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## Pathophysiological changes occurring during *Escherichia coli* endotoxin and *Pasteurella multocida* challenge in piglets: relationship with cough and temperature and predictive value for intensity of lesions

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**Abstract** – The aims of this study were (1) to correlate cough and body temperature (BT) with the severity of bronchopneumonia in pigs, (2) to determine whether these clinical signs can be used to early diagnose bronchopneumonia and (3) to assess the predictive values of cough and BT regarding lung lesions. Bronchopneumonia was induced by administering *E. coli* endotoxin (LPS) combined with *Pasteurella multocida* type A (PmA) in the trachea of 13 piglets. Saline-instilled negative controls ( $n = 8$ ), PmA inoculated ( $n = 6$ ) and LPS instilled ( $n = 5$ ) groups were also constituted. Cough and BT were recorded daily while the bronchopneumonia severity was assessed using bronchoalveolar lavage fluid (BALF) cytology, cytokines and measurement of lung lesion volume. Changes in expiratory breathing pattern were also measured (Penh). The combination of LPS and PmA induced a subacute bronchopneumonia characterised by macrophage, neutrophil, and lymphocyte infiltration, changes in Penh and an increase in the mRNA level of IFN- $\gamma$  while IL8, IL-18 and TNF- $\alpha$  mRNA levels remained unchanged. The daily body weight gain of infected animals was significantly reduced. Cough and BT changes were proportional to the intensity of the lung inflammatory process, functional respiratory changes and to the extent of macroscopic lesions. When comparing the individual values of cough and BT to thresholds defined for both parameters, an early diagnosis of pneumonia was possible. Considering the pooled data of each group, it was possible to define thresholds allowing an early segregation between the groups of diseased and healthy piglets. The daily values of cough and BT were predictive for the volume of lung lesions recorded at the end of the trial. In conclusion, cough and BT appear as potential indicators for the intensity and the evolution of the respiratory disease. They also seem to be good predictors for the magnitude of lung lesions and weight gain recorded at the study endpoint.

**lipopolysaccharides / bronchopneumonia / breathing pattern / whole body barometric plethysmography / cytokines**

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## 1. INTRODUCTION

In pigs, *Pasteurella multocida* type A (PmA) is the most frequent secondary pathogen, which can generate a respiratory disorder called swine pneumonic pasteurellosis [31]. This disease represents the most common final stage of pig respiratory mycoplasmosis which causes important economic losses in swine herds [9, 22]. Only a few moderate clinical signs like coughing and hyperthermia are expressed during subacute to chronic respiratory infections with PmA explaining why infected animals are often only detected in slaughterhouses with extended lung lesions [25]. These clinical signs can also be detected with a variable intensity during other respiratory diseases induced by *Mycoplasma hyopneumoniae* and *Bordetella bronchiseptica* possibly combined with PmA and also in response to several viral infections often complicated with bacteria [1, 5, 9, 23, 26]. In order to prevent the development of these pneumonic lung lesions and the associated weight gain losses by appropriate curative treatments, an early detection of these diseases is needed.

The quantitative assessment of some clinical signs could be very useful to determine the respiratory status in swine herds, to detect diseased animals and also to record the response of infected animals to treatments. Recently, cough detection measurement using an on-line cough recogniser system has been described in pigs [24, 28]. If such techniques become commonly available in the future, the cough and body temperature could be used to improve the management of respiratory diseases in swine. The relationship between the cough, body temperature and lung lesions is poorly documented. The relationship between cough and lung damage has been investigated in pigs with enzootic pneumonia by scoring the cough and assessing the extent of lung lesions at the slaughterhouse [25] but no significant correlation has been obtained. It was suggested that a daily monitoring rather than a weekly recording procedure would

have strengthened this study. Hyperthermia has also been monitored during swine respiratory infections involving *Pasteurella multocida* but the relationship between the body temperature with lung lesions was not investigated [6, 9, 15]. Finally, the relationships between the cough, body temperature and other pathological parameters, scarcely measured in clinical conditions, as the magnitude of the pulmonary inflammatory process and the pulmonary functional changes, have never been investigated so far.

In this study, *E. coli* endotoxin, which is known to be a common airborne pollutant in swine housings [12] has been intratracheally administered to predispose the animals to a *Pasteurella multocida* type A infection. Cough frequency and body temperature were monitored during the course of this experimental subacute bronchopneumonia with the aim to correlate these clinical events with cytokine production, bronchoalveolar lavage fluid composition, respiratory function, weight gain and macroscopic lung lesions. The relationship between these clinical signs and the severity of the disease was especially investigated to determine whether an early diagnosis of pneumonia was possible using these parameters. Moreover, the relationships between the cough, body temperature and the major clinical end-points recorded 14 days after the inoculation were also assessed to check the predictive values of the cough and body temperature regarding the final issue of the disease.

## 2. MATERIALS AND METHODS

### 2.1. Animals

A total of 32 conventional piglets ( $9.05 \pm 2.82$  kg) free from *Mycoplasma hyopneumoniae* infection according to polymerase chain reaction (PCR) and the bacteriological culture of bronchoalveolar lavage fluid and lung tissue samples were used in this

study. The pigs originated from the University of Liège, Belgium, swine herd. The animal experimental protocol was approved by the local animal care committee.

## 2.2. Experimental design

The study was conducted to evaluate the relationship between the cough, body temperature, pulmonary inflammatory process, functional changes and lung lesions during the course of a pneumonic pasteurellosis model combining an intra-tracheal *E. coli* endotoxin administration followed by a subsequent intra-tracheal inoculation with *Pasteurella multocida*. Pigs were randomly allocated into four groups, which were treated according to the following schedule: group 1: negative controls corresponding to two successive inoculations with *E. coli* endotoxin free saline and sterile growth medium for *Pasteurella multocida* ( $n = 8$ ); group 2: inoculation with *E. coli* endotoxin free saline plus *Pasteurella multocida* ( $n = 6$ ); group 3: inoculation with *E. coli* endotoxin enriched saline plus sterile growth medium for *Pasteurella multocida* ( $n = 5$ ); group 4: combined inoculations with *E. coli* endotoxin plus *Pasteurella multocida* ( $n = 13$ ). This experimental design was selected to investigate the individual role of endotoxins and PmA in our model.

