Clinical Strains of *Pseudomonas aeruginosa* Secrete LasB Elastase to Induce Hemorrhagic Diffuse Alveolar Damage in Mice

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**Background:** Acute lung injury and acute respiratory distress syndrome (ALI/ARDS) are most often caused by bacterial pneumonia and characterized by severe dyspnea and high mortality. Knowledge about the lung injury effects of current clinical bacterial strains is lacking. The aim of this study was to investigate the ability of representative pathogenic bacteria isolated from patients to cause ALI/ARDS in mice and identify the major virulence factor.

**Methods:** Seven major bacterial species were isolated from clinical sputum and unilaterally instilled into the mouse airway. A histology study was performed to determine the lung injury effect. Virulence genes were examined by PCR. Sequence types of *P. aeruginosa* strains were identified by MLST. LC-MS/MS was used to analyse the bacterial exoproducts proteome. LasB was purified through a DEAE-cellulose column, and its toxicity was tested both in vitro and in vivo.

**Results:** *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus agalactiae, Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* were randomly separated and tested 3 times. Among them, gram-negative bacteria have much more potential to cause acute lung injury than gram-positive bacteria. However, *P. aeruginosa* is the only pathogen that induces diffuse alveolar damage, hemorrhage and hyaline membranes in the lungs of mice. The lung injury effect is associated with the excreted LasB elastase. Purified LasB recapitulated lung injury similar to *P. aeruginosa* infection in vivo. We found that this was due to the powerful degradation effect of LasB on the extracellular matrix of the lung and key proteins in the coagulation cascade without inducing obvious cellular apoptosis. We also report for the first time that LasB could induce DIC-like coagulopathy in vitro.

**Conclusion:** *P. aeruginosa* strains are most capable of inducing ALI/ARDS in mice among major clinical pathogenic bacteria tested, and this ability is specifically attributed to their LasB production.

**Keywords:** unilateral lung injury, LasB elastase, *Pseudomonas aeruginosa*, ALI/ARDS

**Introduction**

Acute lung injury (ALI) and its more serious form acute respiratory distress syndrome (ARDS) are major causes of death in the intensive care unit (ICU). Despite decades of efforts, the pathogenesis of ALI/ARDS remains unclear and an effective treatment has not been developed.1 Diffuse alveolar damage (DAD), hyaline membrane formation, alveolar bleeding and bilateral lung inflammation, which are typical pathological features found in ALI/ARDS patients, were poorly recapitulated in animal models. Meanwhile, although ARDS is defined as
a syndrome of similar clinical signs associated with damage to the alveolar–capillary membrane, heterogeneity in its etiology has been increasingly recognized.²

The major risk factor for ALI/ARDS is pneumonia caused by various microorganisms, accounting for nearly 60% of the cases.³ Pathogens such as bacteria evolve rapidly in response to environmental stress for better adaptation and survival.⁴⁻⁶ Compared to the type strains or standard strains preserved and studied in the laboratory for decades, clinical isolates of many bacteria currently exhibit significant differences in phenotype, drug resistance and invasiveness.⁷⁻⁹ However, the virulence of this “new generation” of bacteria in causing lung injury is unknown and has not been comparatively explored.

According to the report of the China Antimicrobial Surveillance Network (CHINET) 2019 and 2020, 6 species, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae and Escherichia coli, have become the most commonly found bacteria in the lung, accounting for more than 70% of the nearly 100,000 strains separated from clinical respiratory specimens nationwide. Are these pathogenic bacteria equally virulent? This has led us to hypothesize that some bacteria may be more harmful in the lung than others, leading to ALI or even ARDS. As a member of CHINET,¹⁰ the bacterial prevalence in our hospital is a representative part of the status in China. Thus, we isolated the 6 major bacterial species stated above and Streptococcus agalactiae (commonly found in the neonatal ICU) from daily sputum samples. Since the most relevant features of ALI/ARDS in animal models are rapid onset (within 24 h) and histological evidence of tissue injury,¹¹ our research has focused on the pathological manifestation of the lungs of mice within 24 h post treatment. To better visualize the damage, we applied a unilateral lung injury model by inoculating bacteria specifically into the left lung of the animal while keeping their right lungs intact as the control. This method relieved dyspnea, allowed mice to survive until the intended time and avoided systemic influences such as cytokine storm.

Materials and Methods
Ethical Statement
All experiments referring to the use of animals in this study were approved by the Institutional Animal Care and Use Committee of Shanghai Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-F-2018-047). All animal protocols were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used and their suffering.

Animals
Pathogen-free C57BL/6 mice (female, 10 weeks old, 21±1 g) and SD rats (female, 6–8 weeks old, 200±10 g) were purchased from Sippr-BK Laboratory Animal Co. Ltd, Shanghai. All animals were accommodated at the Model Animal Research Center of Xinhua Hospital in a specific pathogen-free animal facility under constant temperature and humidity, with sufficient qualified food and water for 1 week before use.

Unilateral Intratracheal Instillation
In mice, we selected the left lung for bacterial or exoproducts administration. Meanwhile, the right lung was set as the control lung within each individual. Unilateral lung intubation and instillation are described in the Supplemental Information.

