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Relationship between the inoculum dose of *Streptococcus pneumoniae* and pneumonia onset in a rabbit model

A.L. Yershov, B.S. Jordan, C.H. Guymon and M.A. Dubick

ABSTRACT: It is generally assumed that the development of bacterial pneumonia becomes possible when the dose of inhaled or aspirated pathogens overwhelms the respiratory tract host defence system, but this hypothesis has not yet been tested either clinically or experimentally.

This study evaluated inoculum dose in relation to onset of experimental pneumococcal pneumonia, and estimated the median effective dose resulting in pneumonia in healthy New Zealand White rabbits (mean ± SD 4.75 ± 0.25 kg (n=27)). Rabbits were endobronchially inoculated with increasing doses of *Streptococcus pneumoniae* and pneumonia onset was observed over the following 96 h. The diagnostic approach was based on the Clinical Pulmonary Infection Score, modified for use in rabbits.

Inoculation of *S. pneumoniae* at doses of >4.60 $\log_{10}$ cfu made the development of pneumonia in rabbits more predictable (up to 90%). Lower doses of bacteria failed to cause pneumonia in 80% of inoculated animals. The median effective dose was estimated by means of logistical regression, probit analyses and the Reed–Muench method, and corresponded to 4.32, 4.38 and 4.67 $\log_{10}$ cfu, respectively.

It is speculated that development of pneumococcal pneumonia becomes more likely when the inoculum dose exceeds a threshold of antibacterial protection, making inoculum dose a risk factor for disease onset.

KEYWORDS: Experimental pneumonia, inoculum dose, rabbits, *Streptococcus pneumoniae*

Community-acquired pneumonia remains a common and potentially life-threatening disease [1]. In the USA, there are 2 3 million cases each year [2], accounting for 500,000 hospitalisations, which are not only costly [3], but also associated with significant mortality [4]. Such trends show no signs of slowing. *Streptococcus pneumoniae* is the most common bacterial cause of community-acquired pneumonia, being identified in 20 75% of cases [5]. Widespread disease and increasing incidence of antibiotic resistance in these microbes dictates an urgent need to investigate the pathogenic and host defence mechanisms that influence onset of pneumococcal pneumonia [6].

Animal models of pneumococcal pneumonia have often been used in the evaluation of pulmonary antibacterial host defence mechanisms and for pharmacological studies in pneumonias. Previous experiments in rabbits used inoculum doses of *S. pneumoniae* which ranged 3.70 9.18 $\log_{10}$ cfu [7, 8]. Even when the same serotypes of pneumococcus were used in experiments, such a wide range of inoculum dose and the large differences in mortality make these studies difficult to interpret and compare. A detailed review of the literature did not reveal studies specifically investigating the relationship between bacterial dose and development of pneumonia in either clinical or experimental medicine. Although it is often speculated that the bacterial inoculum dose must overwhelm the respiratory tract host defence system for pneumonia onset [9, 10], this hypothesis has not been tested. Thus, the present study tested this hypothesis by investigating the relationship between inoculum dose of *S. pneumoniae* and probability of pneumonia onset. A second objective was to estimate the dose of this microbe that provoked pneumonia development in 50% of healthy New Zealand White rabbits (ED_{50}).

MATERIALS AND METHODS

Animals and study design

The study was reviewed and approved by the US Army Institute of Surgical Research (Fort Sam Houston, San Antonio, TX, USA) Research

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Council and Animal Care and Use Committee, the manuscript was reviewed for compliance prior to submission for publication, and guidelines for the care and use of laboratory animals were followed [11].

Male specific pathogen-free New Zealand White rabbits (mean±SD 4.75±0.25 kg (n=27)) were obtained from Myrtle's Rabbitry, Inc. (Thompson Station, TN, USA). After arrival, all animals were placed in quarantine for 7 days in individual cages and fed *ad libitum* with water and commercial feed according to current recommendations. The health status of each rabbit was confirmed by the US Army Institute of Surgical Research veterinary staff before commencing each experiment.