One day before inoculation with *Pasteurella multocida*, pulmonary function was investigated using whole body barometric plethysmography and pigs were then anaesthetised. A bronchoalveolar lavage (BAL) was performed as described below and saline or *E. coli* enriched saline was intra-tracheally instilled. A second anaesthesia was performed at day 0 to inoculate the animals with sterile growth medium or growth medium with *Pasteurella multocida*. Then, pulmonary function measurements were repeated at days 1, 6, 8 and 14 post-inoculation (dpi). The animals were anaesthetised again at 14 dpi to perform a last BAL, which was followed by euthanasia. Cough and body temperature were recorded each day from -2 to 14 dpi.

## 2.3. Experimental procedure

### 2.3.1. Anaesthesia

The piglets were anaesthetised with xylazine 2 mg/kg IM (Rompun<sup>®</sup>, Bayer, Brussels, Belgium), ketamin 10 mg/kg IM (Imalgene<sup>®</sup>1000, Merial, Brussels, Belgium), and thiopental 10 mg/kg IV (Pentothal<sup>®</sup>, Abott, Louvain-La-Neuve, Belgium).

### 2.3.2. Administration of *Escherichia coli* endotoxins and challenge with *Pasteurella multocida*

A 0.2 mg/mL endotoxin solution was prepared by diluting purified endotoxins (*E. coli* lipopolysaccharides, serotype O127:B8, Sigma Chemical CO, St Louis, USA) in sterile saline (9‰) and it was instilled in the trachea by bronchoscopy (70 cm × 6.0 mm × 2 mm, ETM GMBH, Germany) in anaesthetised animals at a rate of 1 mL/kg of the solution so that each animal received 100 µg/kg of endotoxins.

A field isolate of non-toxicogenic *Pasteurella multocida* type A (PmA) from a pig with bronchopneumonia was grown overnight in a tryptose sterile broth (tryptose 10 g, glucose 0.5 g, NaCl 2.5 g, yeast extract 1.25 g, 500 mL water, pH = 7.4) with 10% sterile horse serum at 37 °C. The piglets were anaesthetised and 5 mL of the broth culture ( $> 2 \times 10^9$  Colony Forming Unit/mL) was intra-tracheally injected through a needle (No. 21G) introduced in the middle extra-thoracic segment of the trachea.

### 2.3.3. Bronchoalveolar lavage (BAL) and lung cell isolation

Bronchoalveolar lavages (BAL) were performed in anaesthetised animals by bronchoscopy. Twenty milliliters of sterile phosphate-buffered saline were injected in the right principal bronchi and at least 5 mL of BAL fluid (BALF) were recovered. One-hundred microliters of BALF were mixed

with 900  $\mu$ L Turk solution. Fifty microliters of this solution were placed on a Thoma cell (Superior, Germany) for a total cell count. Cytospin (Cytospin 2, Shandon (1400 tours/min, 6 min)) slides were stained with Hemacolor<sup>®</sup> (Merck, Diagnostica, Darmstadt, Germany) and cytology was performed by light microscopy (at least 100 cells were counted).

#### **2.3.4. Clinical observations: cough, temperature and daily weight gain**

Every day, the cough frequency was individually counted between 8.30 am and 9.00 am by one trained person able to simultaneously observe a maximum of 10 animals clearly identified by a number on their back. The rectal temperature was measured between 9.30 am and 10.00 am with a digital thermometer. The cumulative cough count (CCC) was calculated daily by adding the daily cough counts from the beginning of the experiment ( $-2$  dpi) with the aim to obtain a clinical parameter integrating the evolution of the pathological process. All the animals were weighed at  $-2$  and 14 dpi in order to calculate the daily weight gain of each animal.

#### **2.3.5. Whole body barometric plethysmography: the Penh measurement**

A whole body barometric plethysmograph (WBBP) adapted to the size of piglets was built to assess the pulmonary function as previously described by Halloy et al. [16]. Briefly, the pig was introduced in the main chamber of the plethysmograph and a differential pressure signal (pressure transducer, Emka Technologies, Paris, France) was measured between this WBBP box and a reference box maintained at atmospheric pressure. The pressure waveform signal generated by the respiratory cycles was amplified (AC264, Emka Technologies, Paris, France) and analysed by the IOX software (Emka Technologies, Paris, France) before

the Penh was calculated on a breath-by-breath basis using the following formula [18]:

$$\text{Penh} = (\text{PEP}/\text{PIP}) \times \text{Pause}$$

where Pause =  $((\text{Te}-\text{RT})/\text{RT})$ ; Te = expiration time; RT = relaxation time i.e. the time corresponding to pressure decay to 36% of the total box pressure during expiration; PEP = peak of expiratory pressure; PIP = peak of inspiratory pressure.

Penh is an index validated in mice and currently used to assess airway responsiveness in laboratory species [7, 11, 17, 19]. In pigs, Penh has been demonstrated to be a screening index allowing to quantify respiratory pattern disorders and is currently used to investigate airway reactivity to pharmacological agents in freely moving pigs but also to predict the amplitude of the pulmonary functional changes occurring during pulmonary diseases especially those of airway resistance [16].

To minimise the influence of stress due to handling and the new environment, the piglets were previously trained by placing them for 15 min during three consecutive days into the WBBP. Each measurement was performed during 10 min when the animal was breathing regularly and quietly.

#### **2.3.6. Pathology and histology**

Euthanasia was performed by an IV overdose of barbiturate at 14 dpi. The extent of macroscopic lesions was assessed by estimating the lung volume showing abnormalities such as congestion and red or grey hepatisation. The volume of macroscopic pneumonic lesions was determined by adapting a previously described method [15]. The lung volume ( $V_L$ ) was determined by water displacement before the lungs were transversely cut into 2 cm slices. A 0.2 cm  $\times$  0.2 cm grid was applied to the flat side of the cranial transverse section to assess the proportion of injured lung tissue. The counts of squares corresponding to injured

tissue and covering the total lung section were measured for each section and added. The relative proportion of injured parenchyma ( $V_p$ ) was then calculated by dividing total lesion grid points by the total grid points counted. An approximate volume of pneumonic lung was determined by multiplying  $V_L$  by  $V_p$ .