Lung Histopathology
Mice were sacrificed 24 h post infection. The lung was inflated with 4% paraformaldehyde fixative under constant pressure of 15 to 25 cm H₂O via the trachea.¹² The lung was embedded in paraffin block for tissue sections (5 μm), and hematoxylin and eosin (HE) staining or TUNEL staining was subsequently performed. The ALI score was performed as described.¹¹ In the modified ALI score, we substituted the item “Neutrophils in the interstitial space” with “Red blood cells in the alveolar space” to better differentiate the severity of lung injury resulting from P. aeruginosa exoproducts.

Terminal Deoxynucleotidyl Transferase-dUTP Nick-End Labeling (TUNEL) Analysis
Cellular apoptosis in the lung tissues was detected using a TUNEL staining kit (Roche, Switzerland) according to the manufacturer’s instructions. Briefly, the paraffin sections were dewaxed to hydration. Proteinase K liquid was added to incubate the sections for 30 mins at 37°C and then washed away. TUNEL reagent (TdT: dUTP=2:29, mixed) was added and incubated with the sections for 120 minutes at 37°C.
The sections were washed with PBS and subsequently counterstained with DAPI. The stained sections were observed and evaluated using an Olympus IX 70 inverted fluorescence microscope (Olympus, Japan).

Clinical Isolates of Major Bacteria Species
Between April and July 2020, 7 major bacterial species were nonspecifically separated from the clinical sputum specimen (part of the routine hospital laboratory procedure): Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae and Streptococcus agalactiae were isolated according to morphology, confirmed by mass spectrometry and stored at −70°C. Details of the enrichment culture are described in the Supplemental Information.

P. aeruginosa Strains and Exoproducts
A total of 8 P. aeruginosa strains were isolated from 8 clinical respiratory tract specimens between August and September 2020. Pa 1–6 was named according to the separation time. A mucoid strain was named Pa M, and a strain used in earlier experiments was Pa J (isolated in July). The growth medium for routine harvesting of P. aeruginosa exoproducts was Columbia agar supplied with 5% sheep blood (90 mm, Comagal, Shanghai). The entire agar surface was inoculated with a cotton swab. Plates were incubated at 36±1°C for 3 days. Bacteria and exoproducts on the plate were dissolved in sterile NS (5 mL per plate), scratched off and collected. Bacterial cells were removed after centrifugation at 3200 × g for 30 min and further cleared by 0.22 μm filters. Then, the exoproducts acquired were concentrated by ultracentrifugal filters (10 kDa cutoff value, Amicon Ultra, Merck) at 3000 × g for 30 min. The protein level of the exoproducts was measured through the BCA method. All exoproducts were adjusted to the same protein concentration by adding sterile normal saline (NS).

PCR and MLST
Virulence genes were detected by PCR. The primer sequences used are listed in Table S4, and details and results are given in the Supplemental Information.

Gelatin Zymography
Gelatin zymography was performed as reported by Kessler et al. Details are described in the Supplemental Information.

Fibrinogenolytic and Fibrinolytic Activities and Thrombin Degradation Assay
Proteolysis activity was tested on bovine fibrinogen, fibrin and thrombin. Details are given in the Supplemental Information.

Protease Assays
P. aeruginosa exoproducts and purified LasB were tested for elastase activity using the elastin-Congo red (Sigma, US) method. For exoproducts inhibition assay, protease inhibitors cocktail (PIR) composed of 17 mM AEBSF, 2.5 μM Aprotinin, 1 mM Bestatin, 0.1 mM E64, 0.1 mM Leupeptin in DMSO with 8.5 mM EDTA added (Beyotime Biotechnology, Hangzhou) was used to preincubated with 1mg/mL exoproducts at room temperature for 30 min.

Proteomic Analysis of Exoproducts
Exoproducts from different strains were separated by 10% polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. The whole lane or certain protein bands of interest were excised from the gel and processed by mass spectrometry (LTQ Orbitrap Velos Pro, Thermo Finnigan) coupled to liquid chromatography as previously described. Mascot 2.3 software was used for data analysis.

Purification of LasB
The protease was purified from exoproducts by ammonium sulfate precipitation followed by DEAE-cellulose chromatography. Further details are given in the Supplemental Information.

Cytotoxicity Assay
THP-1 cells (TIB 202, ATCC) were seeded in 96-well plates with 1640 medium containing 10% fetal bovine serum. Purified LasB at a concentration of 100 μg/mL, Pa 4 exoproducts at a concentration of 200 μg/mL or Pa 4 bacteria at a concentration of 7.5×10^7 CFU/mL were added to the wells. Cytotoxicity was measured by LDH release 4 hours post incubation using LDH cytotoxicity assay kits (Beyotime Biotechnology, Hangzhou) according to the manufacturer’s instructions.

Hemostasis Function
To examine the effect on hemostasis function, 20 μg LasB was added to 2 mL blood (1.8 mL blood + 0.2 mL sodium citrate anticoagulant) driven from healthy volunteers and
incubated for 35 min at room temperature. The samples were then subjected to automatic hemostasis tests by ACL TOP (550 CTS, Werfen, US).

