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Development of a Reproducible Model of Murine Pneumonia with Diverse Strains of Penicillin-Resistant *Streptococcus pneumoniae* (PRSP)

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ABSTRACT

Background: experimental murine pneumonia can be induced with most susceptible strains, but results with PRSP are much harder to reproduce. By optimizing in vitro growth of PRSP, we developed a reproducible murine model of pneumonia, useful to evaluate in vivo efficacy of antibiotics. Methods: we used 6 clinical strains of PRSP representing serotypes 19F, 9V, 14 and 6B as well as *S. pneumoniae* ATCC 49619 as standard strain. After optimization of culture conditions to obtain maximal growth of PRSP, neutropenic animals were infected by nasal instillation of 50 µL log-phase bacteria. MPF Udea:ICR(CD-1) female mice were 6 weeks-old and weighted 25±2g when inoculated with $10^{7.7\text{-}8.4}$ $\log_{10}$ CFU/mL of each strain. Groups of 3 mice were sacrificed and their lungs removed at 1, 2, 4, 6, 12, 14, 16, 24, 36, and 48 h after infection in order to determine the dynamics of bacterial infection by appropriate homogenization, dilution, serial plating, and culture on TSA 5% blood agar. A second model, in which 50% of the inoculum was replaced by 10% porcine mucin, was similarly evaluated. Results: culture optimization led to growth of 9-10 $\log_{10}$ CFU/mL, avoiding autolysis. One hour after infection, mice had 6.20-7.79 $\log_{10}$ CFU/g of lung, a count that decreased to 3.6-7.6 $\log_{10}$ CFU/g at 17±5 h (nadir) and then increased up to 6.8-9.6 $\log_{10}$ CFU/g at 42±6 h (zenith), giving a net growth of 2.0-3.2 logs in 25 h. Histopathology confirmed pneumonia in all animals with all PRSP strains, but most mice recovered from the infection after 5 days. Mucin enhanced the virulence of all PRSP strains by transforming the model into a uniformly lethal pneumonia by 29 to 79 hours, with bacterial dissemination to all vital organs. Conclusion: reproducible induction of PRSP pneumonia was attained with diverse clinical strains. Virulence enhancement with 10% porcine mucin allowed a lethal pneumonia model.

INTRODUCTION

Streptococcus pneumoniae leads the etiology of acute respiratory tract infection and invasive disease, killing each year more than a million children under 5 years of age. Although this organism was susceptible to penicillin for almost half a century, the past two decades have witnessed an alarming increase in the number of strains resistant to this and other antibiotics. In Colombia, penicillin resistance among pediatric invasive isolates increased from 10 to 56% between 1994 and 2001. Although excellent animal models of pneumonia and sepsis are available for penicillin-susceptible strains, models of pneumonia with PRSP lack reproducibility, mainly because PRSP strains display low virulence in rodents. In the present study, we addressed this particular problem to obtain a model of PRSP pneumonia that is simple, practical, reliable and reproducible for in vivo evaluation of antibiotics. Fundamental for success with this model was the development of a supplemented culture medium to optimize bacterial growth in the infecting inoculum, and its further modification to enhance virulence and induce an infection closely resembling pneumococcal pneumonia in humans.