Pneumococcal pneumonia was induced as previously described [7, 8]. Briefly, on the day of the experiment (day 1), rabbits were anaesthetised intramuscularly with a ketamine xylazine mixture and orally intubated with a 3.5-mm cuffed endotracheal tube. For inoculation of bacteria, a sterile silicone catheter (diameter 1 mm) was introduced aseptically through the endotracheal tube into the trachea and gently inserted until it reached the bronchi, and 1 mL of freshly prepared pneumococcal inoculum (see below) was flushed slowly through the catheter. Animals were placed in the upright position for 15 s in order to facilitate distal alveolar migration by gravity. Every effort was made to minimise the potential adverse effects of anaesthesia and intubation. After awakening and extubation, the animals were placed back in their individual cages separate from other rabbits and given free access to food and water. The inoculum doses used ranged 3.27 7.56 $\log_{10}$ cfu·mL^{-1}. Higher doses were not used as they always result in pneumonia onset in rabbits [7]. In total, 20 different inoculum doses were used in 24 rabbits. A negative control group included three rabbits that were intubated but not inoculated with *S. pneumoniae*.

Serotype 3 *S. pneumoniae* (American Type Culture Collection (ATCC®) 6303; ATCC, Manassas, VA, USA) was used for preparation of the inoculum in the present study. This serotype is frequently seen in patients with community-acquired pneumonia [9], and is widely used in experimental medicine, including in animal pneumonia models [10, 12]. A working stock of the organism was made by growing the organism overnight in nutrient broth and then freezing 1-mL aliquots in evaporated milk at -80℃ in accordance with standard operating procedures at the US Army Institute of Surgical Research.

One day prior to inoculation, a vial of *S. pneumonia* ATCC 6303 was removed from the freezer and thawed at room temperature, and 1 mL of the stock pipetted into 25 mL of brain heart infusion broth. The culture was incubated for 18 h at 37℃ in 5% CO_2. On the day of inoculation, the overnight culture was serially diluted in physiological saline to the desired concentration, again according to standard operating procedures of the US Army Institute of Surgical Research.

In order to confirm that live bacteria were inoculated into rabbits, a viable plate count was also performed at this time, for the determination of inoculum concentration. Plate counts were performed in duplicate, using the standard spread plate method on tryptic soy agar. All plates were incubated for 18 24 h at 37℃ in 5% CO_2. Colonies were then counted and the number of cfu per mL calculated. Only one strain was used in all experiments.

Rabbits were observed for pneumonia onset over the 96 h after inoculation (days 2 5). The diagnostic criteria for pneumonia were based on the DENNESEN *et al*. [13] approach and on the Clinical Pulmonary Infection Score [14], modified slightly to account for rabbit physiology (higher normal body temperature, *etc*.) along with tissue microbiological and morphological findings. In the present study, a diagnosis of pneumonia was accepted when at least four of six (*i.e.* >50%) diagnostic criteria group results were positive (table 1). The accuracy of this diagnostic approach was verified by comparing the results obtained with the histological data. In previous publications, histological results were considered the gold standard for pneumonia verification [15, 16].

Procedures

Computed tomographic (CT) imaging (Aquilion 16; Toshiba America, Inc., New York, NY, USA) of the rabbit thorax was performed under intravenous ketamine xylazine anaesthesia and mechanical ventilation for ~10 min at 24, 48, 72 and 96 h after inoculation. The width of the slices was 0.5 mm. CT scans were performed during breath-hold (inspiratory phase) and always before bronchoalveolar lavage (BAL) in order to avoid the possible appearance of artefacts caused by any lavage fluid remaining.

BAL was performed according to previously published recommendations for paediatricians [19]. Briefly, BAL was performed immediately after each CT scan under ketamine xylazine anaesthesia. Sterile technique was utilised during all manipulations. BAL fluid (BALF) was collected by using up to five separate 8-mL aliquots of 0.9% sodium chloride (38℃). BAL was considered complete when 5 mL of the fluid was obtained back. Acquired BALF specimens were transferred to the laboratory within 5 min for microbiological and cellular evaluation. Cells in BALF were separated using a Millipore filter (Millipore Corp., Bedford, MA, USA) and cytocentrifugation. Cell counts and distributions in BALF were determined using a ABX Pentra 120 Blood Analyzer (HORIBA ABX, Montpellier, France).

For microbiological evaluation of BALF, specimens were serially diluted in physiological saline and then spread on tryptic soy agar plates, using 100 μL per plate and two plates per dilution [20]. The lavage sample was also streaked on trypticase soy agar plates containing 5% sheep blood and MacConkey II agar plates for culture isolation. All plates were incubated overnight at 37℃ in 5% CO_2. All isolates were identified using standard microbiological methods [21]. *S. pneumoniae* identification was based on the presence of alpha haemolysis on trypticase soy agar plates containing 5% sheep blood and the production of a zone of inhibition of ⩾14 mm against a 5-μg ethylhydrocupreine disc (optochin test; BBL; Becton Dickinson, Sparks, MD, USA).