Statistics
The data are expressed as the mean ± standard error of the mean. Statistical analysis was performed in GraphPad Prism 8 software using one-way or two-way t-tests or log rank tests as appropriate. Statistical significance is indicated as (*P < 0.05; **P < 0.005; ***P < 0.0005). Data are representative of at least three independent experiments.

Results
Mice Model of Unilateral Lung Injury
We used a unilateral lung injury model in this study through tracheotomy and intubation into the left lung of the mice. We verified the method through LPS instillation. CT reconstruction (PINGSHENG, Shanghai) in Figure 1A shows that instillation of 30 μL of 5 mg/mL LPS successfully induced consolidation of the whole left lung in 4 hours.

This animal model was initially designed to see if heavy unilateral lung injury would provoke bilateral alveolar damage through systemic impacts such as cytokine storm, whereas we could find no such phenomenon in mice tested with lipopolysaccharide, acid, porcine elastase or mechanical ventilation, even if severe inflammation and animal death were observed (unpublished data). The untreated lungs of unilaterally injured mice exhibited no difference from the lungs of intact normal mice (Figure S1A).

Clinically Isolated Gram-Negative Bacteria Induced More Severe Lung Injury Than Gram-Positive Bacteria
We first tested the major gram-negative bacterial species, which together accounted for 60% of all clinical respiratory isolates. Mice were instilled with 25 μL bacterial suspensions (2.25×10⁷ CFU) of Klebsiella pneumoniae, Acinetobacter baumannii, Escherichia coli or Pseudomonas aeruginosa. As shown in Figure 1B, alveolar injury occurred in all groups within 24 h. Lung tissue injury caused by K. pneumoniae was noted because a large number of leukocytes accumulated in the interstitial tissue, while the alveolar space was filled with mucus. However, the mucus was later proven to be hypermucoviscous K. pneumoniae bacteria instead of protein debris or edema from the hosts (Figure S1C). Lungs infected with A. baumannii and E. coli both triggered neutrophil accumulation, which was patchy in the A. baumannii group but more diffused in the E. coli group. However, no hyaline membrane, red blood cell effusion or septal thickening was found in either group. Strikingly, the 3 independently isolated P. aeruginosa strains all caused hemorrhage, severe hyaline membrane formation, neutrophilic infiltration, parenchymal edema and proteinaceous debris deposits in the infected lungs, fitting the histological change of DAD (Figure 1B).

Next, to study the lung injury effect of gram-positive bacteria in vivo, 2.25×10⁷ CFU of S. aureus, S. agalactiae or S. pneumoniae suspended in 25 μL PBS was instilled into the left lung of mice. Much to our surprise, all mouse groups remained healthy 24 h post inoculation. Lung histology found no obvious alveolar damage (Figure 1C). Considering that these daily isolated gram-positive bacteria may have relatively low virulence, we further tested a hypervirulent mucoid serotype 3 S. pneumoniae stored earlier, of which we had to reduce the bacteria number to 2.25×10⁵ CFU to keep half of the inoculated mice live to the 24 h endpoint. However, no visible alveolar damage occurred, even though 50% of mice died or developed sepsis and multiple organ infection within 24 h (Figure S1B). A large number of neutrophils were found, but the accumulation was limited to the perivascular space of lung arteries (Figure 1C). These results showed that irrelevant to their virulence, the major gram-positive bacteria tested evoked bronchial pneumonia-like lung injury rather than the DAD found in ALI/ARDS.

We then compared the ALI score caused by each species (Figure 1D). Generally, gram-negative bacteria achieved much higher scores than gram-positive bacteria. P. aeruginosa acquired the highest score, indicating its great potency in ALI/ARDS pathogenesis. Thus, we isolated more strains of P. aeruginosa to analyze their injury mechanism. The baseline characteristics of the patients from whom the strains were separated are provided in Table 1. The strains are different in morphology (Figure S3) and have different multilocus sequence typing profile (Table S3).

Hypervirulent P. aeruginosa Strains Infected Unilaterally Leads to Bilateral DAD
Eight P. aeruginosa strains, Pa 1–6, Pa M and Pa J, were stilled unilaterally in mice, and survival curves were established. Quality control strain ATCC27853 was used as a comparison. As shown in Figure 2A, Pa 4, 5, and 6 are the most pernicious. Pa 3 was the least virulent among clinical isolates, while ATCC27853 was the least virulent
in all strains. Pa 2 was less toxic than Pa 4, 5, and 6 (p=0.003) but more toxic than Pa 1, 3, M and J (p<0.0001).

Mice instilled with Pa 2 and Pa 4, 5, and 6 all died within 24 h. They also achieved higher ALI score (Figure 2B).

Meanwhile, pink frothy sputum and bloody sputum which resemble the symptoms of ALI/ARDS patients, were seen from the nose and mouth in 50% of the animals (Figure 2E and F). Autopsy found that Pa 4, 5, and 6 caused a similar pathological change, prominent with lung hemorrhage,
DAD, neutrophil infiltration and septal thickening (Figure 2D). To our surprise, 87% of mice infected unilaterally with these 3 strains developed bilateral lung injury (Figure 2C), which is an important clinical feature of ARDS patients but was poorly recapitulated in animals before. However, in the *P. aeruginosa* uninoculated lungs, DAD was mostly observed around the bronchus or bronchia instead of the alveoli, indicating that it was likely caused by aspiration of the bloody sputum or bacteria from the infected lung instead of systemic influence.