After examining the macroscopic lung lesions, specimens for light microscopy were sampled and fixed in 4% formaldehyde. The fixed specimens were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

### **2.3.7. RNA extraction, RT-PCR detection of cytokine mRNA and quantification of PCR products**

Pneumonic lung samples collected after euthanasia in the right cardiac lobe were maintained in Trizol (Life Technologies, Eragny, France) at  $-80^\circ\text{C}$  before they were homogenised using a Cat homogeniser. Total RNA was extracted as recommended by the manufacturer. The RNA was resuspended in 50 to 200  $\mu\text{L}$  of ultrapure water containing 0.02% (wt/vol) diethyl pyrocarbonate (Sigma, St. Quentin Fallavier, France) and 1 mM EDTA. Total RNA was quantified using a spectrophotometer at an optical density of 260 nm ( $\text{OD}_{260}$ ) and the purity was assessed by determining the  $\text{OD}_{260}/\text{OD}_{280}$  ratio. All of the samples had an  $\text{OD}_{260}/\text{OD}_{280}$  ratio above 1.8.

An RT-PCR procedure was performed as previously described [13] with minor modifications. Briefly, 1.5  $\mu\text{g}$  of RNA were reverse transcribed using murine moloney leukaemia virus reverse transcriptase (Point Mutant; Promega, Charbonnières, France) and suspended to a final volume of 100  $\mu\text{L}$ . Then, 5  $\mu\text{L}$  of cDNA were amplified for cyclophilin and tested for cytokines using 2.5 U of DNA *Taq* Polymerase enzyme (Invitrogen, Cergy Pontoise, France). The primer sequences, annealing temperatures and the number of PCR cycles for IFN- $\gamma$ , TNF- $\alpha$ , IL-18 and the house-keeping gene

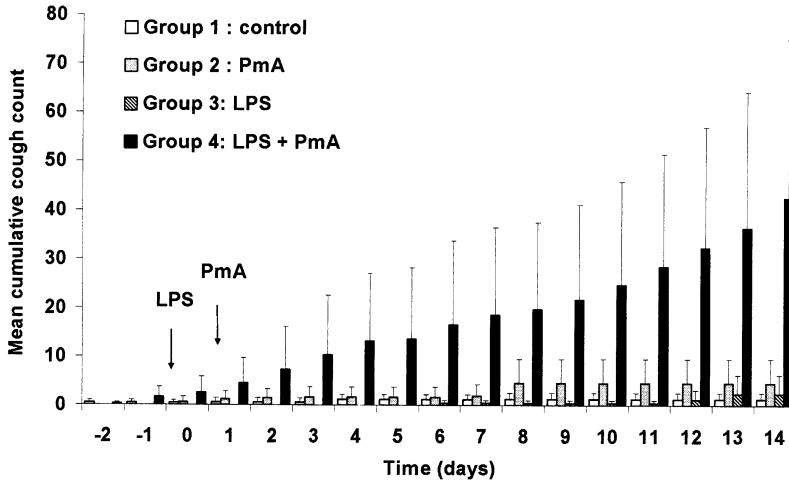
cyclophilin have already been described [10, 13, 14]. For IL-8, the primers used were 5'-TCTCTGGCAACCCTATGTC-3' (sense) and 5'-AGGACCAGAGCCAGGAAGA-3' (antisense), the annealing temperatures was  $55^\circ\text{C}$  and 30 PCR cycles were realized. Amplified DNA were analysed following electrophoresis on 1% TBE (Tris-Borate-EDTA) agarose gels, which were stained with ethidium bromide. The level of each PCR product was quantified by densitometry using a Quantity One programme (Bio-Rad, Hercules, USA). To compare the relative cytokine mRNA expression levels among samples, the values were presented as the ratio of the band intensity of the cyclophilin-specific RT-PCR product over that of the corresponding constitutively expressed "house-keeping" gene, cyclophilin.

### **2.3.8. *Pasteurella multocida* isolation and identification**

At necropsy, samples of pneumonic lungs were collected from caudal and cranial lobes and homogenised within a sterile mixer (Servall, Norwalk, USA) in 4 mL of sterile saline. One-hundred microliters of the homogenised solution were placed on Columbia agar with 5% sheep blood (BD, Erembodegem, Belgium), Muller-Hinton chocolate agar (BD, Erembodegem, Belgium) and Gassner agar (Oxoid, Drogen, Belgium). After overnight growth at  $37^\circ\text{C}$  in aerobic conditions, the colonies were examined using standard laboratory procedures and identified using the API 20 NE kit (Bio-Mérieux, Marcy-l'Étoile, France).

## **2.4. Data analysis and statistics**

The normal and variance homogeneity hypotheses were tested by using Kolmogorov-Smirnov and Levene tests, respectively. When both hypotheses were significantly ( $p < 0.05$ ) confirmed, data within and between groups were subjected to one-way or two-way analysis of variance (ANOVA). When the F test was significant ( $p < 0.05$ ), further differences between means were



**Figure 1.** Evolution of the mean cumulative cough count (CCC) during the course of the disease. Control: group 1 ( $n = 8$ ); *Pasteurella multocida* type A (PmA): group 2 ( $n = 6$ ); *E. coli* lipopolysaccharides (LPS): group 3 ( $n = 5$ ); *E. coli* lipopolysaccharides plus *Pasteurella multocida* type A: group 4 ( $n = 13$ ). CCC was significantly increased ( $p < 0.05$ ) from 1 to 14 dpi in animals inoculated with *E. coli* endotoxin and PmA. No significant changes were detected in other groups.

determined by the least square difference (LSD) Fisher procedure. The Pearson linear correlation coefficient was calculated to establish statistical relationships between parameters. When non-normal data distribution was obtained, unpaired data were subjected to Kruskal-Wallis analysis of variance and when the H was significant ( $p < 0.05$ ), further differences between the raw sums were established using Mann-Whitney U test. The non-parametric paired data were analysed with Friedman analysis of variance before they were compared with the Wilcoxon test. The Spearman linear correlation coefficient was used to calculate statistical relationships between non-parametric data and other parameters. All data were expressed as means  $\pm$  standard errors on the mean (SEM) while the p value defining significance was set at 0.05.