For determination of total blood cell count and arterial blood gas levels, 1 mL of blood was obtained from the arterial catheter during initial catheterisation and 24, 48, 72 and 96 h after inoculation. Total cell count and arterial blood gas analyses were performed immediately after sample collection

TABLE 1 Diagnostic criteria for pneumonia used in the present study

Criterion	Parameters	Evaluation/comments
Clinical	Body temperature >40℃ Purulent mucus in the endotracheal tube Difficulty breathing and tachypnoea Cyanosis of the ears, lips and tongue	Admitted as positive when three of four (>50%) are present. The initial diagnosis of pneumonia is usually based on clinical features and all listed criteria are recognised as important constituents of this approach [13, 14]
Radiological	Progressive infiltrate Consolidation Cavitations Pleural effusion	Positive when at least one sign is present. Each of these signs alone is sensitive and indicative of pneumonia diagnosis in experiments [17]
Laboratory	Impairment of pulmonary function as defined by $P_{a,O_2}/F_{I,O_2}$ ratio of <200 mmHg White cell density >11000 or <4000 cells·μL^{-1} on ⩾2 consecutive days BALF cellular content ⩾1.0×10^3 cells·mm^{-3}	Considered positive when at least two of three signs (>50%) are present. These signs of pneumonia are considered reliable and are widely used in clinical and experimental medicine [18]
Microbiological	*S. pneumoniae* burden of ⩾1×10^4 cfu·g lung tissue^{-1} in at least one sample. BALF *S. pneumoniae* concentration of ⩾1×10^4 cfu·mL^{-1}	Considered positive when at least one of two signs is present. Microbiological evaluation of lung tissue and especially BALF represent the cornerstones of pneumonia diagnosis and are commonly used in both clinical and experimental medicine [18]
Macroscopic	Lung lesion scoring ⩾3 (red congestion or higher#) in at least one lobe Pleural exudate or fibrin deposits (or both) in at least one pleural cavity	Considered positive when at least one of two signs is present
Histological	Combination of accumulation of polymorphonuclear leukocytes in the capillaries and adjacent alveolar spaces in at least one sample	In many previous studies, *post mortem* morphological observations were considered the most dependable criteria for pneumonia [15, 18]

P_{a,O_2}: arterial oxygen tension; F_{I,O_2}: inspiratory oxygen fraction; BALF: bronchoalveolar lavage fluid; *S. pneumoniae: Streptococcus pneumoniae*; cfu: colony forming unit. #: see table 2. 1 mmHg=0.133 kPa.

using the ABX Pentra 120 Blood Analyzer and the AVL Omni Modular System (AVL Medical Instruments; Roche Omni Systems, Indianapolis, IN, USA), respectively. All blood samples were collected and analysed according to current clinical standards [24].

Lung tissue specimens were collected from each animal at the time of death or euthanasia. Euthanasia was performed *via* intravenous administration of a veterinary euthanasia solution (Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI, USA). Autopsy was performed such that the lungs were aseptically removed. For each pulmonary lobe, the macroscopic aspect was noted using a scoring grid [7] based upon human morphological findings (table 2). An overall macroscopic score was calculated as the sum of all lobar macroscopic scores, plus 2 points in the case of pleural effusion (range 0 29). This method has already been successfully used in a rabbit model of pneumonia [7, 8]. Macroscopic diagnosis of pneumonia was defined as positive when the score in one lobe was >2.

Two portions of each lobe with the maximum macroscopic lesions were harvested aseptically (one for quantitative cultures and one for histological study) in order to compare histological and bacteriological findings. For pathological studies, specimens were fixed in buffered formalin according to standard methods. A veterinary pathologist, without knowledge of the animal and bacterial data, evaluated the specimens. The lesions were graded into five categories: oedema, alveolitis, bronchiolitis/bronchopneumonia, necrosis, and fibrin vasculitis. Only bronchiolitis/bronchopneumonia and necrosis were accepted as histological confirmation of pneumonia consistent with previous studies [18].