MATERIALS AND METHODS

Bacteria and culture media: eight invasive strains of *S. pneumoniae* tested the reproducibility of this pneumonia model; *S. pneumoniae* ATCC 49619 was included as a reference penicillin-resistant strain. These isolates belong to a nationwide collection administered by *Instituto Nacional de Salud* in Bogotá, Colombia; all came from CSF or blood of sick patients, and included serotypes 19F, 9V, 14, and 6B; 4 were penicillin-resistant, 2 penicillin-intermediate, and 2 penicillin-susceptible. Pneumococcal strains were kept frozen at -70ºC in skim milk and resuscitated as needed. Previous to animal experiments, we optimized culture conditions for PRSP under different combinations and found a three-phased (Phases 0, 1 and 2) culture method that produced an inoculum large and young enough to cause pneumonia in the mice before activation of autolysis mechanisms [1]. **Susceptibility tests:** MIC and MBC were determined by broth microdilution following CLSI and ASM guidelines for penicillin, vancomycin and ceftriaxone. **Animals:** 6 week-old, female, murine pathogen free (MPF) Swiss mice from the strain Udea:ICR(CD-1) weighting 23-27 g were rendered neutropenic by two intraperitoneal injections of cyclophosphamide (Cytoxan®, BMS, Princeton, NJ) 4 days (150 mg/kg) and 1 day (100 mg/kg) before infection. The animals were maintained and cared in accordance with international requirements and were provided sterile food and vitaminized water ad libitum. **Murine virulence:** to see if successful induction of pneumonia was dependent on murine virulence, we determined such trait by 3 successive passes of each strain, inoculating ~10^7 CFU in the thighs of immunocompetent male mice of the same strain, age and weight of those employed in the pneumonia model. Bacteria were quantified 24 h later in the thighs, lungs, liver, kidneys and spleen to verify local growth and dissemination capacity of each strain; furthermore, we designed a virulence scale based in clinical signs of dissemination (local infection, edema, sepsis, or death within 24 h), grading dead mice with 4+, sick mice with signs of systemic infection with 3+, mice with local but not systemic signs of infection with 2+, and healthy mice with 1+. **Experimental pneumonia:** mice were first anesthetized by 0.1 mL intramuscular injection of a mixture containing respectively 100+10 mg/kg ketamine (Ketalar®, Parke-Davis, Quito, Ecuador) and xylazine (Rompun®, Bayer, Sao Paulo, Brazil), then inoculated by intranasal instillation of 50 µL bacterial suspension from Phase 2, and finally suspended vertically for 10 minutes hanging from their incisors on a nylon string. Infected mice were sacrificed in groups of 3 animals at 1, 2, 3, 4, 5, 6, 12, 18, 24, 32, 40, and 48 h after infection to determine the dynamics of bacterial growth in the lung; another group of 3 mice was left to evaluate survival 120 h after infection. Once the nadir and the zenith of bacterial growth in the lung were established, additional time-points included 14, 16, 21, 30, 36, 42, 60, and 120 h after infection. **Histopathology studies:** lungs were removed at the time-point defined as the zenith of bacterial growth, fixed in 10% formalin buffer, sectioned, and stained with hematoxilin-eosin and Gram for microscopic confirmation of the pneumonia process.

RESULTS

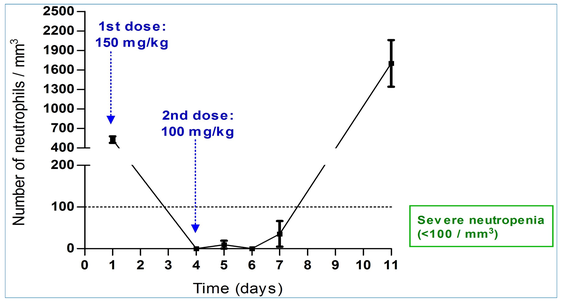
Mouse virulence varied widely among PRSP strains and was not correlated with the pattern of susceptibility to penicillin or the degree of human virulence, as judged by the serotype and the specimen from which the different strains were isolated (Table 1). Also, low virulence in immunocompetent mice did not predict failure to induce pneumonia with this model (see below).

Table 1. Microbiological characteristics of PRSP strains employed in the animal model

S. pneumoniae Strain	Strain Serotype	Diagnostic Specimen	MIC/MBC (mg/L)			Mouse Virulence
			Penicillin	Ceftriaxone	Vancomycin	
INS-E611	6B	Blood	2.00/2.00	1.00/1.41	0.35/0.35	++++
INS-E674	14	Blood	2.00/2.00	1.00/1.00	0.25/0.25	+
INS-E676	14	CSF	1.00/1.41	1.00/1.41	0.35/0.70	+
INS-E678	14	CSF	0.12/0.12	0.03/0.03	0.25/0.25	+
INS-E682	6B	CSF	0.01/0.01	0.01/0.01	0.25/0.25	++++
INS-E683	9V	CSF	1.00/1.00	0.35/0.35	0.35/0.35	++
INS-E684	14	CSF	1.00/1.00	0.50/0.50	0.25/0.25	++
INS-E685	1	Blood	0.01/0.01	0.01/0.01	0.25/0.25	+
ATCC 49619	19F	Sputum	0.46/0.46	0.06/0.12	0.33/0.44	+++