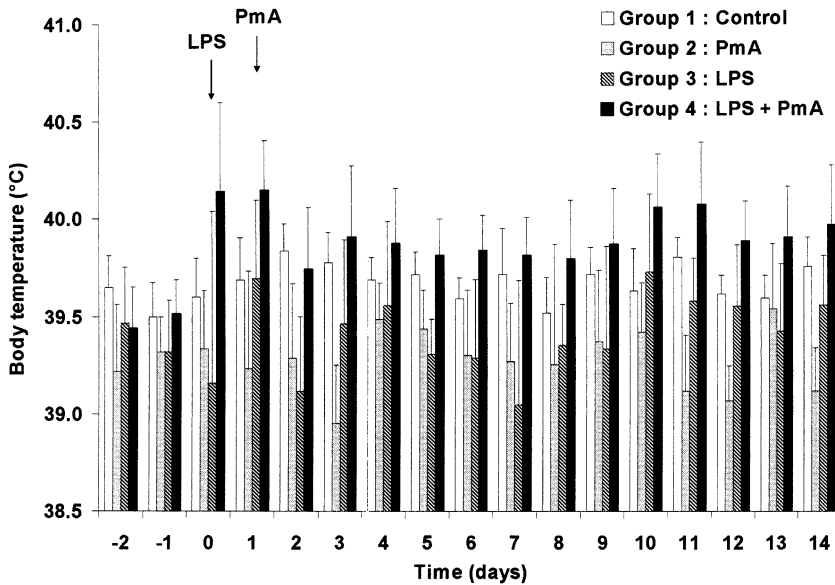
To determine whether CCC and body temperature measured during the course of pneumonia could be used to predict the magnitude of lung lesions, the severity of the pulmonary inflammation or the impact of the respiratory disease on weight gain at

the end of the experimental protocol, the individual daily data of CCC and body temperature obtained from 0 up to 14 dpi were correlated with end-point values of the parameters measured at 14 dpi.

### 3. RESULTS

#### 3.1. Changes induced in inoculated piglets

Cumulative cough count (CCC) was increased significantly ( $p < 0.05$ ) from 1 to 14 dpi in the animals inoculated with *E. coli* endotoxin and PmA (group 4) (Fig. 1). No significant changes were detected in the other groups. Similar results were obtained when data were expressed as the daily mean of cough counted one hour per day ( $2.1 \pm 2.7$  and  $8.1 \pm 9.4$  on 1 and 14 dpi respectively versus  $0.2 \pm 0.4$  at  $-2$  dpi). A significant ( $p < 0.05$ ) hyperthermia was detected in group 4 between 0 and 14 dpi while the body temperature recorded in groups 1, 2 and 3 remained within a physiological



**Figure 2.** Evolution of the mean rectal temperature in the four groups during the course of the disease. Control: group 1 ( $n = 8$ ); *Pasteurella multocida* type A (PmA): group 2 ( $n = 6$ ); *E. coli* lipopolysaccharides (LPS): group 3 ( $n = 5$ ); *E. coli* lipopolysaccharides plus *Pasteurella multocida* type A: group 4 ( $n = 13$ ). A significant ( $p < 0.05$ ) hyperthermia was detected in group 4 between 0 and 14 dpi while the body temperature recorded in groups 1, 2 and 3 remained within a physiological range.

range (Fig. 2). Except coughing and hyperthermia observed in group 4, no other clinical signs appeared in any group.

Piglets receiving LPS alone (group 3) ( $0.24 \pm 0.02$  kg) or in combination with PmA (group 4) ( $0.19 \pm 0.07$  kg) presented a similar but significant ( $p < 0.05$ ) reduction in daily weight gains compared to those of control ( $0.45 \pm 0.12$  kg) and PmA inoculated groups ( $0.31 \pm 0.15$  kg).

The whole body barometric plethysmography measurements revealed a significant Penh increase from 8 to 14 dpi in group 4 as presented in Figure 3. No significant changes were observed in other groups.

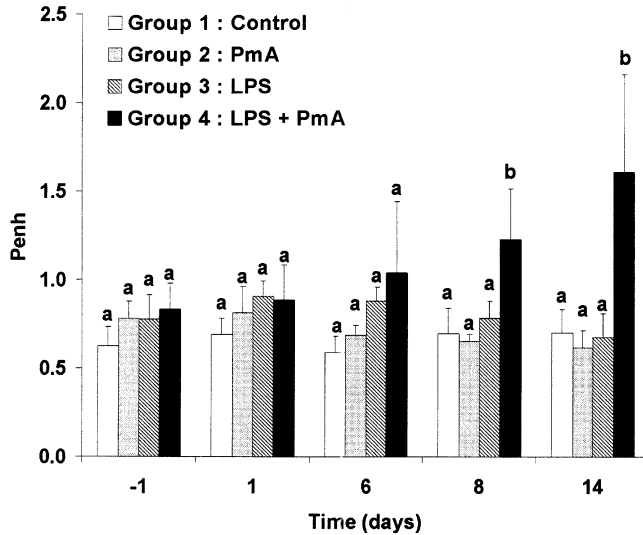
Cell counts measured at -1 and 14 dpi are presented in Figure 4. Significant increases in total lung cells, macrophages, neutrophils and lymphocytes were measured in group 4 while no significant changes occurred in

other groups except in group 1 exhibiting a slight but significant increase in total lung cells, and macrophages.

Fourteen days post-inoculation, the cytokine/cyclophilin mRNA absorbance ratio of IFN $\gamma$  was significantly ( $p < 0.05$ ) increased in group 4 compared to the control group (Fig. 5). In group 3 inoculated with LPS alone, a slight but significant ( $p < 0.05$ ) increase was also observed. No significant changes in IL-8, IL-18 and TNF $\alpha$  levels were observed in any groups.