For bacteriological processing of specimens, tissue samples, aseptically taken at necropsy, were flamed quickly in order to remove any surface contaminants and then placed in pre-weighed Stomacher® 80 bags (Seward, London, UK) and weighed to determine tissue weight. Physiological saline was added to the Stomacher bags and the tissue blended in a Stomacher 80 laboratory blender for 60 s at normal speed. The resulting solution was serially diluted and quantitative plate counts and culture isolation were performed as previously

TABLE 2 Lung macroscopic scoring grid

Aspect	Score
Normal	0
Scar	1
Slight congestion	2
Red congestion	3
Grey congestion	4
Yellowish congestion	5

TABLE 3 Relationship between inoculum size and diagnostic criteria in the rabbit model of pneumococcal pneumonia

Inoculum size $\log_{10}$ cfu	Diagnostic criterion						Pneumonia
	Clinical	Radiological	Laboratory	Microbiological	Macroscopic	Histological	
0#	0	0	0	0	0	0	0
3.267	1	1	0	0	1	1	1
3.279	0	0	1	1	0	0	0
4.389	0	0	1	0	0	0	0
4.505	0	0	0	0	1	1	0
4.544	0	1	0	1	0	0	1
4.602	1	1	0	1	1	1	1
4.724	0	0	0	0	0	0	0
5.342	1	1	0	1	1	1	1
5.389	0	1	0	1	1	1	1
5.477	1	1	0	0	1	1	1
5.505	1	1	0	0	1	1	1
5.525	1	1	0	1	1	1	1
5.550	0	1	0	1	1	1	1
5.550	1	1	0	1	1	1	1
6.182	1	1	1	1	1	1	1
6.182	0	0	0	0	0	0	0
6.204	1	1	0	1	1	1	1
6.433	1	1	0	1	1	1	1
6.608	1	1	0	0	1	1	1
6.608	1	1	0	1	1	1	1
6.633	1	1	0	0	1	1	1
6.633	0	1	1	1	1	1	1
6.796	1	1	0	0	1	1	1
7.562	1	1	0	1	1	1	1

cfu: colony forming unit; 0: negative result; 1: positive result. #: three control rabbits.

described [20, 21]. All isolates were identified using standard microbiological methods. *S. pneumoniae* identification was based on the presence of alpha haemolysis on trypticase soy agar plates containing 5% sheep blood and the production of a zone of inhibition of ⩾14 mm against a 5-μg ethylhydrocupreine disc (optochin test).

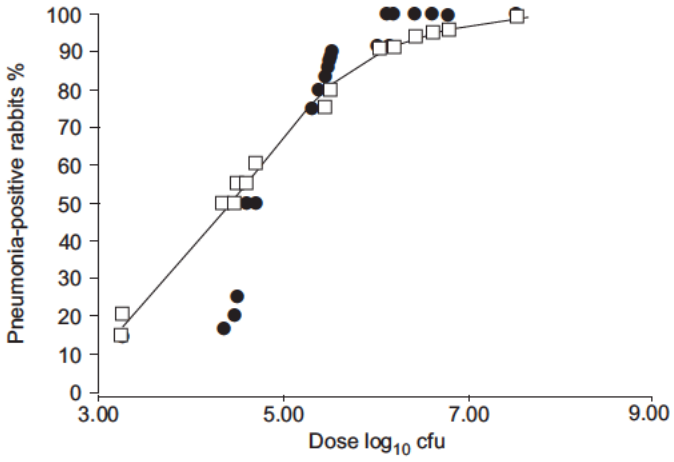

FIGURE 1. Probability of pneumonia onset as a function of dose using Reed Muench (●), probit (□) and logistical regression ( ) analysis. cfu: colony forming unit.

Data analysis

The independent variable was microbe dose and the dependent variable morbidity. For estimation of the inoculum size corresponding to ED50 in rabbits, the Reed Muench method

TABLE 4 Sensitivity, specificity, positive and negative predictive values, and accuracy of the diagnostic criteria, for pneumonia#

Criterion	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
Clinical	83	100	100	67	24
Radiological	90	100	100	67	27
Laboratory	11	67	50	20	26
Microbiological	67	67	86	40	24
Macroscopic	100	83	95	100	24
Histological	100	83	95	100	24

PPV: positive predictive value; NPV: negative predictive value. #: estimated by the method of SACKETT *et al.* [24].