### Exoproducts from *P. aeruginosa* Reproduced Hemorrhagic DAD Caused by Bacterial Infection

We examined the virulence genes (Table S1), drug-resistance and pyocyanin production of all 8 *P. aeruginosa* strains (Table S2), however, they did not show a clear relation with the virulence. Since all 8 *P. aeruginosa* strains tested were positive for the T1SS and T2SS genes but not the T3SS genes, we collected their extracellular products and examined virulence in vivo and...
in vitro. As shown in Figure 3A, the exoproducts (1 mg protein/mL) reproduced the typical histologic change of *P. aeruginosa* lung infection. The gross appearance of the left lungs showed different severities of injury, which was largely alleviated by proteinase inhibitor cocktail with EDTA added. The modified ALI score clearly shows that exoproducts from Pa 4, 5, and 6 are the strongest in inducing ALI, while Pa 3 is the weakest (Figure 3C). This tendency is consistent with the virulence of the corresponding bacteria (Figure 2A). To our surprise, when the concentration of the exoproducts was elevated to 4 mg protein/mL, it not only caused severe hemorrhage and DAD in all animal groups (n=3 mice, 8 group) but also the death of 14 mice in merely 3 hours. Autopsy found tension pneumothorax and lung compression in these mice. In contrast, surviving mice showed emphysematous alveolar destruction without lung consolidation (Figure 3B).

Interestingly, exoproducts lost the ability to cause alveolar hemorrhage or hyaline membranes after heating at 100°C for 10 mins (Figure S2A). We then found that the extracted bacterial composition of *P. aeruginosa* could not provoke hemorrhage or hyaline membranes either (Figure S2B). These findings suggest that *P. aeruginosa* mainly injured the lung through heat labile extracellular products, which were most likely proteases instead of heat stable substances, such as DNA or LPS.

*P. aeruginosa* induced cell apoptosis is the most widely studied cell death in ALI/ARDS. To determine whether and which pulmonary cell apoptosis was provoked, TUNEL labeling was used to stain *P. aeruginosa* bacteria or exoproducts injured lung tissue (Figure 3D and E). To our surprise, although both caused severe DAD, the exoproducts only induced apoptosis of 5.19% pulmonary cells compared to the 71.7% apoptosis rate by live bacteria. This result revealed that cell damage was present but probably not essential for the appearance of DAD in *P. aeruginosa* infection.

Consequently, we infer that hemorrhagic lung injury may have resulted from the destruction of extracellular matrix and disturbance of hemostasis. Collagen and elastin are the major protein components of the pulmonary extracellular matrix and are essential for physiological function and the initiation of blood coagulation. First, we applied gelatin zymography to visualize the degradation of collagen by exoproducts (Figure 3F). All exoproducts from *P. aeruginosa* exhibited multiple bright bands on zymography, especially hypervirulent strains Pa 4, 5 and 6. Most exoproducts showed a major band at the same level at 120 kDa, while Pa 2 manifested as a major band near 80 kDa. The activity was largely inhibited by EDTA. We then measured the elastin lytic activity of each exoproducts using the elastin-Congo red method (Table 2). Exoproducts exhibited different elastinolytic activities. The level has a tendency to be positively related to the ALI score of exoproducts and the virulence of the corresponding *P. aeruginosa* strains. Apparently, exoproducts from *P. aeruginosa* are powerful in destroying pulmonary structural compositions, especially Pa 4, 5 and 6.

As fibrinogen and thrombin are the two most important protein factors in coagulation, their degradation could have contributed to hemorrhagic lung injury. Thus, we examined the proteolytic ability of exoproducts. As shown in Figure 3G, all 8 exoproducts from *P. aeruginosa* strains hydrolyzed fibrinogen efficiently. We used several protease inhibitors and found that fibrinogen lysis was most significantly suppressed by the MMP inhibitor Ilomastat. Freshly formed fibrin clots were digested by all exoproducts as well (data not shown). The exoproducts also exhibited a strong ability to hydrolyze thrombin, as shown in the thrombin degradation assay (Figure 3H). The effect was partially inhibited by EDTA (EDTA usage was much larger due to the Ca$^{2+}$ ions contained in commercially acquired thrombin). These findings suggest that virulence effectors in the exoproducts of *P. aeruginosa* are most likely matrix metalloproteases.

### Proteomic Analysis of Exoproducts and Purification of the Virulent Protease LasB

*P. aeruginosa* exoproducts were then subjected to SDS-PAGE to analyze the secretory proteome (Figure 4A). LC-MS/MS analysis found that the most abundant protein secreted by Pa 4, 5 and 6 at 35 kDa is LasB elastase. Relative LasB expression in the exoproducts of 8 *P. aeruginosa* strains fits their lung damage ability (Figure 4B). The intense band appearing at a molecular mass of 70 kDa from less virulent strains also caught our attention, which, however, was found to be catalase or acetylated catalase. Meanwhile, no LasA was detected from Pa 2 or Pa 4 exoproducts.

