Impact of Multidrug Resistance on Experimental Empyema by *Pseudomonas aeruginosa*

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**Key Words**
Empyema • Multidrug resistance • *Pseudomonas aeruginosa* • Apoptosis

**Abstract**

**Background:** *Pseudomonas aeruginosa* is a cause of infections of the lower respiratory tract among patients with chronic lung disorders. It is questionable whether virulence of this species may be influenced by multidrug resistance (MDR). **Objectives:** To define the impact of MDR in experimental lung infection. **Methods:** Experimental empyema was induced in rabbits by MDR (group A, \( n = 16 \)) and by susceptible isolates (group B, \( n = 10 \)). Pleural fluid was sampled for quantitative culture and estimation of cell apoptosis and of tumor necrosis factor-alpha (TNF\( \alpha \)) and malondialdehyde (MDA). Survival was recorded. Cytokine production was stimulated in U937 monocytes by samples of pleural fluid. Whole blood of rabbits was incubated with the isolates; induction of apoptosis was assessed. **Results:** Survival of group A was prolonged compared to group B. This was accompanied by lower bacterial counts of the inoculated pathogens in pleural fluid and in the lungs of group A compared with group B. Early apoptosis of neutrophils of pleural fluid of group A was lower compared with group B. Pleural fluid concentrations of TNF\( \alpha \) and MDA did not differ between the groups. Cytokine production by U937 monocytes after stimulation with pleural fluid was greater in group B than in group A. The susceptible isolate induced apoptosis of neutrophils in vitro at a greater rate than the MDR isolate. **Conclusions:** Experimental empyema by susceptible *P. aeruginosa* is accompanied by greater mortality compared with MDR *P. aeruginosa*. This phenomenon may be attributed to the different growth pattern of the pathogens or to their interaction with the innate immune system.

**Introduction**

The human opportunistic pathogen *Pseudomonas aeruginosa* is a major cause of pulmonary damage and mortality in patients with cystic fibrosis and bronchiectasis [1] and a relatively common cause of hospital-acquired pneumonia [2]. The airways are colonized by this species, which is often multidrug resistant (MDR). MDR usually occurs after previous administration of antimicrobials. It is believed that when a bacterial species becomes MDR, it adopts altered virulence properties [3]. In a previous experimental study by our group, MDR isolates of *P. ae-
P. aeruginosa stimulated a lower burden of proinflammatory cytokines by human monocytes compared with susceptible isolates. Mice inoculated with the MDR isolates survived longer than those inoculated with susceptible isolates [4].

It is questionable if these results coming from a murine animal model of monomicrobial infection may also apply for acute lower respiratory tract infections (LRTIs) in humans. P. aeruginosa causes acute exacerbations of bronchiectasis and cystic fibrosis. If inflammatory responses stimulated in LRTIs depend on the antimicrobial resistance of the implicated pathogen, the impact on therapeutics may be considerable. Mice are not a suitable model for such studies where the host’s response should be intensively monitored. Induction of empyema seems a reasonable experimental approach. In the event of empyema, neutrophils and mononuclear cells migrate in large numbers from the systemic circulation in the pleural space. With this model, the viability of neutrophils and mononuclear cells to contain the offending pathogens should be closely monitored. As a consequence, the study of empyema with intense monitoring necessitates larger animals than mice, i.e. rabbits.

Apoptosis of neutrophils is crucial for the physical course of any LRTI. This is of major importance for pneumonia caused by P. aeruginosa which induces apoptosis of neutrophils [5, 7]. It is also important for isolates that express the type III secretion system [7]. Apoptosis of neutrophils in the lung is greatly influenced by the inflammatory process per se. It has recently been reported that tumor necrosis factor-alpha (TNFα) produced during acute inflammation of the airways inhibits apoptosis of neutrophils in the lung and contributes to the vicious cycle of inflammation [8].