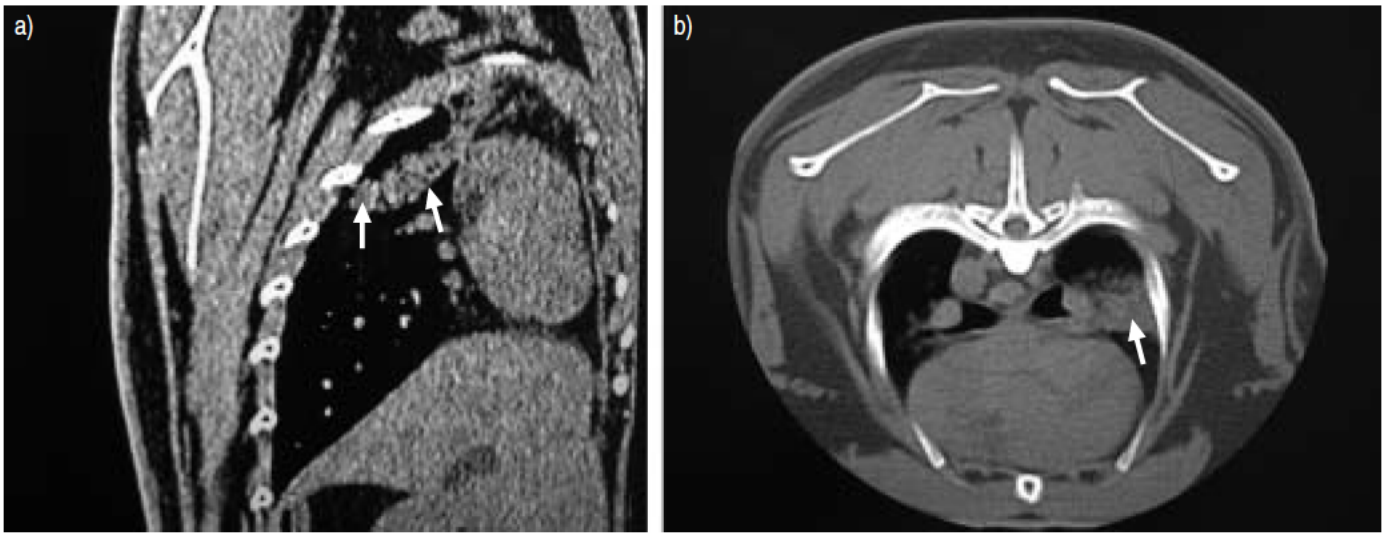

FIGURE 2. Computed tomographic scan of the rabbit thorax showing pneumonia in the right upper lobe (arrows): a) lateral position, and b) cross sectional image.

was used [23]. Logistical regression and probit were also used to evaluate the relationship between *S. pneumoniae* dose and morbidity in the rabbit pneumonia model and to confirm ED_{50} estimation using the Reed Muench method. The sensitivity and specificity of the diagnostic criteria for pneumonia, as well as prevalence of pneumonia, were calculated according to the method of SACKETT *et al.* [24]. Fisher's exact test was used to compare categorical variables. Correlation was assessed using the Spearman rank test. A p-value of <0.05 was considered significant. Where appropriate, data are presented as mean±SD.

RESULTS

Based on the present diagnostic approach, 18 of 24 (75%) inoculated animals developed *S. pneumoniae* pneumonia. No signs of pneumonia were found in the control group. Inoculum doses and distributions of the clinical, radiological, laboratory, microbiological, macroscopic and microscopic signs of disease resulting in pneumonia confirmation are shown in table 3. It was observed that most rabbits developed pneumonia when inoculum doses of *S. pneumoniae* were ≥ 4.60 $\log_{10}$ cfu, suggesting that, in the current model, this inoculum level represents a critical value that makes pneumonia onset most predictable. In order to test this assumption statistically, all inoculated animals were divided into two subgroups: those with an inoculum dose of <4.60 $\log_{10}$ cfu, and those with doses of ≥ 4.60 $\log_{10}$ cfu. In the first subgroup, only one of five (20%) rabbits developed pneumonia, whereas, in the second subgroup, 17 of 19 (89.5%) rabbits were pneumonia positive. According to Fisher's exact test, the difference in morbidity between the two subgroups was significant ($p=0.0021$). In order to confirm the accuracy of the diagnostic approach

