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PERACUTE INFECTION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* SEROTYPE 1 IN GUINEA PIGS

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Abstract

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This experiment was performed to study gross and microscopic lung lesions after endotracheal inoculation with Actinobacillus pleuropneumoniae (*A. pleuropneumoniae*) serotype 1 in twenty-four guinea pigs. Four-six hours post inoculation, clinical signs of respiratory distress and epistasis were observed. Most of the infected animals suddenly died during this time. The differences between the pulmonary lesions were evaluated. In this experiment, the lesions of peracute infection by *A. pleuropneumoniae* were induced within 4-6 hours and the lesions of subacute infection, characterized by fibrinopurulent pleuritis were observed in surviving animals 18-20 hours post inoculation. The results suggested that guinea pigs could be used as a model for the study the pathogenesis of *A. pleuropneumoniae* infection in pigs.

Keywords : *Actinobacillus pleuropneumoniae*, guinea pigs, peracute infection

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การติดเชื้อ *Actinobacillus pleuropneumoniae serotype* 1 แบบเฉียบพลันในหนูตะเภา

ตรวจสอบอาการทางคลินิกและศึกษารอยโรคปอดที่สำคัญทั้งมหพยาธิและจุลพยาธิวิทยาของหนูตะเภา หลังจากฉีด Actinobacillus pleuropneumoniae serotype 1 ปริมาณ 3.7x10^8 โคโลนี/มล. เข้าหลอดลมหนูตะเภา จำนวน 24 ตัว ผลการทดลองพบว่าหนูตะเภาในกลุ่มที่ติดเชื้อเริ่มแสดงอาการหายใจลำบาก มีเลือดออกจากจมูกและตายอย่างเฉียบพลันตั้งแต่ชั่วโมงที่ 4-6 หลังจากฉีดเชื้อ รอยโรคปอดที่พบมีลักษณะแตกต่างกัน จากการศึกษาครั้งนี้พบว่าสามารถเหนี่ยวนำให้เกิดรูปแบบการติดเชื้อเอพีพีแบบเฉียบพลันภายใน 4-6 ชม. ตามด้วยรอยโรคแบบกึ่งเฉียบพลันภายใน 18-20 ชม. ซึ่งพบเยื่อหุ้มปอดอักเสบชนิดมีหนองและไฟบริน จากการศึกษาครั้งนี้พบว่าหนูตะเภาเป็นสัตว์ทดลองเพื่อศึกษาพยาธิกำเนิดของโรคปอดและเยื่อหุ้มปอดอักเสบในสุกรได้

คำสำคัญ : *Actinobacillus pleuropneumoniae* หนูตะเภา การติดเชื้อแบบเฉียบพลัน

Introduction

An outbreak of porcine pleuropneumonia in pigs was first described in detail by Pattison et al. (1957). *Actinobacillus pleuropneumoniae* is the etiological agent of pleuropneumonia. This pathogen has played a substantial role as one of the most severe respiratory bacterial pathogens in growing and finishing pigs, affecting pig production enterprises worldwide (Sebunya and Saunders, 1983; Barnum 1990; Nicolet, 1992; Chiers et al., 1999; Marsteller and Finwick, 1999). It causes acute death from pneumonia and pleurisy, and was a big impacts on production and economic losses due to the associated depression of growth performance in chronically infected herds (Barnum, 1990; Taylor, 1999). The severity and variety of the clinical signs in infected pigs, depends on the serotypes existing in the herd, the weight of infection and the susceptibility of the animals. At least 15 serotypes of *A. pleuropneumoniae* have been described (Haesebrouck et al., 1997; Nielsen et al., 1997; Blackall et al., 2002). Serotypes 1, 5, 9, 10 and 11 are highly virulent (Taylor, 1999). The remainder are less. *A. pleuropneumoniae* infection in Thai pig populations mostly involve cross-reacting serotypes 1/9/11, serotype 5a, 3/6/8 and 4/7 (Neramitmansook et al., 1990; Assavacheep et al., 2003). Clinical signs are divided into 4 forms; peracute, acute, chronic and sub-clinical. Following infection, pigs may die within 12 hours (Veenhuizen, 1998). In a previous study, it has been implied endotoxin play a role in the pathogenesis of *A. pleuro- pneumoniae* (Sebunya and Saunders, 1983), although the pathogenesis has not been clearly illustrated even now. A range of pathogenicity, from no signs of disease to severe disease with high mortality, has been described (Thacker, 1998). These bacteria can only infect pigs, but guinea pigs may also be experimentally infected and it has been suggested that guinea pigs are useful laboratory animals for the study of the pathogenesis of *A. pleuropneumoniae* (Perfumo et al., 1999). This experiment was performed to compare the lung lesions in guinea pigs infected with *A. pleuropneumoniae* serotype 1 (strain CU-001) at different times after infection.