(1) Linking the antimicrobial resistance of P. aeruginosa with the innate immune responses (based on previous findings [4]), and (2) the complex interaction of P. aeruginosa with the apoptotic process, we studied an experimental model of empyema by P. aeruginosa in rabbits. The impact of multidrug resistance of P. aeruginosa on survival was examined and findings were correlated with intense monitoring of the innate immune responses.

Animals and Methods

Animals

A total of 37 white New Zealand male rabbits of a mean (± SD) weight of 3.12 ± 0.34 kg was studied. The study received a permit from the Veterinary Directorate of the Prefecture of Athens according to the Greek legislation in conformance with the 160/1991 Council Directive of the EU. Animals were housed in single metal cages and had access to tap water and standard balanced rabbit chow ad libitum. Room temperature ranged between 18 and 22°C, relative humidity between 55 and 65% and the light/dark cycle was 6 a.m./6 p.m.

Bacterial Isolates

Two isolates of P. aeruginosa derived from different patients with LRTIs were applied: isolate 26 (susceptible to antibiotics), and isolate 2 (MDR to ticarcillin/clavulanate, piperacillin, cefazidime, imipenem, meropenem, ciprofloxacin and amikacin, as defined by well-defined criteria [9]).

The isolates were stored as multiple aliquots in skim milk (Oxoid Ltd, London, UK) at −70°C. Before each experiment, one aliquot was thawed and cultured onto MacConkey agar (Becton Dickinson, Cockeysville, Md., USA). Single colonies were suspended in Mueller-Hinton broth (Oxoid) and incubated for 12 h at 37°C in a shaking water bath. The inoculum was then washed 3 times with NaCl 0.9% and it was adjusted to 5 × 10⁷ CFU/ml by 0.5 of the McFarland climax. This allowed discarding of endotoxin-free supernatants.

Induction of Empyema

Empyema was induced by a modification of the protocol described in Strahilevitz et al. [10]. The rabbits were initially sedated by the intramuscular injection of 25 mg/kg of ketamine and 5 mg/kg of xylazine. The sixth right intercostal space was recognized after percussion on the lateral right chest wall. A puncture was performed under aseptic conditions with a 16-gauge needle. The needle was inserted 3 mm deep and it was applied as a driver for the sequential insertion of a plastic catheter tapped into its end. If the tip of the catheter followed respiratory movements, this assured that the catheter was correctly placed. The catheter was then secured subcutaneously. Then, 0.3 ml of turpentine (Sigma, St. Louis, Mo., USA) was injected through the catheter. The next day, 0.3 ml of the prepared inoculum containing 1 × 10⁷ CFU was injected through the catheter. The inoculum applied for bacterial challenge was determined after preliminary experiments with 6 rabbits.

Animal Follow-Up

Animals were placed in their cages. On days 1, 3, 5 and 7 after bacterial challenge, 1 ml of pleural fluid was sampled from the inserted catheter; 0.5 ml were collected into EDTA-coated tubes for flow cytometry and 0.5 ml into pyrogen-free tubes for quantitative cultures and for the measurement of proinflammatory mediators (Vacutainer, Becton Dickinson, Cockeysville, Md., USA). After centrifugation, pleural fluid was kept refrigerated at −70°C until assayed.

The primary clinical endpoint was 7-day survival. To this end, survival was recorded every 12 h for 7 days. After death, autopsy was performed; animals remaining alive after 7 days of follow-up were sacrificed by the intravenous administration of sodium thiopental. Under sterile conditions, segments of the lower lobe of the right lung were taken and placed into separate sterile plastic containers for quantitative cultures.

Evaluation of Apoptosis

Samples were incubated for 15 min in the dark with the protein Annexin V at the fluorocolor fluorescein isothiocyanate (emis-
1,1,3,3-tetramethoxypropane (Merck). All determinations were run through the EPICS XL/MSL flow cytometer (Beckman Coulter Co., Miami, Fla., USA). Cells staining positive for both Annexin V and PI were considered late apoptotic. Separate gating for neutrophils and lymphocytes was performed by their characteristic FS/SS scattering.