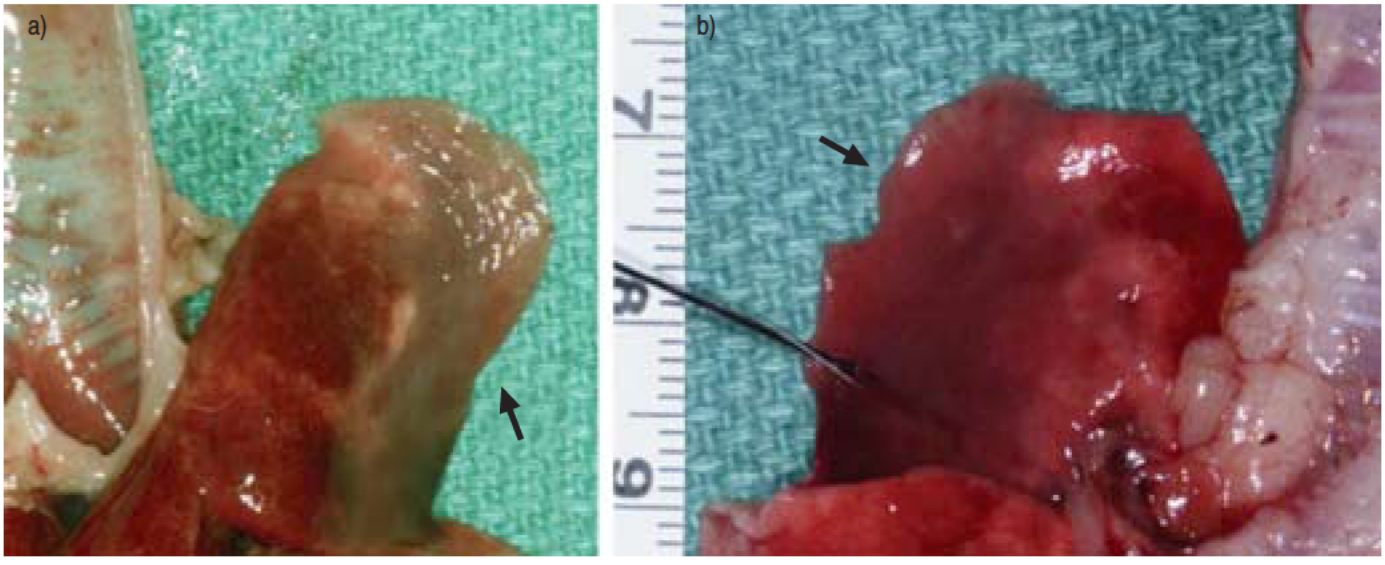

FIGURE 3. Rabbit lung lobes from the same animal as used for computed tomographic imaging (see fig. 2) showing pneumonia in upper right lobe (arrows): a) anterior view, and b) posterior view.

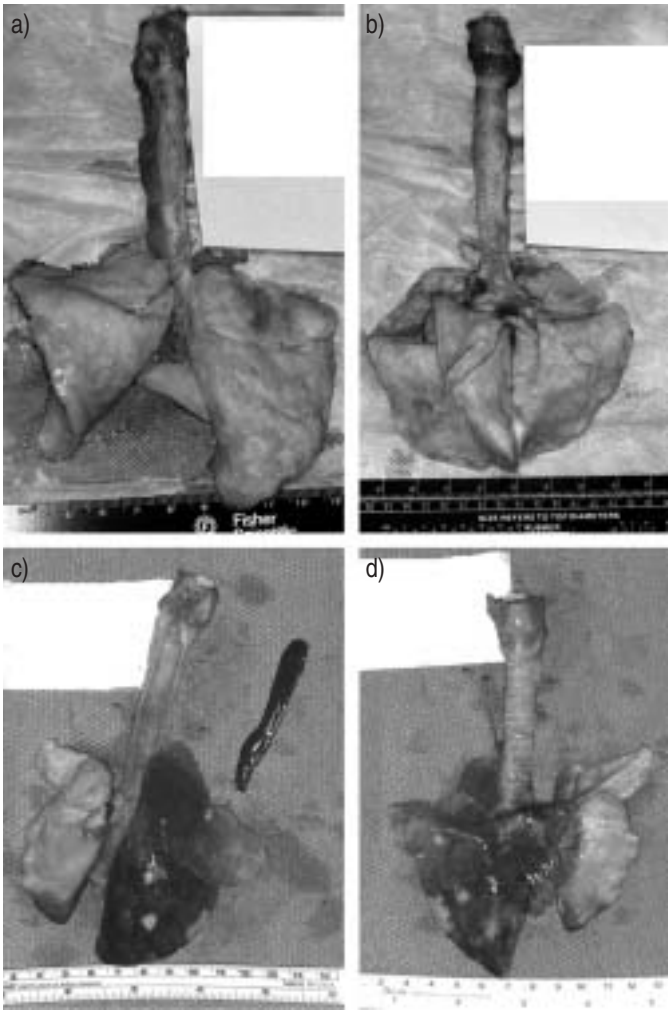

FIGURE 4. a, b) Normal rabbit lungs: a) posterior view, and b) anterior view. The heart, vessels and oesophagus are ablated; the trachea is not dissected lengthwise. c, d) Sample of the subtotal pneumonia (red congestion) in the right upper, middle and lower lobes: c) posterior view, and d) anterior view. The trachea's posterior wall is dissected lengthways along the middle line. Purulent sputum is present in the upper third of the trachea. The spleen is of normal shape and size.