Low volume macroscopic lung lesions preferentially located in caudal lung lobes were detected in group 1 (controls) ( $34.4 \pm 23.3$  cm $^3$ ), group 2 ( $11.4 \pm 6.2$  cm $^3$ ) and group 3 ( $7.0 \pm 3.6$  cm $^3$ ). In group 1, the alveolar walls likely consisted of flattened epithelial cells in close contact with capillaries and of a delicate connective tissue framework. It also contained scanty macrophages.



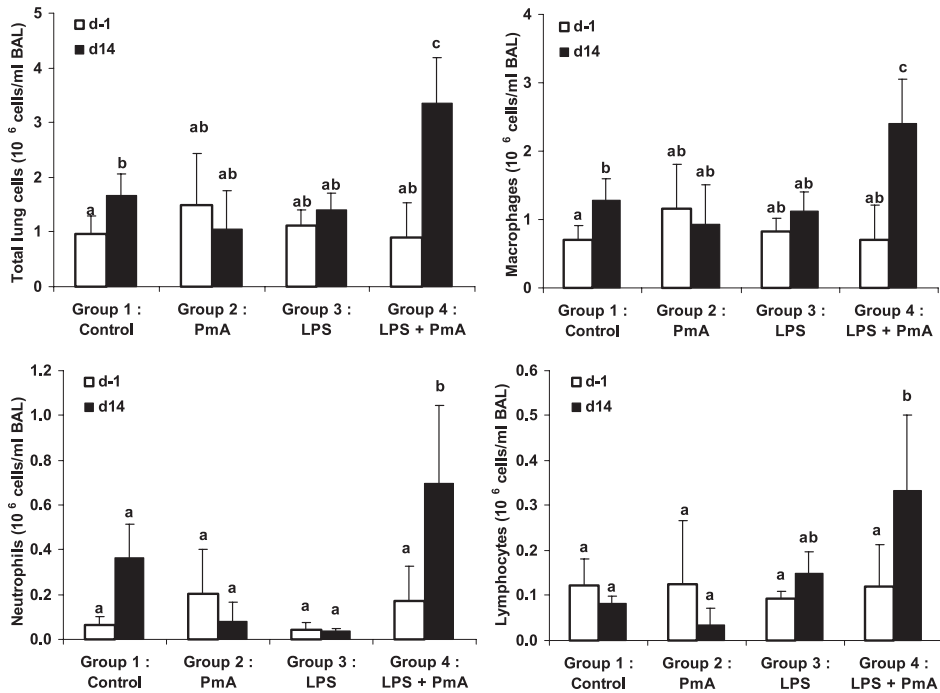


**Figure 3.** Evolution of the mean Penh value recorded in the different groups (unit: -). Control: group 1 ( $n = 8$ ); *Pasteurella multocida* type A (PmA): group 2 ( $n = 6$ ); *E. coli* lipopolysaccharides (LPS): group 3 ( $n = 5$ ); *E. coli* lipopolysaccharides plus *Pasteurella multocida* type A: group 4 ( $n = 13$ ). Letters differing between groups indicate a significant difference ( $p < 0.05$ ). When at least one letter is common between the two groups, the difference is not significant. Within a group, the values were compared to those obtained on -2 dpi. Comparisons between groups were performed by testing the values measured the same day.

The alveolar spaces were well expanded and, as the bronchiolar lumina, were free of inflammatory cells (Fig. 6A). Sometimes, the alveolar walls were enlarged by the presence of a slight lymphocytic and a moderate macrophage infiltrate. However, no inflammatory infiltrate was found in the bronchi. In group 2, the alveolar framework was preserved but the walls were enlarged by the presence of a slight lymphocytic and a moderate macrophage infiltrate. A subtle lymphocytic infiltrate was also seen in the bronchiolar and bronchial walls (Fig. 6B). In group 3, there was a minimal enlargement of the alveolar septa due to the increase of macrophages and lymphocyte number. Scanty macrophages were free in the alveoli. There was no inflammation of the bronchial wall (Fig. 6C). In group 4 where LPS and PmA were combined, significantly more extended lung damage

( $119.7 \pm 39.2 \text{ cm}^3$ ) was measured. These lesions were located in cranial lobes as well as in caudal lobes. The lung parenchyma was characterised by an accumulation of intra-alveolar fluid, extravasated red cells and some fibrin together with polymorphs and macrophages (Fig. 6D). A moderate bronchial inflammatory infiltrate and a pleuritis characterised by a lympho-plasmocytic infiltrate and a florid reactive mesothelial proliferation were observed. The lesions of pleurisy were recorded in 4 of the 13 animals of group 4.

No *Pasteurella multocida* or other swine respiratory tract pathogens were isolated from the control, LPS and PmA groups. In group 4, *Pasteurella multocida* was isolated from one pig (cranial lung lobe) while no other swine respiratory tract pathogen was founded.