**Measurements of TNFα and MDA**

TNFα was measured by a bioassay on the L929 fibrosarcoma cell line, as already described [11, 12]. Briefly, confluent cells were thoroughly washed with Hanks' solution and harvested with 0.25% trypsin/0.02% EDTA (Biochrom AG, Berlin, Germany). Cells were centrifuged, resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM of glutamine (Biochrom AG) and distributed into a 96-well cell culture plate at a density of 1 × 10⁵ cells/well. The final volume of fluid in each well was 0.05 ml. After incubation for 2–3 h at 37°C at 5% CO₂, 0.06 ml of pleural fluid or of standard dilutions of known concentrations of human TNFα (Sigma, range 5.75–375.00 pg/ml) were added into each well followed by 0.05 ml of a 0.3 mg/ml dilution of cycloheximide (Sigma) to inhibit any de novo protein synthesis. After overnight incubation, the supernatant of each well was discarded by aspiration and 0.1 ml of a 0.5 mg/ml methylene blue solution in methanol 99% was added. After 10 min, the dye was removed and the wells were washed thoroughly 3 times with 0.9% NaCl. The wells were left to dry and remnants of the dye in each well became soluble by the addition of 0.01 ml of 50% glacial acetic acid (Merck, Darmstadt, Germany). Optical density in each well was read at 495 nm (Hitachi Spectophotometer, Tokyo, Japan) against blank wells and control wells without added serum. Concentrations of TNFα were estimated by the reduction of the optical density of the control wells by unknown samples applying a standard curve generated by standard concentrations. All determinations were performed in quadruplicate. The interday variation of the assay was 13.75%.

MDA was measured as already described [13]. Briefly, a 0.1-ml aliquot of each sample was mixed with 0.9 ml of trichloroacetic acid 20% (Merck) and centrifuged at 12,000 g for 10 min. The supernatant was removed and incubated with 2 ml of thiobarbituric acid 0.2% (Merck) for 60 min at 90°C. After centrifugation, a volume of 10 µl of the supernatant was injected into a high-pressure liquid chromatography system (HPLC, Agilent 1100 Series, Waldbronn, Germany) with the following characteristics of elution: Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 µm) column under 37°C; mobile phase consisting of a 50 mM K₂PO₄ (pH 6.8) buffer and methanol 99% at a 60/40 ratio with a flow rate of 1 ml/min. fluorometric detection with signals of excitation at 515 nm and emission at 535 nm. The retention time of MDA was 3.5 min and it was estimated as µmol/ml by a standard curve created with 1,1,3,3-tetramethoxypropane (Merck). All determinations were performed in duplicate.

**Cultures of Pleural Fluid and Lung Tissue**

A 0.1-ml aliquot of pleural fluid was diluted 1:10 into sterile NaCl 0.9% 4 times consecutively. Another aliquot of 0.1 ml of each dilution was plated onto MacConkey agar (Becton Dickinson) and incubated at 35°C for a total period of 2 days. Plates were incubated at 35°C and viable colonies were counted into each dilution and multiplied by the appropriate dilution factor. The lower detection limit was 10 CFU/ml. The number of bacterial cells was expressed by their log₁₀ value. Segments of lung were weighted and homogenized by a mortar and pestle. Quantitative cultures were done as described for pleural fluid.

**Comparative Growth Rate of the Studied Isolates**

A 1 × 10⁶ CFU/ml log-phase inoculum of every isolate was incubated in 10 ml of Mueller-Hinton broth at 37°C in a shaking water bath. The inoculum was prepared by suspending single bacterial colonies in 3 ml of Mueller-Hinton broth (Becton Dickinson) for 12 h at 37°C and subsequent adjustment with 0.5 of the McFarland climax. At 0, 2, 4, 6 and 24 h of incubation, aliquots of 0.1 ml were removed and serially diluted 1:10 in sterile NaCl 0.9%. An aliquot of 0.1 ml of each dilution was plated onto MacConkey agar. The bacterial growth at each time of sampling was estimated after multiplying the number of grown colonies with the appropriate dilution factor and expressed by their log₁₀ value.