The duration of agranulocytosis is critical for this model. Figure 1 shows that our protocol for mouse immunosuppression was successful at rendering the animals absolutely neutropenic by the day of intranasal instillation of *S. pneumoniae*, but this effect lasted only 4 days. It implies that pneumonia should be firmly established in that period, and that mortality should be assessed not later than day 4 after infection if selected as an endpoint [2].

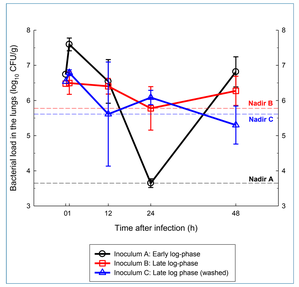
Figure 1. Neutrophil count obtained from 6 week-old Swiss Udea:ICR(CD-1) MPF mice after immunosuppression with 2 intraperitoneal injections of cyclophosphamide (n=10 female mice)



We also found that early log-phase bacteria must be inoculated to the neutropenic animals in order to induce pneumonia. Bacteria in early log-phase started at 7.02 $\log_{10}$ CFU per mouse (50 µL inoculum), decreased to a nadir 24 h later (3.65 ± 0.12 $\log_{10}$ CFU/g), and reached the zenith at 48 h (6.18 ± 0.43 $\log_{10}$ CFU/g), with a net growth of 3.16 $\log_{10}$ CFU/g between hours 24 and 48. On the contrary, late log-phase bacteria started at 6.39 $\log_{10}$ CFU per mouse, but grew only 0.49 $\log_{10}$ CFU/g between hours 24 (nadir: 5.78 ± 0.62) and 48 (zenith: 6.27 ± 0.42 $\log_{10}$ CFU/g). This incapacity of late log-phase bacteria to grow in lung tissue did not change by washing the cells and replacing their supernatant by sterile saline: each mouse was inoculated with 7.05 $\log_{10}$ CFU, but bacterial growth in lung tissue did not take place (Figure 2).

RESULTS (CONT.)

Figure 2. Impact of inoculum growth phase on virulence of *S. pneumoniae* INS-E611 in the lungs of neutropenic mice after intranasal instillation



Pneumonia was effectively established with all PRSP strains tested (INS-E611, E674, E683, E684, and ATCC 49619) with an optimized early log-phase inoculum of 6.25 - 7.32 $\log_{10}$ CFU per mouse. The nadir and the zenith were observed respectively 12-18 and 36-42 hours after infection, with a growth of 1.51 to 3.08 $\log_{10}$ CFU/g. However, most mice cleared the lung infection and recovered their health without treatment after 120 hours. Lethal pneumonia was induced in 80-100% of the animals infected by adding 10% porcine mucin to the bacterial inoculum in a 1:1 proportion (final mucin concentration, 5%), and in most cases it also improved the net growth of PRSP in the lungs (Figure 3).

Figure 3. Growth dynamics of 5 PRSP strains in the lungs of neutropenic mice after intranasal instillation of a pure culture (blue squares and line) or a 1:1 mix of the same inoculum plus 10% porcine mucin (red circles and line)

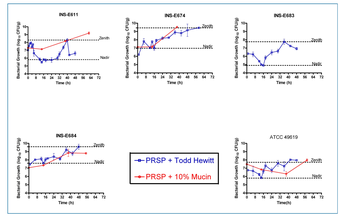


Table 2 shows the morphological study of lung tissues from agranulocytic mice with clinical signs of pneumonia sacrificed 38 hours after intranasal instillation of (A) 5% mucin, slight histological changes compatible with aspiration chemical pneumonitis, these animals never showed signs of disease; (B) *S. pneumoniae* INS-E611, lymphocytic interstitial and hemorrhagic pneumonitis, these animals looked sick, but recovered spontaneously 5 days after infection; and (C) *S. pneumoniae* INS-E611 plus 5% mucin, the lungs showed much more severe damage than that described for mice instilled with PRSP alone, with extensive septum edema, necrosis, and destruction of the alveolar structure. Polymorphonuclear infiltrate and subsequent lung consolidation is not seen in this model because the animals are rendered severely neutropenic with cyclophosphamide, as described above.