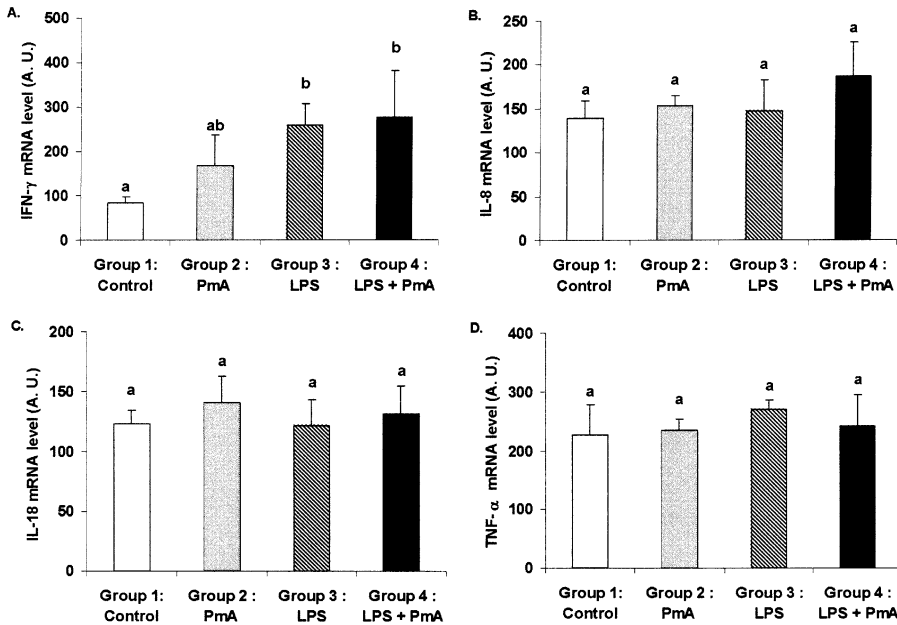
**Figure 4.** Inflammatory lung cells counted in bronchoalveolar lavage fluids. Control: group 1 ( $n = 8$ ); *Pasteurella multocida* type A (PmA): group 2 ( $n = 6$ ); *E. coli* lipopolysaccharides (LPS): group 3 ( $n = 5$ ); *E. coli* lipopolysaccharides plus *Pasteurella multocida* type A: group 4 ( $n = 13$ ). Letters differing between groups indicate a significant difference ( $p < 0.05$ ). When at least one letter is common between two groups, the difference is not significant. Within a group, the values were compared to those obtained on  $-2$  dpi. Comparisons between groups were performed by testing the values measured the same day.

### 3.2. Relationship between pathophysiological changes and clinical signs

Significant linear correlation coefficients were obtained when individual values of the volume of lung lesions, Penh, number of BALF macrophages and neutrophils were correlated with the corresponding individual values of CCC measured simultaneously during the protocol. Penh, BALF macrophage, neutrophil and lymphocyte numbers were also significantly ( $p < 0.01$ ) correlated with body temperature (Tab. I). Individual values of Penh were correlated with the BALF neutrophil number ( $r = 0.64$ ;  $p > 0.0001$ ) and the volume of lung lesions ( $r = 0.35$ ;  $p < 0.05$ ) was measured simultaneously.

### 3.3. Use of cough and body temperature for the detection of diseased animals

When selecting a threshold for CCC of five coughs per animal, it appeared that among the animals from group 4 ( $n = 13$ ), CCC exceeded this threshold at least one day during the course of the disease in 12 piglets (92.3%), all of them exhibiting severe lung lesions at the end of the protocol (lung lesions volume:  $119.6 \pm 42.4 \text{ cm}^3$ ). The mean detection time corresponding to the moment when the threshold was exceeded was  $4.5 \pm 3.1$  dpi. In this group, only one pig with extended lung lesions ( $120 \text{ cm}^3$ ) showed CCC values remaining under the selected threshold. In animals

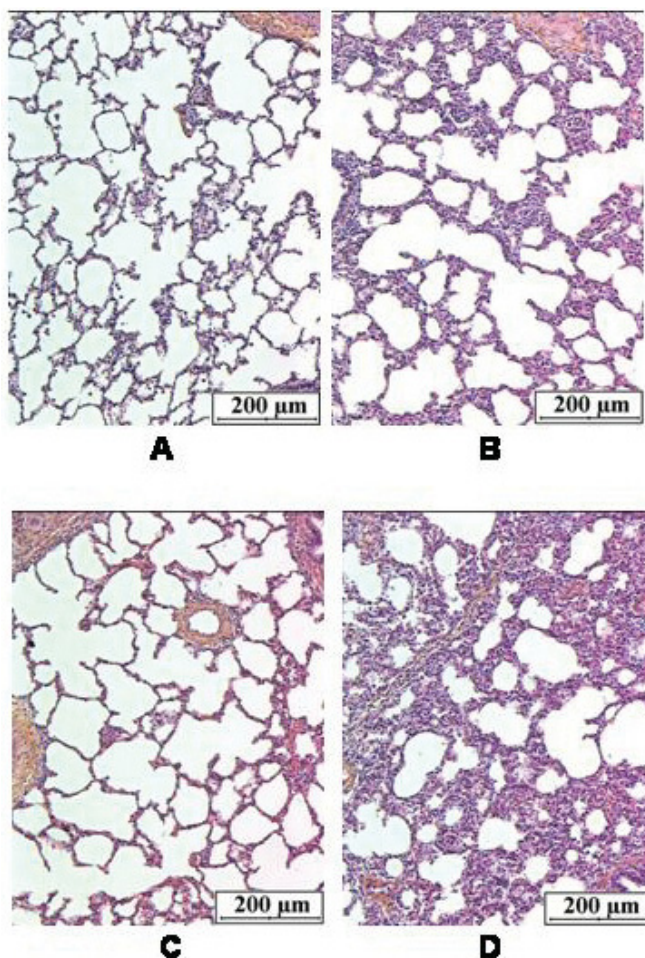


**Figure 5.** Cytokine production in lung tissue sampled at 14 dpi in: control: group 1 ( $n = 6$ ); *Pasteurella multocida* type A (PmA): group 2 ( $n = 5$ ); *E. coli* lipopolysaccharides (LPS): group 3 ( $n = 5$ ); *E. coli* lipopolysaccharides plus *Pasteurella multocida* type A: group 4 ( $n = 13$ ). Total RNA was isolated and assayed for expression of (A) IFN- $\gamma$ , (B) IL-8, (C) IL-18, (D) TNF- $\alpha$  and the cyclophilin housekeeping genes by RT-PCR. Quantification of these cytokine mRNA is shown. Letters differing between groups indicate a significant difference ( $p < 0.05$ ). When at least one letter is common between two groups, the difference is not significant.

from other groups (1, 2, 3) ( $n = 19$ ), only three (16%) developed coughs while their mean lung lesion volume was very low ( $11.7 \pm 5.1 \text{ cm}^3$ ). The others (84%) exhibited low values of CCC and lung lesion volumes ( $21.5 \pm 16.5 \text{ cm}^3$ ). When a threshold for body temperature was considered ( $> 40.0 \text{ }^\circ\text{C}$ ), it appeared that all animals from group 4 (100%) developed hyperthermia at least one day during the protocol (mean detection time:  $2.6 \pm 2.3$  dpi), all of them having severe lung lesions ( $119.7 \pm 39.2 \text{ cm}^3$ ). However, in the other groups, 11 animals exceeded the threshold (57%) while their lung lesion volume was not significantly increased ( $21.5 \pm 15.7 \text{ cm}^3$ ). When both criteria were combined (CCC  $> 5$  and body temperature  $> 40.0 \text{ }^\circ\text{C}$ ), only one pig

from groups 1, 2 and 3 (5%) was considered as a diseased animal while its lung lesion volume was very low ( $4.1 \text{ cm}^3$ ). In group 4 ( $n = 13$ ), 11 animals were detected on the basis of this combined criteria.