applied, the difference between these two subgroups was tested using *post mortem* lung tissue histopathological findings, considered to be one of the most reliable criteria for the diagnosis of ventilator-associated pneumonia [15]. Microscopic evaluation revealed that one of five animals developed pneumonia when the inoculum dose was <4.60 $\log_{10}$ cfu and 17 of 19 were pneumonia positive when the inoculum dose was $\geqslant 4.60$ $\log_{10}$ cfu (table 3). The difference between these two subgroups measured by the Fisher's exact test was also significant ($p<0.05$).

A significant relationship between $\log_{10}$ dose and probability of pneumonia onset was observed using logistic regression and probit analyses ($p<0.05$; fig. 1). Calculation of ED_{50} using the Reed Muench method gave a value of 4.67 $\log_{10}$ cfu in the present rabbit model. This approximation was validated by logistic regression and probit analyses, which showed ED_{50} of 4.32 and 4.38 $\log_{10}$ cfu, respectively (fig. 1).

Based on the data shown in table 3, the sensitivity, specificity, positive and negative predictive values, and accuracy of the different diagnostic criteria in pneumonia confirmation were tested according to SACKETT *et al*. [24]. Three criteria showed the greatest reliability in the present study: CT, and microscopic and macroscopic morphological signs (table 4). Red congestion was the most common macroscopic lesion in ill animals (16 of 18 (89%)). Histological manifestations of pneumonia were observed in all ill rabbits. Thoracic CT scans were highly sensitive and specific in the present study. The usage of contemporary high-resolution tomography revealed comparatively small infiltrates in rabbit lungs and had obvious diagnostic advantages over traditional thoracic radiography, which, for anatomical reasons, is very complicated in rabbits. Representative images of CT scan, macroscopic and histological changes in pneumonia-positive rabbits are shown in figures 2 5.

DISCUSSION

The present study demonstrated a relationship between *S. pneumoniae* inoculum dose and the development of pneumonia. The probability of pneumonia onset significantly increased when the inoculum dose exceeded 4.60 $\log_{10}$ cfu. Doses below this level failed to cause disease in the majority (80%) of healthy New Zealand White rabbits. According to the Reed Muench and logistical regression methods, under the present experimental conditions, the ED_{50} was estimated to be ~4.32 4.67 $\log_{10}$ cfu for this rabbit model. A new diagnostic approach for pneumonia verification in rabbits was also tested in the present study and appeared to be acceptable.

These observations made it possible to speculate that the development of experimental *S. pneumoniae* pneumonia in the majority of healthy New Zealand White rabbits may require a threshold dose. In the present study, doses of >7.56 $\log_{10}$ cfu were not used, since they have previously been shown to produce 100% morbidity and 100% mortality in animals [7, 8, 17]. The present histological data were similar to the description of lung tissue samples seen in rabbits inoculated with a 100% lethal dose of *S. pneumoniae* [7].

The diagnostic approach used in the present study for pneumonia confirmation employed several criteria routinely used in clinical practice. In previous studies, it was shown that any of the criteria used alone for diagnosing ventilator-associated pneumonia, such as BAL, chest radiography, quantitative bacterial culture of lung tissues and even histological findings, failed to be highly sensitive and specific diagnostic tools [25]. In the absence of a universally recognised diagnostic gold standard [18], the true accuracy of the various diagnostic procedures for experimental pneumonia remains unknown. Histological signs of pneumonia are generally considered the most reliable [18, 25], but are used most commonly for *post mortem* pneumonia confirmation. Consequently, better methods of pneumonia diagnosis in live patients are required. In the present rabbit model, the assumption was made that several different criteria for pneumonia would increase the reliability of the diagnosis. Thus, the present diagnostic approach appeared to be sensitive and acceptable for pneumonia confirmation in rabbits, and should be adaptable to other experimental models. Among the criteria used, high-resolution CT scans were found to be the