Materials and Methods

Pathogens: *A. pleuropneumoniae* serotype 1 (strain CU-001, field isolated from an outbreak in fattening pigs

in Thailand) was inoculated onto chicken meat infusion agar, with 5-10 % CO_2 in the atmosphere, for 18-24 hours, at 37 ^{o}C The concentration of the inoculum was adjusted to $3.7x10^8$ CFU/ml.

Experimental design: Thirty-one Dunkin Hartley, guinea pigs,* 10 weeks of age, weighing 400-500 g/animal and of both sexes, were purchased from the National Laboratory Animal Center, Mahidol University (NLAC-MU) and were randomly divided into 7 groups and acclimatized in challenge unit for one week. Group 1 (non-infected group) comprised of seven animals. One animal in this non-infected group was sacrificed using an overdose of Pentobarbiturate sodium (Nembutal®) before starting inoculation, to check the lung condition. The remaining six animals were endotracheally inoculated with 1 ml normal saline solution (placebo) under anesthetic. Group 2 (infected group) consisted of twenty-four animals which were endotracheally inoculated with 1 ml of $3.7x10^8$ CFU/ml *A.pleuropneumoniae* (Figs.1a and 1b).Thereafter, one animal in the non-infected group and four randomized animals in the infected group were sacrificed at 6, 12, 24, 36, 48 and 72 hours post inoculation. Clinical signs were observed (Fig. 1c). The number of sick and dead animals and their lung lesions were recorded (Fig. 1d). All animals were examined for the predominant pathological lesions in their lungs. The right lung lobes from each animal were fixed in 10% neutral buffer formalin and paraffin sections were stained with hematoxylin and eosin (H&E) using Brown and Brenn method for the identification of bacteria in the sections (Luna, 1968). Routine bacteriological examination was performed on the left lung lobes samples of each animal.

Pneumonia lesions were generally divided into four types, as lung edema with congestion, red hepatization, grey hepatization, and lung resolution. In the present study, the disease proceeded rapidly within the first twenty-four hours post inoculation. A s a consequence, the gradation of the lung lesions in such peracute stages was modified to follow a previous study (Perfumo et al., 1999). The gross pulmonary lesions were graded into 4 categories as, no remarkable lesions, emphysema, hemorrhage and pneumonia (+1 to +3). The microscopic lesions were also divided into 4 categories as, pulmonary hemorrhage, pulmonary edema with inflammatory cell infiltration, pneumonia and pleurisy.

Results

To verify the health status of the animals used in this experiment, one randomized animal was sacrificed and the lung condition evaluated before inoculation. Only acute pulmonary edema and congestion were found. It was concluded that this was a lesion from the shock of euthanasia an no evidence of *A. pleuropneumoniae* infection was seen (Fig. 1e).

Unexpectedly, most of the animals in the infected group expressed clinical signs of respiratory distress, such as dyspnea, open mouth and abdominal breathing and a suddenly massive bloody nasal discharge resulting in death 4-14 hours post inoculation. Only one surviving animal in this group had no major signs of infection and was subsequently sacrificed 2 weeks post inoculation. According to the time period after inoculation, the number of dead animals and the major lung lesions were analyzed and calculated using a pneumonic score system. As shown in Table 1, the pneumonic score ranged from 0.2-3.0. Most of the infected animals had a pneumonic score lower than 1.5, except the last two animals that died more than 18 hours post inoculation and had the highest pneumonic score and the most pleuritis lesions. From Table 2, it can be seen that most of the gross lung lesions involved hemorrhage (75%) pneumonia +1 (33.3%) and emphysema (25%), whereas the non-infected group showed no dominant lung lesions.