**Induction of Neutrophil Apoptosis**

Two milliliters of blood were sampled under aseptic conditions through venipuncture of the left ear vein of 5 rabbits. Aliquots of 0.4 ml of heparinized venous blood were distributed into wells of a 12-well plate; growth medium was added at a final volume of 2.4 ml. Growth medium was RPMI 1640 enriched with 10% FBS and 2 mM of glutamine. The final bacterial inoculum was 1 × 10⁵ CFU/ml. After incubation for 4 h at 37°C in a 5% CO₂ atmosphere, wells were centrifuged and the cell pellet was removed after treatment with 0.25% trypsin/0.02% EDTA. The rate of early-apoptotic neutrophils was assayed, as previously defined.

**Effect of Pleural Fluid on Cytokine Production**

Cells of the U937 human monocytic cell line were incubated with pleural samples from animals of groups A and B. Confluent cells were thoroughly washed with Hanks' solution and distributed in a 96-well plate at a density of 5 × 10⁵ cells per well at a volume of 100 µl in the growth medium. This growth medium was RPMI 1640 supplemented with 2 mM of glutamine; 100 µl of pleural fluid were added to each well. After incubation at 37°C for a period of 24 h, the plate was centrifuged for 8 min at 1,400 g. After centrifugation, aliquots of 0.1 ml of each well were transferred to 12-well plates and distributed into wells of a 12-well plate; growth medium was added at a final volume of 1 ml. After incubation for 4 h at 37°C, supernatants of the pleural fluid and lung tissue were added to each well. The plate was incubated at 37°C in a 5% CO₂ atmosphere. After 24 h, the plate was centrifuged for 8 min at 1,400 g at room temperature. Concentrations of TNFα, interleukin (IL)-1β and IL-10 were estimated in supernatants by an enzyme-immunoabsorbent assay (R&D, Minneapolis, Minn., USA). The lowest limits of detection were 16 pg/ml for TNFα, 20 pg/ml for IL-1β and 20 pg/ml for IL-10.

**Statistical Analysis**

Results of pleural fluid cultures and lung cultures and of the percentages of neutrophils in pleural fluid were expressed by their mean ± SD, those of apoptosis and of cytokine stimulations by their mean ± SE, and those of TNFα and MDA in pleural fluid and in serum by their median and range. Comparisons were done by the Mann-Whitney U test. Survival was estimated by Kaplan-Meier analysis; comparisons were done by the log-rank test. Any value of p < 0.05 was considered significant.
Results

Experimental empyema was induced by the MDR isolate 2 in 16 rabbits (group A) and by the susceptible isolate 2 in 10 rabbits (group B). Initial experimental design comprised induction of empyema at a 1:1 ratio in 20 rabbits. Since none of the first tested 10 rabbits assigned to group A died after 7 days, another 6 animals were assigned to group A to ascertain the result. One animal from group A with empyema by the MDR isolate 2 died (6.25%) compared with 4 animals from group B with empyema by the susceptible isolate 26 (40%). The survival of group A was prolonged compared to group B (fig. 1).

We tried to provide a mechanism explaining the difference in survival after challenge by the MDR isolate and by the susceptible isolate. Our hypothesis was that the observed difference may be related to different growth rates of the studied isolates and/or an effect on the innate immune response.

The in vitro growth rate of the 2 isolates studied did not differ (fig. 2). To define the existence of differences in bacterial growth rate, the number of bacteria in pleural fluid in the lung was estimated over follow-up. Counts of bacteria were greater in group B than in group A on days 1 and 5 (table 1). Mean ± SD log_{10} of bacteria in the lung were 4.19 ± 2.80 CFU/g for group A and 6.56 ± 0.98 CFU/g for group B (p = 0.006 between groups).

In order to define any effect on the innate immune response, concentrations of TNFα and of MDA (which is an index of lipid peroxidation) and cellular apoptosis were estimated in pleural fluid. No differences were found between the groups (table 1). On day 1, early apoptosis of neutrophils was greater in group B compared to group A (fig. 3).