To check whether the total cumulative cough counted daily in a group could be used to differentiate groups composed of diseased or healthy animals, a threshold of five coughs per animal multiplied by the number of animals included in the group was selected. The thresholds of CCC calculated for group 1 ( $n = 8$ ), 2 ( $n = 6$ ), 3 ( $n = 5$ ) and 4 ( $n = 13$ ) were 40, 30, 25 and 75, respectively. These thresholds were never reached for groups 1, 2 and 3 while pneumonia was diagnosed at 2 dpi in group 4.



**Figure 6.** Lung parenchyma of a control animal (group 1, A), an animal inoculated with *Pasteurella multocida* only (group 2, B), an animal instilled with *E. coli* lipopolysaccharides only (group 3, C) and of an animal instilled with *E. coli* lipopolysaccharides and inoculated with *Pasteurella multocida* (group 4, D). (A) Free of inflammatory cells in alveolar wall and lumina. Respiratory bronchiole with smooth muscle lined by flattened epithelial cells and a normal arteriole. (B) Thickening of the alveolar septa by a moderate macrophage infiltrate and a slight lymphocytic inflammatory infiltrate. (C) Minimal inflammatory infiltrate consisting of macrophages and lymphocytes observed in the alveolar wall; presence of scanty alveolar macrophages. (D) Alveolar framework is modified by the presence of a massive confluent exudation with red cells, neutrophils, macrophages and some fibrin filling the alveolar spaces. Alveolar wall congestion and inflammatory infiltrate are also seen ( $\times 248$ ).

### 3.4. Predictive value of CCC and body temperature

CCC and body temperature measured from 1 to 14 dpi were significantly ( $p < 0.05$ )

correlated with the lung lesion volume and BALF cells recorded at the end of the protocol while only CCC was significantly ( $p < 0.05$ ) correlated with the daily body weight gain from 12 to 14 dpi (Tab. II).

#### 4. DISCUSSION

In this model of subacute bronchopneumonia, daily weight gain reduction and only a few clinical signs such as cough and hyperthermia were detected. A pulmonary inflammatory process involving macrophages, neutrophils, lymphocytes and IFN $\gamma$  was responsible for the lung lesions observed at the end of this study. Functional disorders consisting of the alteration of the expiratory airflow pattern also occurred. While cough and body temperature were moderately expressed, their magnitude was proportional to the intensity of the lung inflammatory process, the pulmonary functional changes and the extent of gross lesions. Moreover, cough and body temperature measured during the course of the disease were also related to the severity of the inflammation and lung injuries recorded at the end of the pathological process. Only cough was related to the reduction of the daily weight gain.

Airway clearance of non-attached organisms is very effective in pigs so that the epithelium integrity needs to be impaired to successfully induce pneumonic pasteurellosis. *Mycoplasma hyopneumoniae*, Aujeszky disease virus, porcine reproductive and respiratory syndrome virus with *Bordetella bronchiseptica* and crude *Actinobacillus pleuropneumoniae* cytotoxin are very effective agents to promote PmA-induced pneumonia [5, 8, 9, 22]. In our study, *E. coli* endotoxins, which have already been shown to inhibit mucociliary clearance in rats and to induce a transient acute lung inflammation in pigs which remains in the lung for a maximum of 48 h, were intratracheally injected to promote PmA infection [20, 27]. The lung injuries observed in the present study, especially subacute exudative bronchopneumonia involving migration of neutrophils in alveoli and bronchi, marked alveolar macrophage proliferation, and peribronchiolar and alveolar lymphocytic infiltration were in full agreement with studies devoted to *Pasteurella multo-*

*cida* infection [9, 15]. However, PmA was scarcely isolated from lung lesions suggesting that a prolonged colonisation of the respiratory tract was not necessary to strongly worsen the pulmonary inflammatory process initiated by *E. coli* endotoxins. The fact that PmA was cleared from the lungs during the experiment might explain the baseline levels of IL-8, IL-18 and TNF- $\alpha$  (Fig. 5) observed at 14 dpi which illustrated that the inflammatory process was decreasing in intensity. Indeed, inflammatory cytokines have been shown to be expressed during acute *Pasteurella multocida* infection [3]. However in the latter study, the animals were infected with a higher dose of bacteria (5 mL of  $4.8 \times 10^9$  CFU mL $^{-1}$  versus 5 mL of  $2 \times 10^9$  CFU mL $^{-1}$  in the present study) and were killed at an earlier time point of the respiratory infection (5 versus 14 dpi). A very transient production of cytokines within the first eight hours post infection has also been described during *Actinobacillus pleuropneumoniae* infection [2]. The mRNA synthesis of IFN- $\gamma$  detected suggests that lymphocytes observed within the lung tissue and the BALF participate in the regulation of both innate and acquired antimicrobial immunity [4, 30].

The Penh measured by whole body plethysmography is a functional parameter which has previously shown changes in the expiratory airflow pattern during cholinergic or endotoxin challenges [16]. In this experiment, the increase of Penh recorded on 8 and 14 dpi in group 4, was likely due to airway obstruction and/or to a decrease in the lung elastic recoil induced by the pulmonary inflammatory process as reported in anaesthetised piglets with other infectious pulmonary diseases [21]. To the best of our knowledge, it is the first time that such pulmonary functional alterations are reported in non-sedated, freely moving piglets with bronchopneumonia. Since the raise in Penh was proportional to the intensity of the lung inflammatory reaction and to the extent of lung lesions, measurement of this functional parameter might be an

**Table I.** Relationship between individual values of cumulative cough count (CCC) or body temperature (BT) and the corresponding individual values of bronchoalveolar lavage fluid cell numbers, Penh and volume of lung damage measured simultaneously. Regression equations with significant linear correlation coefficients are only indicated in this figure. The corresponding determination coefficients are also presented.