Regarding the microscopic pulmonary lesions seen in the infected group, pulmonary emphysema, edema and hemorrhage were the most common lesions as is presented in Table 3.

According to the time of onset, most of the pulmonary lesions in the infected animals, 4-8 hours post inoculation, were defined as toxic shock. The main lesions

Table 1. The number of infected animals with gross pulmonary lesions in different time periods after inoculation (n = 24)

Periods after inoculation	Gross lesions (No. of animals with lesions/Total died)	Pneumonic score[b] (mean±SD)
4-5 hrs	pneumonia[a] +1 (4/10)	0.4
5-6 hrs	pneumonia +1 (1/5)	0.2
6-7 hrs	pneumonia +1 (1/3)	0.2
8 hrs	pneumonia +1 (1/2), pneumonia +2 (1/2)	1.5±0.7
12-14 hrs	pneumonia +1 (1/1)	1.0
18-20 hrs	fibrinous pleuritis (1/1), pneumonia +3 (1/1)	3.0
22-24 hrs	fibrinous pleuritis (1/1), pneumonia +3 (1/1)	3.0
14 days	no remarkable lesions (1/1)	-

[a] Pneumonia was defined as pulmonary congestion, edema and hemorrhage; [b] The severity of pneumonia was measured by the distribution of pneumonia in the lung; pneumonia +1 = focal pneumonia, pneumonia +2 = patchy pneumonia, pneumonia +3 = lobar pneumonia; The calculation of pneumonic score was based on the number of animals with pneumonic lesions multiplied by the observed score (+1 to +3) and divided by total of deaths in each period

Table 2. The number of infected (n = 24) and non-infected (n = 6) animals with gross pulmonary lesions

Gross lesions	No. of animals/Total (%)	
	Non-infected group	Infected group
Emphysema	6/24 (25.0%)	0/6
Hemorrhage	18/24 (75.0%)	0/6
Pneumonia +1[a]	8/24 (33.3%)	0/6
Pneumonia +2[b]	1/24 (4.2%)	0/6
Pneumonia +3[c]	2/24 (8.3%)	0/6
No remarkable lesions	13/24 (54.2%)	0/6

[a] Pneumonia +1 = focal pneumonia, [b] Pneumonia +2 = patchy pneumonia, [c] Pneumonia +3 = lobar pneumonia

Table 3. The number of infected (n = 24) and non-infected (n = 6) animals with microscopic pulmonary lesions

Microscopic lesions	No. of animals/Total (%)	
	Infected group	Non-infected group
Diffuse hemorrhage	8/24 (33.3%)	3/6 (50%)
Multifocal hemorrhage	8/24 (33.3%)	0/6
Subpleural hemorrhage	1/24 (4.2%)	0/6
Edema	10/24 (41.7%)	1/6 (16.7%)
Cellular infiltration	5/24 (20.8%)	0/6
Congestion	4/24 (16.6%)	2/6 (33.3%)
Emphysema	12/24 (50%)	4/6 (66.67%)
Bacterial clumps	3/24 (12.5%)	0/6
Focal pneumonia	7/24 (29.2%)	1/6 (16.7%)
Patchy pneumonia	1/24 (4.2%)	0/6
Lobar pneumonia	2/24 (8.3%)	0/6
Fibrinopurulent pleuritis	2/24 (8.3%)	0/6