Fig. 1. Survival after induction of experimental empyema. Experimental empyema was induced in 16 rabbits by the MDR isolate 2 (group A) and in 10 rabbits with experimental empyema by the susceptible isolate 26 (group B).

Fig. 2. Comparative in vitro growth over time of the 2 P. aeruginosa isolates studied, MDR isolate 2 and susceptible isolate 26.

Table 1. Quantitative cultures and concentrations of TNFα and of MDA of pleural fluid

<table>
<thead>
<tr>
<th>Time</th>
<th>Group A</th>
<th>Group B</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>log_{10} of bacteria (mean ± SD), CFU/ml</td>
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<tr>
<td>Day 1</td>
<td>4.12 ± 1.84</td>
<td>6.04 ± 1.07</td>
<td>0.031</td>
</tr>
<tr>
<td>Day 3</td>
<td>4.32 ± 1.98</td>
<td>5.79 ± 1.06</td>
<td>NS</td>
</tr>
<tr>
<td>Day 5</td>
<td>3.05 ± 1.11</td>
<td>5.04 ± 0.83</td>
<td>0.020</td>
</tr>
<tr>
<td>Day 7</td>
<td>3.56 ± 2.31</td>
<td>5.12 ± 1.11</td>
<td>NS</td>
</tr>
</tbody>
</table>

| TNFα (median, range), pg/ml |
| Day 1    | 17.67 (<5.75–4,515.69) | 14.08 (11.50–53.12) | NS    |
| Day 3    | <5.75                  | 29.10 (11.50–89.39)  | NS    |
| Day 5    | <5.75                  | 11.50 (11.50–45.66)  | NS    |
| Day 7    | 15.76 (<5.75–155.88)   | 11.50 (11.50–45.50)  | NS    |

| MDA (median, range), μmol/ml |
| Day 1    | 0.56 (0.09–5.45)       | 0.20 (0.09–1.12)     | NS    |
| Day 3    | 1.66 (0.50–5.18)       | 0.20 (0.19–7.35)     | NS    |
| Day 5    | 0.59 (0.46–3.66)       | 1.78 (0.22–7.05)     | NS    |
| Day 7    | 0.63 (0.28–4.28)       | 1.68 (0.51–8.87)     | NS    |

Experimental empyema was induced in 16 rabbits by the multidrug-resistant isolate 2 (group A) and in 10 rabbits with experimental empyema by the susceptible isolate 26 (group B). NS = Not significant.
We assumed that the observed differences may also be related to either a circulating factor in pleural fluid affecting innate immune responses or to a direct effect of the studied isolates in apoptosis. To study these hypotheses, cytokine production from U937 monocytes was stimulated by samples of pleural fluid collected from groups A and B and apoptosis of neutrophils was induced by the studied isolates in rabbit whole blood.

Concentrations of TNFα, of IL-1β and of IL-10 in the supernatants of U937 monocytes after stimulation with sampled pleural fluid are shown in figure 4. Stimulation with fluid sampled from rabbits infected by the susceptible isolate (group B) produced greater concentrations of all three cytokines compared with concentrations produced after stimulation with fluid sampled from rabbits infected by the MDR isolate (group A).

Induction of apoptosis of neutrophils after incubation of whole blood of healthy rabbits with the studied isolates is shown in figure 5. Early apoptosis by the susceptible isolate 26 was greater compared with the MDR isolate 2.

We tried to evaluate histopathology of the lung after challenge by both the isolates tested. To this end, histopa-
Histology of the lung was tested in 5 rabbits injected with turpentine into the pleural space and not challenged by whole bacteria; these rabbits were sacrificed 7 days after the injection of turpentine. Histological sections of the lungs stained by H&E showed mild infiltration by lymphocytes and by neutrophils (data not shown). These findings led to the assumption that histopathology of the lung should be evaluated neither in group A nor in group B, since turpentine may act as a confounder for the interpretation of results.