Taken together, the results of LC-MS/MS and gelatin zymography indicated that strain Pa 4 could be used to culture and harvest LasB. LasB was separated from the exoproducts of Pa 4 by DEAE-cellulose chromatography.
As shown in the elution profile (Figure 4C), the only active enzyme was eluted between 0.1 M to 0.25 M NaCl (fractions 5–15). Fraction 10 was collected and concentrated to 2 mg/mL protein. The purity of protease was verified by SDS-PAGE as a single band at 35 kDa, which was confirmed as LasB elastase (Figure 4D). Purified LasB exhibited an increase in elastase-specific activity (Table 2). As shown previously on zymography (Figure 3), Pa 2 exoproducts presented a clear band at 80 kDa, different from the rest. However, LC-MS/MS
Table 2 Elastase Activity of Exoproducts Measured by Elastin-Congo Assay

| Elastase activity (U) | Pa 1 | Pa 2 | Pa 3 | Pa 4 | Pa 5 | Pa 6 | Pa M | Pa J | Purified LasB |
|----------------------|------|------|------|------|------|------|------|------|--------------|
|                      | 1.59 | 3.23 | 0.57 | 12.5 | 12.6 | 15.1 | 3.33 | 2.18 | 90.96        |

analysis found no proteases other than LasB (Figure 4E). Marquart et al reported the same phenomenon. Some studies point out that this might be due to differences in polymer conformation, since aggregation of LasB monomers to different molecular weight polymer forms is commonly found on gelatin zymograms.

LasB Elastase Provoked Hemorrhagic DAD Through Degradation of the Alveolar Matrix and Key Proteins in the Coagulation Cascade

Figure 5A presents the histological changes in the lungs of mice after being instilled unilaterally with 3 μg LasB for 24 h. Significant DAD, hemorrhage, hyaline membrane and emphysematous alveolar destruction were formed in the left lung. The histopathological change is consistent with the injury caused by *P. aeruginosa* bacteria and exoproducts. Lung injury could be alleviated by EDTA and to a greater extent by MMP inhibitor ilomastat (Figure 5B). Additionally, we found 70% of the mice died in 3 hours from tension pneumothorax when LasB usage was higher than 6 μg (n=10).

LasB showed high activity on gelatin zymography, which was manifested as a single clear band at a molecular weight of 120 kDa, thus proving that it is powerful for cleaving collagen (Figure 5C). Similar to the exoproducts, LasB exhibited a strong capability to hydrolyze fibrinogen and thrombin (Figure 5D). The process was attenuated by both EDTA and ilomastat but not by TLCK. To examine the overall effect of LasB on clotting, we incubated LasB with citrate-anticoagulated healthy human blood. As shown in Figure 5E, LasB drastically reduced fibrinogen levels, increased FDP and D-dimer levels and elongated thrombin time (TT), causing DIC-like coagulopathy in vitro. However, it did not change the indicators of the endogenous coagulation pathway, such as APTT and PT, or affected the activity of antithrombin (AT) or plasminogen (PA). We then studied the fibrinogen lysis effect in vivo by injecting LasB intravenously into rats (2 mg/kg). Significant fibrinogen reduction was also observed 1 h post injection (Figure 5F). However, no difference was detected in thrombin time or other indicators.

Finally, we applied TUNEL staining to look for possible cellular apoptosis. Similar to the exoproducts, no significant difference in pulmonary cell apoptosis was observed between LasB-damaged lungs and intact lungs (Figure 5G and H), meaning that LasB has little cytotoxicity toward pulmonary cells in vivo. Additionally, the THP-1 cytotoxicity assay found that purified LasB was not apparently virulent to cells, unlike the bacterium (Figure 5I).

Discussion

Pneumonia is the major risk factor for ALI/ARDS. In most patients suspected or diagnosed with pneumonia, infection of bacteria is most commonly found, sometimes even secondary to virus or fungus infection. In this study, we examined the capability of 7 major pathogenic bacteria isolated from patients to induce ALI in mice. The 3 major gram-positive bacterial species did not cause obvious alveolar injury. Although *S. pneumoniae* is considered a primary pathogen of ARDS, we found that it mainly induced inflammation in the periarterial space. In comparison, gram-negative bacteria are much more deleterious. *P. aeruginosa* was found to be the most destructive, rapidly inducing hemorrhagic DAD, pink frothy sputum and bilateral lung infiltration similar to the clinical features of ALI/ARDS patients in animals. In this study, we isolated 8 clinical strains of *P. aeruginosa* that are different in morphology, pigment production, T3SS genes and many aspects. LasB production was the only factor we found that correlated in proportion with virulence in the lung. A high dosage of LasB even caused pneumothorax, bullae formation and death in mice in merely 3 hours.