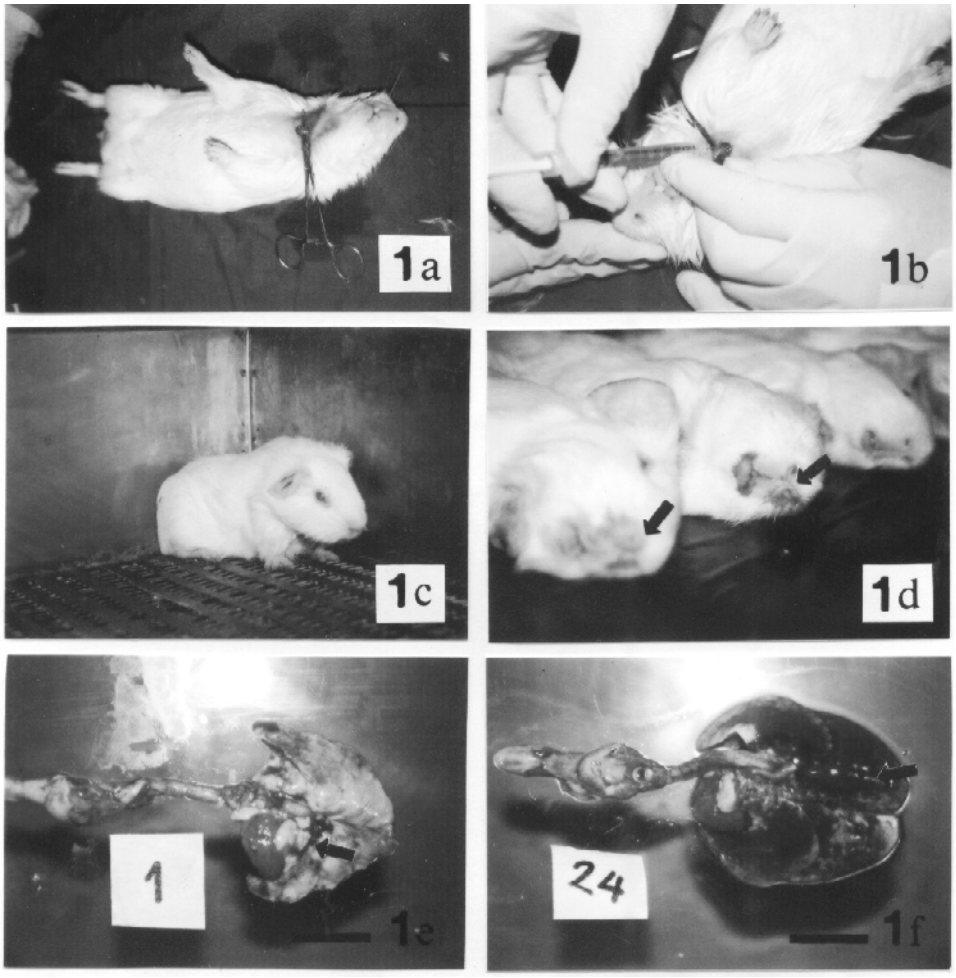

Figure 1. The procedure for endotracheal inoculation in experimental animals with *A. pleuropneumoniae* (1a and 1b), clinical signs of respiratory distress in infected animals (1c), bloody nasal discharge in dead animals (1d), pulmonary hemorrhage and emphysema in non-infected animal (1e), and multifocal hemorrhage in infected an animal that died 8 hours post inoculation (1f), bar = 1.5 cm.

were characterized by pulmonary emphysema, edema and congestion, included the presence of gram negative bacterial clumps in alveolar spaces and bronchioles.

The infected animals died within 6-7 hours post inoculation and had microscopic lesions including diffused pneumonia, severe congestion, with compensatory emphysema, subpleural hemorrhages and bacterial clumps in the alveoli (Figs. 2a, 2b and 2c). The pulmonary lesions in animals that died 12-14 hours post inoculation were focal pneumonia.

The severity of pneumonia increased in animals that died 18-20 hours post inoculation (Fig. 2d). These pulmonary lesions included pulmonary hemorrhage and pneumonia +3 with lymphocyte and neutrophil infiltration. In addition, fibrinous pleuritis was common during this period.

Twenty two-twenty four hours post inoculation, the most severe pneumonic lungs were predominantly focal pneumonia, necrosis and inflammatory cell (neutrophils, lymphocytes and macrophages) infiltration (Fig. 2f), including severe fibrinous pleuritis (Fig. 2e).