**Discussion**

Infections of the lower respiratory tract by *P. aeruginosa* are often problematic. They usually complicate intubated patients in intensive care units causing ventilator-associated pneumonias, or they generate LRTIs among patients with chronic lung disorders, mainly cystic fibrosis and bronchiectasis [14]. The MDR status of these pathogens creates difficulties about the correct choice of antimicrobials. Despite this difficulty, there is clinical speculation that MDR isolates lead to deterioration of the host at a slower rate and with more indolent clinical presentation compared with susceptible isolates [4]. This may in part be due to the virulence properties of susceptible and MDR isolates, and in part to the response of the host to susceptible and MDR isolates.

We report that in an experimental model of LRTIs and empyema caused by *P. aeruginosa*, survival was longer after challenge by an MDR isolate than after challenge by a susceptible isolate. The mechanisms explaining this phenomenon may either be related to the replication rate of the studied isolates or to their interaction with the innate immune system. Our findings indicate that both mechanisms may be present. Although both isolates studied had a similar growth rate in vitro, viable counts of the susceptible isolate in the lung and in the pleural fluid were greater than counts of the MDR isolate. This

**Fig. 4.** Concentrations of TNFα, interleukin (IL)-1β and IL-10 in the supernatants of U937 monocytes after stimulation with pleural fluid sampled at sequential time intervals (mean ± SE). Group A: 16 rabbits with experimental empyema by the MDR isolate 2. Group B: 10 rabbits with experimental empyema by the susceptible isolate 26. Comparisons between groups at the indicated time of sampling are shown as p values. NS = Not significant.

**Fig. 5.** Induction of neutrophil apoptosis after incubation of whole blood collected from 5 rabbits with one $1 \times 10^4$ CFU/ml inoculum of the isolates studied, MDR isolate 2 and susceptible isolate 26 (mean ± SE).
indicates that MDR isolates are slower to adapt to the environment of the host.

Our findings also support the notion that some of the differences in the virulence of MDR and susceptible *P. aeruginosa* are due to interactions with the innate immune system. Interactions may involve cytokine stimulation and induction of apoptosis. Stimulation data of U937 monocytes show that after infection, pleural fluid contains molecular patterns able to stimulate production of proinflammatory and anti-inflammatory cytokines. Cytokine release was greater after stimulation with pleural fluid sampled after infection with the susceptible isolate compared with cytokine release with pleural fluid sampled after infection with the MDR isolate. One limitation is that the exact nature of the molecular patterns contained in the pleural fluid is not defined. They may be constituents of the offending pathogens, animal cytokines or other animal danger signals.

This study also provided evidence which indicates that part of the difference in virulence between susceptible and MDR *P. aeruginosa* is due to the ability of isolates to induce apoptosis of neutrophils. Early apoptosis of neutrophils contained in the pleural fluid was greater in the event of infection by the susceptible isolate than in the event of infection by the MDR isolate. In vitro data of whole blood incubation of rabbits with the viable isolates indicates that susceptible isolate is a more potent inducer of apoptosis of neutrophils compared to the MDR isolate.

Apoptosis of neutrophils may be a clinically important mechanism for the persistence of *P. aeruginosa* in human tissue. Apoptosis of neutrophils is a key step in the resolution of acute inflammation. Inhibition of the apoptotic process may lead to the persistence of infection [7, 15]. In the light of this, many authors have tested the potential therapeutic efficacy of inhibitors of the apoptotic pathways for the management of experimental pneumonia. Le Berre et al. [16] used a broad-spectrum caspase inhibitor to influence lung fluid balance in experimental pneumonia by *P. aeruginosa* in rats. They found that this inhibitor decreased apoptosis in the lung and could, at least partially, modulate fluid transport. Similar results have been reported by Hotchkiss et al. [17] with the administration of caspase inhibitors in an animal model of pneumonia by *P. aeruginosa*.