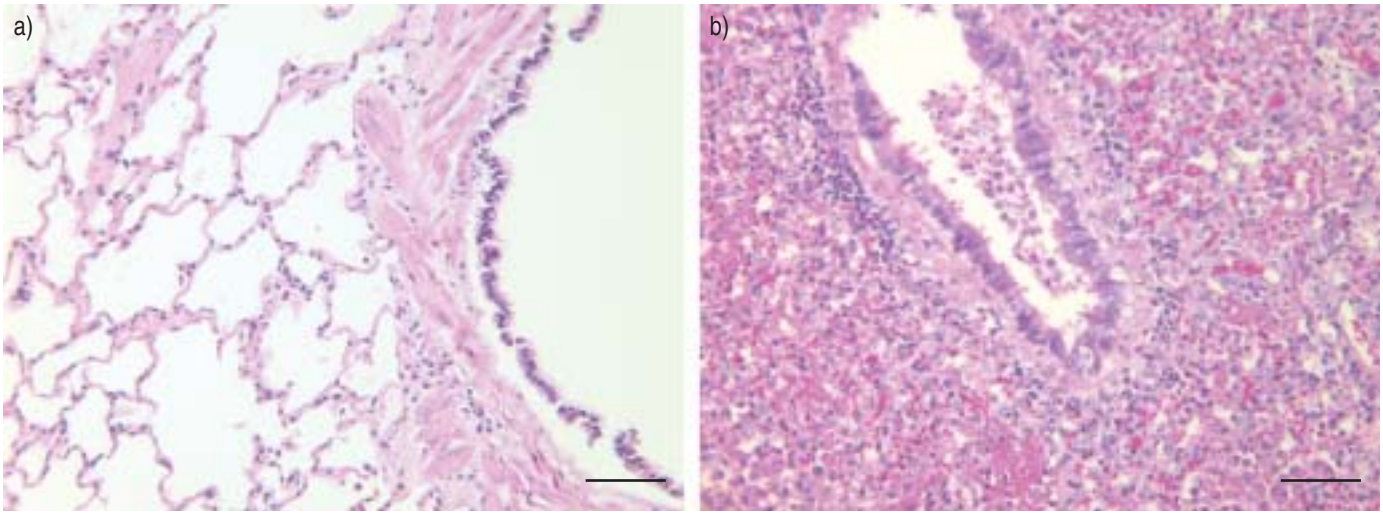

FIGURE 5. Rabbit lung histology: a) normal lung, and b) bronchopneumonia with intrabronchiolar inflammatory cells and debris, peribronchiolar inflammatory cells and alveoli filled with fibrin. Haematoxylin and eosin staining; internal scale bar=35 μm.

most reliable for pneumonia diagnosis in rabbits, with a sensitivity and specificity similar to those of histological data, but with the advantage of allowing diagnosis in live animals.

Adult rabbits appear to be a good model for the study of pneumococcal pneumonia. New Zealand White rabbits are naturally not highly susceptible to *S. pneumoniae* infections [7, 26], which guarantees that the observed pathologies were induced by the experimental manipulation. Also, the size of rabbit used (4.75 ± 0.5 kg) permitted the use of equipment, instruments and techniques intended for clinical paediatric practice, and the rabbits tolerated the daily BAL and blood sampling well. In contrast to previous reports [27, 28], the present study used immunocompetent animals, which made pneumonia development more natural. The strain of *S. pneumoniae* used in the present study was a clinical isolate with a common serotype, and inoculated rabbits were observed over a comparatively long period of time (up to 96 h after inoculation). In addition, the present model employed inoculation through natural airways into bronchi, which has other advantages. It is more reproducible than aerosol instillation [7, 29] and less invasive than transcutaneous intratracheal or transthoracic inoculation [7, 31]. Thus, it is believed that the present rabbit model is a good mimic of pneumonia in humans.

An important feature of the present model was its ability to approximate to severe human lower airways inflammation, especially post-operative pneumonia. This disease is often caused by the dislocation of pathogenic microbes from the upper airways to the trachea. Similarly, the present model also began directly with an invasion phase. Thus, the present experimental model should also be relevant to ventilator-associated pneumonias in which the predominant microbe invasion route is through an endotracheal tube [32].

A successful biological balance between microbe and host is the usual situation in the normal lung, but this relationship can be disrupted easily under various conditions, such as a rapid and massive bacterial invasion that makes the pre-existing host defence inadequate and, thus, allows disease. A better understanding of the local defences of normal lungs in animal models should lead to improved treatments against invading microbes. Thus, the present study supports the hypothesis that inoculum dose should be included as a risk factor for pneumonia development. Additional studies to confirm this supposition appear warranted.

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