Bacteria were cultivated from the left lobes of the lungs in infected groups (23/23) and identified as *A. pleuropneumoniae*, except for one animal sacrificed 14 days post inoculation. Otherwise, results from all lungs in the non-infected animals were negative (7/7).

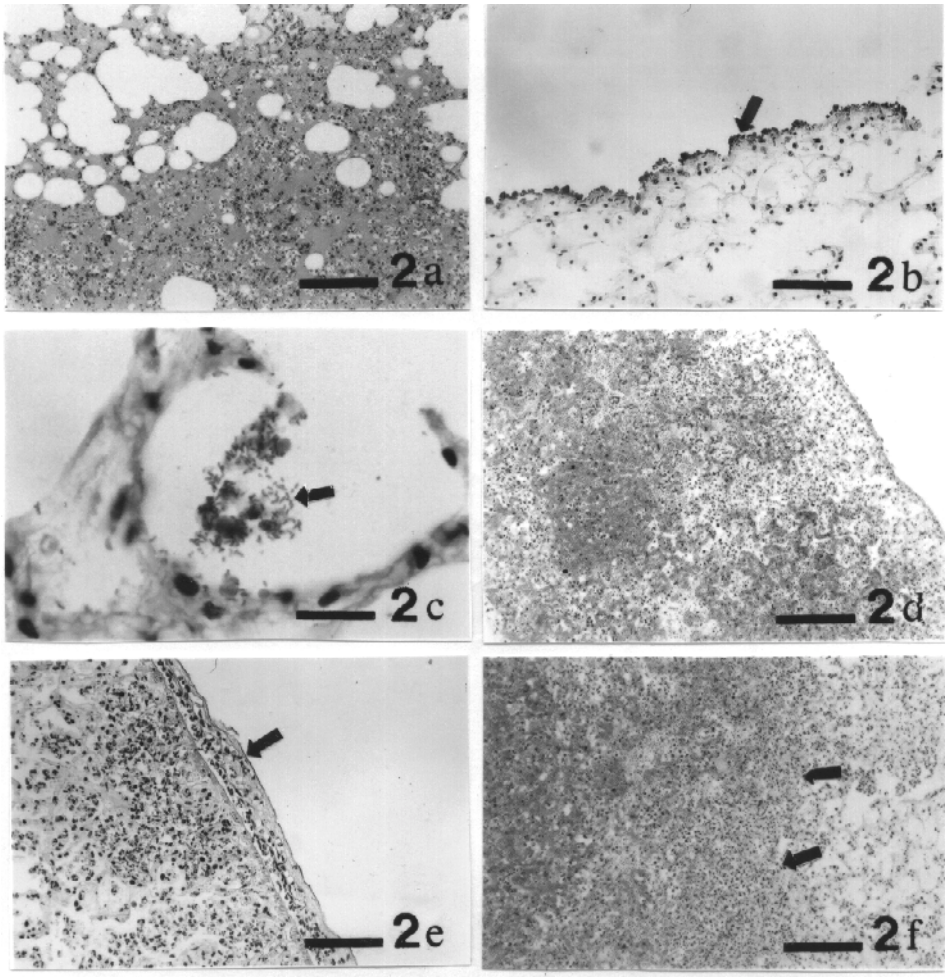

Figure 2. The microscopic pulmonary lesions of infected groups; compensatory emphysema resulting from the euthanasia procedure in infected animals that died 4-8 hours post inoculation, bar=120 μm (2a), subpleural hemorrhage (arrow), bar = 60 μm (2b) and bacterial clump in alveoli showing gram negative-rod shape organisms (red color), Brown and Brenn stain, bar = 4 μm (2c) in infected animals that died 6-7 hours post inoculation, diffused pulmonary hemorrhage in infected animals that died 18-20 hours post inoculation, bar = 120 μm (2d), pleural thickening with fibrin and inflammatory cell infiltration, found in infected animals that died 22-24 hours post inoculation, bar = 60 μm (2e) and focal consolidation and necrosis, including a demarcation line due to inflammatory cell infiltration, bar = 120 μm (2f)

The microscopic pulmonary lesions seen in one sacrificed animal before experiment started showed acute pulmonary edema, diffuse congestion, and compensatory emphysema. In non-infected groups, the pulmonary lesions only signified the effect of shock by the euthanasia procedure and a low degree of non-specific pneumonia. However, these microscopic pulmonary lesions involved pulmonary hemorrhage, congestion and emphysema.

