wells P.W., Burrells C., Dawson A.M. (1976): Maternal immunoglobulins and parainfluenza 3 virus inhibitors in the nasal and lachrymal secretion and serum of newborn lambs. *Clinical and Experimental Immunology*, 23, 544–553.
- Stipkovits L., Miller D., Glavits R., Fodor L., Burch D. (2001): Treatment of pigs experimentally infected with *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae* with various antibiotics. *Canadian Journal of Veterinary Research – Revue Canadienne de Recherche Veterinaire*, 65, 213–222.
- Tanigawa M., Sawada T. (2003): Exposure time-dependent bactericidal activities of amoxicillin against *Actinobacillus pleuropneumoniae*; an *in vitro* and *in vivo* pharmacodynamic model. *Journal of Veterinary Medicine, Series B – Infectious Diseases and Veterinary Public Health*, 50, 436–442.
- Tascon R.I., Vazquez-Boland J.A., Gutierrez-Martin C.B., Rodriguez-Barbosa J.I., Rodriguez-Ferri E.F. (1996): Virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*. *Microbiologia*, 12, 171–184.
- van Leengoed L.A.M.G., Kamp E.M. (1989a): Bronchoalveolar lavage in live pigs. *Veterinary Quarterly*, 11, 65–72.
- van Leengoed L.A.M.G., Kamp E.M. (1989b): Endobronchial inoculation of various doses of *Haemophilus (Actinobacillus) pleuropneumoniae* in pigs. *American Journal Veterinary Research*, 50, 2054–2059.

- Vigre H., Ersboll A.K., Sorensen V. (2003): Decay of acquired colostral antibodies to *Actinobacillus pleuropneumoniae* in pigs. *Journal of Veterinary Medicine Series B – Infectious Diseases and Veterinary Public Health*, 50, 430–435.
- Wallgren P., Segall T., Morner A.P., Gunnarsson A. (1999a): Experimental infections with *Actinobacillus pleuropneumoniae* in pigs – I. Comparison of five different parenteral antibiotic treatments. *Journal of Veterinary Medicine Series B – Infectious Diseases and Veterinary Public Health*, 46, 249–260.
- Wallgren P., Segall T., Morner A.P., Gunnarsson A. (1999b): Experimental infections with *Actinobacillus pleuropneumoniae* in pigs – II. Comparison of antibiotics for oral strategic treatment. *Journal of Veterinary Medicine Series B – Infectious Diseases and Veterinary Public Health*, 46, 261–269.
- Zeidler R.B., Kim H.D. (1985): Phagocytosis, chemiluminescence, and cell-volume of alveolar macrophages from neonatal and adult-pigs. *Journal of Leukocyte Biology*, 37, 29–43.

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