*P. aeruginosa* is an opportunistic pathogen ubiquitously present in the environment. For more than 4 decades, it has held a nearly unshakable position in the rank order of pathogens causing ICU-related infections. The correlation of *P. aeruginosa* to high mortality of patients with hospital-acquired and ventilator-associated pneumonia (VAP) in the ICUs has aroused serious concerns. *P. aeruginosa* are known to have a plethora of virulence
Figure 4 Proteomic analysis of exoproducts and purification of the virulent protease LasB (A) SDS-PAGE of exoproducts from 8 *P. aeruginosa* strains. (B) Relative LasB expression of 8 *P. aeruginosa* strains. *P < 0.05; **P < 0.005; (C) elution profile of LasB protease on DEAE 52 cellulose column. (D) Verification of LasB purity by SDS-PAGE. 10, purified LasB (Fraction 10); 15, impurities (Fraction 15); 35, impurities (Fraction 35); CE, crude enzyme solution. (E) Amino acid sequence alignment of LasB from Pa 4, Pa 2 and PAO1.

Abbreviation: RFU, relative fluorescence unit.
factors, represented by T1SS, T2SS, T3SS and quorum sensing systems. Currently, T3SS, with its four toxins ExoU, ExoT, ExoS and ExoY, is recognized as the most important virulence factor of *P. aeruginosa* responsible for alveolar epithelial injury in patients. Strains possessing the exoU gene are especially hazardous. However, among the 8 strains tested, we found that the exoU+ genotype Pa 2 is not as virulent as the exoU- genotype Pa 4, 5 or 6 in causing DAD, indicating that LasB (T2SS) is probably more important than T3SS in ALI/ARDS pathogenesis.

Exoproduits of *P. aeruginosa* are composed of multiple pathogenic agents, such as alginate, LPS, flagellum,
proteases from T2SS, exotoxins, pyocyanin, siderophores, hemolysins, and phospholipases, and each plays an important role in lung infection. Among them, we discovered that hemorrhagic DAD was mainly caused by proteases. However, _P. aeruginosa_ secretes at least six proteases, including alkaline protease (AprA), elastase A (LasA), elastase B (LasB), large exoprotease A (LepA), protease IV (P1V) and Pseudomonas small protease (PASP). Many studies have reported that _P. aeruginosa_ proteases cause severe hemorrhage or lung injury in animals, but the effect was never specifically attributed to LasB elastase, as opposed to LasA elastase or other proteases. Moreover, most studies investigated the role of LasB in lung infection using defined deletion or insertion mutants of the LasB gene, which may not be assigned unequivocally to the impact of LasB elastase. Instead, our team successfully purified 35 kDa LasB elastase from a virulent _P. aeruginosa_ strain and elucidated the direct causative effect of LasB on alveolar-capillary structure and clotting cascade in vivo and vitro. Furthermore, we ruled out the contribution of LasA, LepA, PASP and alkaline protease using a combined method of LC-MS/MS, SDS-PAGE and zymography. We applied a TLCK inhibition assay to further exclude PIV contamination. Our study strongly suggests that LasB is the most important lung injury protease from _P. aeruginosa_.

_P. aeruginosa_ LasB elastase (pseudolysin) is a neutral Zn²⁺-dependent metalloprotease with both proteolytic and elastolytic activities that can be inhibited by metal chelators, such as EDTA or MMP inhibitors. It has been shown to degrade a vast array of host proteins, including structural components, including elastin, collagen, laminin, immune factors such as IgA, IgG, TNF-α, IFN-γ, IL-2, IL-6, and α1-antiprotease, and proteins involved in coagulation functions, such as fibrin, fibrinogen and thrombin. In this study, we validated that LasB is highly efficient in destroying structural proteins in the lung and key enzymes in clotting. Interestingly, although LasB per se is insufficient to provoke histopathological changes almost identical to DAD in ALI/ARDS, it did not show obvious cytotoxicity to pulmonary cells in vivo or to THP-1 cells in vitro. Jose et al reported similar results that moderate LasB protease is nontoxic to Hep2 cells. However, in real _P. aeruginosa_ infection, not only DAD but also extended cell apoptosis were observed. Since bacteria are highly cytotoxic but not LasB and exoproducts, we suppose that cell death might be caused by T3SS or T6SSs, which are highly efficient but require direct bacteria-to-cell contact. Interestingly, we found that the most abundant protein secreted by less virulent _P. aeruginosa_ strains was catalase, which probably explained their clinical prevalence because catalase offers strong protection over bacteria from H₂O₂ and antibiotic-mediated killing.

FDPs and D-dimers are the products of degraded fibrinogen or fibrin. We discovered for the first time that LasB could significantly increase FDP and D-dimer levels and decrease fibrinogen levels in citrate-anticoagulated blood, causing DIC-like clotting dysfunction in vitro. Interestingly, LasB did not disrupt the anticoagulant process, which helped to explain the heavy bleeding in the lung. LasB administered intravenously to rats decreased fibrinogen levels in vivo as well. However, elongation of thrombin time was not found. We are not sure if this was caused by the strong compensatory capacity of the SD rats or by other reasons yet. More exploration will focus on this matter in the future.