Discussion

It was unfortunate that, the results did not adhere to the experimental design, as the peracute form of *A. pleuropneumoniae* infection, was induced 4-8 hours after endotracheal inoculation. The occurrence of peracute infection, characterized by acute death may be explained because of two things. Formerly, the bacterial concentration of inoculum in the present experiment was a high dose

($3.7x10^8$ CFU/ml.), when compared with previous reports and the pathological findings on infected lungs were differently dependent on bacterial concentrations (Perfumo et al., 1999).

A further factor may be the *A. pleuropneumoniae* strain used in the present study. This strain was isolated from an acute infection in a Thai fattening pig herd and had high virulence. It also induced the disease in a different animal species. The predominant lung lesions in this experiment were very similar to those described in pigs, even though guinea pigs were the animals susceptible to *A. pleuropneumoniae* (Nakai et al., 1984; Perfumo et al., 1999). *A. pleuro- pneumoniae* infection can also be induced in other animal species such as mice (Idris et al., 1993), but hemorrhagic lesions, without fibrinous exudate and pleuritis, were the only lesions demonstrated (Nakai et al., 1984). Guinea pigs can therefore be recommended as laboratory animals to study the pathogenesis of *A. pleuropneumoniae* infection in pigs as suggested earlier .

The prominent lung lesions associated with toxic shock occurred 4-8 hours after inoculation. This period was considered similar to the acute onset of disease in pigs after exposure to a large number of organisms (Bertram, 1985; Taylor, 1999). The severity of the pneumonia lesions increased in parallel to the onset period (Perfumo et al., 1999).

Since, fibrinopurulent pleuritis was demonstrated in two animals that died 18-24 hours post inoculation, this lesion was categorized as an acute response (Bertram, 1988; Taylor, 1999). This lesion has been reported in guinea pigs in only a few publications. It may result in acute massive reaction and death a short time after inoculation without any progression of disease (Nakai et al., 1984). A lower dose of organisms in the future experiments may possibly induce pleuropneumonia in guinea pigs over a longer onset period.

Recently, lobar fibrinous pleuropneumonia was seen in guinea pigs that died 9 days post inoculation, with a median dose of *A. pleuropneumoniae* (Perfumo et al., 1999). Such doses may produce less severity and allow a longer onset time than in our experiment.

Demarcated thickening lines between necrosis and normal lungs 22-24 hours post inoculation, were seen (Fig 2f). They originated from toxic components in the early stages, such as 3 hours after experimental infection and became gradually more visible (Idris et al., 1993; Taylor, 1999). Platelet aggregation and neutrophil accumulation in the alveolar wall are illustrated (Idris et al., 1993). Afterwards, the alveolar wall became necrotic and the boundary of the lesions was filled with damaged and dead inflammatory cells or debris (Bertram, 1985) which was simultaneous involved with pulmonary hemorrhage (Bertram, 1988; Taylor, 1999).

One animal in the infected group survived for 2 weeks post inoculation. No pulmonary lesions and negative results from bacterial culture were recorded for this animal. It was a reflection on a mistaken inoculation procedure i.e. A lower amount of inoculum intake, influenced by oozing from the nose or mouth.

Conclusion

A peracute infection of *A. pleuropneumoniae*, serotype 1, in guinea pigs was induced 4-8 hours after innoculation, followed by a subacute infection characterized by fibrinopurulent pleuritis an seen at 18-24 hours. The severity of the pneumonia increased in parallel to the onset period after inoculation. Since, fibrinopurulent pleuritis the most was marked lesion 18-24 hours post inoculation, this lesion was never seen in the earlier onset period. It was considered that guinea pigs could be used as laboratory animals for a study on the pathogenesis of *A. pleurpneumoniae.*

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