X	Y	<i>r</i>	<i>p</i>	ax + b	<i>R</i> <sup>2</sup>
BT (°C)	Macrophages (10 <sup>6</sup> /mL BALF)	0.41	0.01	0.96 x - 37.09	0.16
	Neutrophils (10 <sup>6</sup> /mL BALF)	0.45	0.001	0.40 x -15.73	0.20
	Lymphocytes (10 <sup>6</sup> /mL BALF)	0.38	0.01	0.17 x - 6.51	0.14
	Penh	0.27	0.001	0.26 x - 9.29	0.07
CCC	Macrophages (10 <sup>6</sup> /mL BALF)	0.42	0.01	0.02 x + 0.98	0.17
	Neutrophils (10 <sup>6</sup> /mL BALF)	0.38	0.01	0.004 x + 0.20	0.14
	Volume of lung lesions (cm <sup>3</sup> )	0.57	0.001	1.12 x + 40.71	0.32
	Penh	0.35	0.0001	0.01 x + 0.77	0.12

**Table II.** Linear correlation coefficients (*r*) between clinical signs and bronchoalveolar lavage fluid cells, volume of lung lesions and daily weight gain. Values of clinical signs were day by day correlated with the clinical end-points recorded at d14. Days corresponding to significant (*p* < 0.05) *r* values are indicated in this table with the range of daily *r* values obtained during the indicated period of time.

		Cumulative cough count	Body temperature
Total lung cells	days p.i.	3 to 14	1 to 14
	<i>r</i> range	0.35 to 0.58	0.37 to 0.64
Macrophages	days p.i.	1 to 14	1 to 14
	<i>r</i> range	0.41 to 0.53	0.43 to 0.63
Neutrophils	days p.i.	4 to 14	1 to 14
	<i>r</i> range	0.38 to 0.50	0.51 to 0.69
Lymphocytes	days p.i.	6 to 14	1 to 14
	<i>r</i> range	0.42 to 0.48	0.48 to 0.68
Volume of lung lesions	days p.i.	1 to 14	1 to 14
	<i>r</i> range	0.31 to 0.59	0.34 to 0.55
Daily weight gain	days p.i.	12 to 14	-
	<i>r</i> range	-0.39 to -0.40	-

interesting tool for a non-invasive follow-up of a lung disease accompanied by air-flow limitation.

Pigs from group 4 exhibited moderate cough and hyperthermia. These clinical findings are in agreement with previous

data and field observations collected in pigs with PmA-induced pneumonia [9]. Without any accurate quantitative recording procedure, these symptoms are not so easy to detect explaining why an early diagnosis of such pulmonary diseases remains difficult

in field conditions. Since other clinical signs are often missing or cannot be detected, the use of quantitative measurement of coughing and body temperature might improve the diagnosis of respiratory disorders in pigs. The relationship between coughing and lung damage has been investigated in pigs with enzootic pneumonia by scoring the cough on a weekly basis and measuring lung lesions at slaughterhouses but no significant correlation was obtained probably due to a non appropriate assessment of cough and lung lesions [25]. Cough was counted by mixing pigs in the pen for 30 s and observing the response (cough or no cough). Moreover, the extent of lung lesions was semi-quantitatively determined by scoring them as a percentage of the damaged lung surface. As concluded by the authors of this trial, a daily monitoring of cough and a better record of the lung lesion volume could improve the predictive value of this symptom regarding the intensity of the pulmonary disease. Hyperthermia has also been monitored during swine respiratory infections involving *Pasteurella multocida* but the relationship between the body temperature and lung lesions was not investigated [6, 9, 15].

The significant linear correlation coefficients obtained in our study between cough, body temperature and the corresponding values of BALF cells, lung lesions and Penh (Tab. I) suggest that these clinical signs could be used to predict the severity and evolution of the inflammatory process and lung lesions during the course of an infectious respiratory disease. To assess the potential use of CCC and body temperature to early diagnose a respiratory disease in a pig, thresholds for both parameters have been defined. Hence, all piglets reaching five coughs or 40 °C at least one time during the protocol were considered as diseased animals and the accuracy of the diagnosis was confirmed by the presence of lung lesions on 14 dpi. Applying this method with CCC as the criteria, 92.3% of the animals with lung lesions were detected early

while only 16% of the pigs with no significant lesions compared to controls were erroneously declared as diseased (false positive response). Considering body temperature as the discriminative criteria, the corresponding percentages were 100 and 57% respectively. A combination of both criteria allowed a marked reduction of the number of false positive cases (85% of positive responses versus 5% of false positives). When a threshold for CCC was defined for a group, the respiratory disease was detected at 2 dpi in group 4 while the others (groups 1, 2, 3) never overcame the threshold in our experimental conditions. Thus, if cough recognition systems are available in field conditions in the future, this might be a real advance to determine the respiratory status of animals, to early and non-invasively diagnose respiratory diseases in herds but also to perform the follow-up of bronchopneumonia [28, 29]. However, a prior validation of these systems is needed to confirm our observations in a large swine population which contains animals with several common respiratory diseases. Data from Table II also show that BT and CCC could be used to predict early-on the extent of major end-points regarding the intensity of respiratory disease in piglets.

We conclude that the *E. coli* lipopolysaccharide-induced lung inflammatory process might be worsened by *Pasteurella multocida* while it does not allow lung colonisation. The resulting inflammatory process is characterised by an inflammatory cell influx into the airways which limits the air-flow and by extended lesions of subacute bronchopneumonia explaining the weight gain reduction. Cough and body temperature, while moderately exhibited, could be used as indicators to assess the intensity and the evolution of the lung inflammatory reaction and functional changes during the course of the disease. They are potential predictors for the magnitude of lung lesions and weight gain reduction assessed at the end of the process.

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