In addition to _P. aeruginosa_, many bacteria secrete extracellular proteases, such as collagenase from _Clostridium histolyticum_ or thermolysin from _Bacillus thermoproteolyticus_. _S. aureus, S. agalactiae_ and _S. pneumoniae_ are also well known for their hyaluronidase production, which might have led to their unique pathological pattern in lung infection. Proteases enable bacteria with extraordinary power to break barriers and spread. However, due to limited time and resources, we were not able to test and identify the virulence factors of all clinically isolated bacteria. Additionally, as our research focused on the major respiratory pathogenic bacterial species in China, it is not clear whether our results are fully applicable to other areas. Thus, further studies are needed.

Zupetic et al recently proved that _P. aeruginosa_ elastase activity was common in ICU respiratory isolates representing 75% of samples and was associated with increased 30-day mortality. In this study, we demonstrated that _P. aeruginosa_ is the queen of pulmonary pathogens. Among all its exoproducts, LasB per se is sufficient and essential to elicit ARDS-like symptoms and histological changes in animals by inducing matrix degradation and coagulopathy. Monitoring LasB levels might be helpful in predicting the outcome of ALI/ARDS patients in the future.

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**Author Contributions**

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, agreed to the submitted journal, and agree to be accountable for all aspects of the work.

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**Disclosure**

The authors declare no conflicts of interest in this work.

**References**

1. Fan E, Brodie D, Slutsky AS. Acute respiratory distress syndrome: advances in diagnosis and treatment. JAMA. 2018;319(7):698–710. doi:10.1001/jama.2017.21907

2. Silva PL, Pelosi P, Roccio PRM. Personalized pharmacological therapy for ARDS: a light at the end of the tunnel. Expert Opin Investig Drugs. 2020;29(1):49–61. doi:10.1080/13543784.2020.1699531

3. Bellani G, Laffey JG, Pham T, et al. Epidemiology, patterns of care, and mortality for patients with acute respiratory distress syndrome in intensive care units in 50 countries. JAMA. 2016;315(8):788–800. doi:10.1001/jama.2016.0291

4. Tanner JR, Kingsley RA. Evolution of salmonella within hosts. Trends Microbiol. 2018;26(12):986–998. doi:10.1016/j.tim.2018.06.001

5. Defaine F, Faure M, Michiels J. Fighting bacterial persistence: current and emerging anti-persister strategies and therapeutics. Drug Resist Updat. 2018;38:12–26. doi:10.1016/j.drup.2018.03.002

6. Bauer TT, Ewig S, Rodloff AC, et al. Acute respiratory distress syndrome and pneumonia: a comprehensive review of clinical data. Clin Infect Dis. 2006;43(6):748–756. doi:10.1086/506430

7. Cepas V, Soto SM. Relationship between virulence and antibiotic resistance among gram-negative bacteria. Antibiotics. 2020;9(10). doi:10.3390/antibiotics9100719

8. Maunders EA, Triniman RC, Western J, et al. Global reprogramming to molecular types. Infection Immun. 2019;87(6):2275–2281. doi:10.1128/iai.02142.x

9. Duan Y, Gao H, Zheng L, et al. Antibiotic resistance and virulence of Extraintestinal Pathogenic Escherichia coli (ExPEC) vary according to molecular types. Front Microbiol. 2020;11:598305. doi:10.3389/fmicb.2020.598305

10. Hu F, Guo Y, Yang Y, et al. Resistance reported from China antimicrobial surveillance network (CHINET) in 2018. Eur J Clin Microbiol Infect Dis. 2019;38(12):2275–2281. doi:10.1007/s10096-019-03673-1

11. Matute-Bello G, Downey G, Moore BB, et al. An official American thoracic society workshop report: features and measurements of experimental acute lung injury in animals. Am J Respir Cell Mol Biol. 2011;44(5):725–738. doi:10.1165/rcmb.2009-0210ST

12. Hsia CCW, Hyde DM, Ochs M, et al. An official research policy statement of the American thoracic society/European respiratory society: standards for quantitative assessment of lung structure. Am J Respir Crit Care Med. 2010;181(4):394–418. doi:10.1164/rccm.200809-1522ST

13. Kessler E, Safrin M. Elastinolytic and proteolytic enzymes. Methods Mol Biol. 2014;1149:135–169. doi:10.1007/978-1-4939-0473-0_13

14. Wiśniewski JR, Zougman A, Nagaraj N, et al. Universal sample preparation method for proteome analysis. Nat Methods. 2009;6(5):359–362. doi:10.1038/nmeth.1322

15. Rubenfeld GD, Thompson T, Ferguson ND, et al. Acute respiratory distress syndrome: the berlin definition. JAMA. 2012;307(23):2526–2532. doi:10.1001/jama.2012.5669

16. Deshpande R, Zou C. Pseudomonas aeruginosaa induced cell death in acute lung injury and acute respiratory distress syndrome. Int J Mol Sci. 2020;21(15):5356. doi:10.3390/ijms21155356

17. Marquart ME, Caballero AR, Chonnawang M, et al. Identification of a novel secreted protease from Pseudomonas aeruginosaa that causes corneal erosions. Invest Ophthalmasl Vis. 2005;46(10):3761–3768. doi:10.1177/04-1483