RESULTS (CONT.)

Table 2. Histopathology analysis of mice lungs after intra-nasal instillation with PRSP, mucin, or PRSP plus mucin

Lung Pathology	5% Mucin	PRSP	PRSP: 5% Mucin
Congestion	Mild	Severe	Severe
Atelectasia	A few foci with lymphocytes	Areas	Extensive, with proteinaceous material with mononuclear infiltrate
Alveolar distension	Absent	Focal	Plus focal alveolar edema
Septum edema with infiltrates of mononuclears and megacariocytes	A few lymphocytes	Focal, perivascular and peribrochiolar	Extensive, gross septum with lymphocyte and mononuclear infiltrate and perisvascular bacteria; dispersed bacterial groups in alveolar septa
Intra-alveolar hemorrhage or erythrocyte extravasation	Absent	Focal intra-alveolar hemorrhage	Extensive, with perivascular erythrocyte extravasation
Terminal bronchial epithelia	Unaffected	Unaffected	Ulcerated with lymphoid infiltrate and bacterial accumulation
Gram-Positive Cocci (GPC)	Absent	2 of 3 mice	Abundant GPC accumulated within fibrin, forming proteinaceous material, heavier around terminal bronchioli, but present intra-alveolar. No epithelial cells mixed with bacteria or in the middle of fibrin
Fibrin	Absent	1 of 3 mice	Abundant, forming focal hyaline membranes full of bacteria
Mononuclear infiltrate	Absent	Only in the septum	Focal, but multiple foci
Necrosis	Absent	Absent	Multiple foci of necrosis in alveolar septa, with severe changes in extension, number and concentration of GPC
Histopathology diagnosis	Non-specific lymphocytic interstitial pneumonitis	Lymphocytic interstitial pneumonitis with alveolar hemorrhage and lung atelectasis	Severe interstitial pneumonitis with abscess formation

CONCLUSION

- **The optimization of PRSP culture conditions allowed us to obtain an inoculum that was highly virulent for mice and less prone to autolysis. After direct nasal instillation to neutropenic mice, these bacteria consistently grew in the lungs more than 2 $\log_{10}$ CFU/g in 24 hours, causing pneumonia with all strains tested. Mixing the inoculum in a 1:1 proportion with 10% porcine mucin caused a progressive, lung destructive, uniformly fatal infection.**
- **The model with PRSP alone can be applied to antibiotic dose-effect studies which endpoint is the bacterial counts remaining in the lungs after treatment. Experiments with survival endpoints cannot be designed with such model, because animals recover spontaneously once the neutrophil count starts to normalize (day 5 after infection).**
- **The model with PRSP plus 10% mucin (1:1) is uniformly lethal with strains belonging to different serotypes and displaying different murine virulence, a useful characteristic for survival or bacterial counts endpoints.**
- **There is a relatively wide variation in the nadir points with this pneumonia model due to the different murine virulence of PRSP strains. Given that the nadir signals the time to start treatment in dose-effect experiments, it is always advisable to run a pilot experiment to determine the nadir and the zenith for each particular strain. Addition of mucin to the bacterial inoculum also helps to reduce this source of variation.**

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REFERENCES

1.Restrepo AV, Salazar B, Agudelo M, Rodriguez CA, Zuluaga AF, O Vesga. Optimization of culture conditions to obtain maximal growth of penicillin resistant *Streptococcus pneumoniae*. BMC Microbiol 2005; 5:34. Available from: URL: http://www.biomedcentral.com/content/pdf/1471-2180-5-34.pdf.

2.Zuluaga AF, Salazar BE, Rodriguez CA, Zapata AX, Agudelo M, Vesga O. Characterization of neutropenia induced by a simplified low-dose cyclophosphamide regimen in outbred mice. BMC Infectious Diseases. Submitted, November 2005.