18. Caballero AR, Moreau JM, Engel LS, et al. Pseudomonas aeruginosaa protease IV enzyme assays and comparison to other Pseudomonas proteases. Anal Biochem. 2001;290(2):330–337. doi:10.1006/abio.2001.4999

19. Rawson TM, Moore LS, Zhu N, et al. Bacterial and fungal coinfection in individuals with coronavirus: a rapid review to support COVID-19 antimicrobial prescribing. Clin Infect Dis. 2020;71(9):2459–2468. doi:10.1093/cid/ciaa530

20. Bakalez LO. Viral-bacterial co-infections in the respiratory tract. Curr Opin Microbiol. 2017;35:30–35. doi:10.1016/j.mib.2016.11.003

21. Mannes GP, Boersma WG, Baur CH, Postmus PE. Adult respiratory distress syndrome (ARDS) due to bacteremic pneumococcal pneumonia. Eur Respir J. 1991;4(4):503–504.

22. Trautmann M, Lepper PM, Haller M. Ecology of Pseudomonas aeruginosaa in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. Am J Infect Control. 2005;33(Suppl 5):S41–S49. doi:10.1016/j.ajic.2005.03.006

23. Botelho J, Grosso F, Peixe L. Antibiotic resistance in Pseudomonas aeruginosaa - mechanisms, epidemiology and evolution. Drug Resist Updat. 2019;44:100640. doi:10.1016/j.drup.2019.07.002

24. Sawa T, Shimizu M, Moriyama K, et al. Association between Pseudomonas aeruginosaa type III secretion, antibiotic resistance, and clinical outcome: a review. Crit Care. 2014;18(6):668. doi:10.1186/s13054-014-0668-9

25. Sauvage S, Hardouin J. Exoproteomics for Better understanding Pseudomonas aeruginosaa virulence. Toxins. 2020;12(9):571. doi:10.3390/toxins12090571

26. Minkin G, Barum J, Rosenberg B, et al. In vivo studies with the partially purified protease (elastase) from Pseudomonas aeruginosaa. Infect Immun. 1970;2(5):583–589. doi:10.1128/iai.2.5.583-589.1970

27. Elsen S, Huber P, Bouillot S, et al. A type III secretion negative Extraintestinal Pathogenic Escherichia coli (ExPEC) vary according to molecular types. Front Microbiol. 2020;11:598305. doi:10.3389/fmicb.2020.598305

28. Williams JC, Lucas BJ, Knee C, Renzetti M, Donahue J. Acute lung injury induced by Pseudomonas aeruginosaa elastase in hamsters. Exp Lung Res. 1992;18(1):155–171. doi:10.3109/109286092092020658

29. Gray L, Kregar A. Microscopic characterization of rabbit lung damage produced by Pseudomonas aeruginosaa proteases. Infect Immun. 1979;23(1):150–159. doi:10.1128/iai.23.1.150-159.1979

30. Wolz C, Hellstern E, Haug M, et al. Pseudomonas aeruginosaa LasB mutant constructed by insertional mutagenesis reveals elastolytic activity due to alkaline proteinase and the LasA fragment. Mol Microbiol. 1991;5(9):2125–2131. doi:10.1111/j.1365-2958.1991.tb02142.x

31. Everett MJ, Davies DT. Pseudomonas aeruginosaa elastase (LasB) as a therapeutic target. Drug Discov Today. 2021. doi:10.1016/j.drudis.2021.02.026

32. Qu Y, Olonisakin T, Bain W, et al. Thrombospondin-1 protects against pathogen-induced lung injury by limiting extracellular matrix proteolysis. JCI Insight. 2018;3(3). doi:10.1172/jci.insight.96914
33. Cowell BA, Twining SS, Hobden JA, et al. Mutation of lasA and lasB reduces Pseudomonas aeruginosa invasion of epithelial cells. Microbiology. 2003;149(8):2291–2299. doi:10.1099/mic.0.26280-0

34. Jose D, Manjusha K, Jose S, Mohandas A, Singh IS. Purification and characterization of highly active LasB protease from pseudomonas aeruginosa MCCB 123. Indian J Exp Biol. 2017;55:303–310.

35. Khakimova M, Ahlgren HG, Harrison JJ, et al. The stringent response controls catalases in Pseudomonas aeruginosa and is required for hydrogen peroxide and antibiotic tolerance. J Bacteriol. 2013;195(9):2011–2020. doi:10.1128/jb.02061-12

36. Duarte AS, Correia A, Esteves AC. Bacterial collagenases – a review. Crit Rev Microbiol. 2016;42(1):106–126. doi:10.3109/1040841X.2014.904270

37. Hynes WL, Walton SL. Hyaluronidases of gram-positive bacteria. FEMS Microbiol Lett. 2000;183(2):201–207. doi:10.1111/j.1574-6968.2000.tb08958.x

38. Matsumoto K. Role of bacterial proteases in pseudomonal and serratial keratitis. Biol Chem. 2004;385(11):1007–1016. doi:10.1515/bc.2004.131

39. Zupetic J, Peñaloza HF, Bain W, et al. Elastase activity from Pseudomonas aeruginosa respiratory isolates and ICU mortality. Chest. 2021. doi:10.1016/j.chest.2021.04.015