The reported results have some similarities with previous findings of our study group in experimental sepsis in rats and in rabbits [3, 18]. In these previous studies, monomicrobial systemic infections were induced by intraperitoneal bacterial challenge in rats and by intravenous administration through a central catheter in rabbits. These models differ considerably with the model of localized infection performed via empyema in rabbits in our study. Our findings, however, coincide with those of the earlier studies where survival after challenge by MDR isolates was prolonged compared to survival after challenge by the susceptible isolates.

Two main limitations of this study should be reported. (1) The lack of application of groups with empyema induced after challenge with the bacterial vehicle. This may not influence the results since the final endpoint was de-

Table 2. Rate of apoptosis of neutrophils and of lymphocytes in the pleural fluid

<table>
<thead>
<tr>
<th>Time</th>
<th>Neutrophils</th>
<th></th>
<th></th>
<th>Lymphocytes</th>
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<tr>
<td></td>
<td>group A</td>
<td>group B</td>
<td>p</td>
<td>group A</td>
<td>group B</td>
<td>p</td>
</tr>
<tr>
<td>Annexin V(+) / PI(–) (mean ± SE), %</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>8.04 ± 4.08</td>
<td>34.45 ± 4.16</td>
<td>0.037</td>
<td>6.59 ± 2.37</td>
<td>17.80 ± 4.89</td>
<td>NS</td>
</tr>
<tr>
<td>Day 3</td>
<td>26.07 ± 10.67</td>
<td>25.29 ± 6.12</td>
<td>NS</td>
<td>10.37 ± 5.88</td>
<td>8.38 ± 2.19</td>
<td>NS</td>
</tr>
<tr>
<td>Day 5</td>
<td>12.98 ± 7.85</td>
<td>11.14 ± 3.40</td>
<td>NS</td>
<td>10.27 ± 2.31</td>
<td>6.58 ± 1.62</td>
<td>NS</td>
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<tr>
<td>Day 7</td>
<td>8.78 ± 5.79</td>
<td>3.86 ± 2.28</td>
<td>NS</td>
<td>10.89 ± 9.81</td>
<td>1.60 ± 0.38</td>
<td>NS</td>
</tr>
<tr>
<td>Annexin V(+) / PI(+) (mean ± SE), %</td>
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<tr>
<td>Day 1</td>
<td>5.05 ± 1.39</td>
<td>9.35 ± 5.12</td>
<td>NS</td>
<td>8.69 ± 4.15</td>
<td>4.38 ± 1.43</td>
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<tr>
<td>Day 3</td>
<td>3.03 ± 2.46</td>
<td>3.64 ± 0.86</td>
<td>NS</td>
<td>16.67 ± 9.32</td>
<td>9.11 ± 1.94</td>
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<tr>
<td>Day 5</td>
<td>32.24 ± 21.89</td>
<td>49.87 ± 27.51</td>
<td>NS</td>
<td>13.55 ± 5.39</td>
<td>26.62 ± 24.65</td>
<td>NS</td>
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<td>Day 7</td>
<td>31.66 ± 16.15</td>
<td>33.49 ± 19.64</td>
<td>NS</td>
<td>15.39 ± 12.75</td>
<td>23.22 ± 19.64</td>
<td>NS</td>
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</table>

Experimental empyema was induced in 16 rabbits by the multidrug-resistant isolate 2 (group A) and in 10 rabbits with experimental empyema by the susceptible isolate 26 (group B). NS = Not significant.
terminated after comparing group A with group B which used the same bacterial vehicle. (2) The recording of the survival for only 7 days, since death in some group A animals might supervene later. Even if this were the case, the results of the survival analysis would not be influenced. On the contrary, if animals were sacrificed later than 7 days, the estimation of lung bacterial growth would perhaps not be comparable between the 2 groups.

The presented results in an experimental setting of empyema reveal that infection by MDR *P. aeruginosa* is accompanied by prolonged survival compared with infection by susceptible *P. aeruginosa*. This phenomenon may be attributed either to the different growth pattern of the pathogens or to their interaction with the innate immune system of the host. These results contribute to our understanding of the pathogenesis of lung infections by MDR *P. aeruginosa*. They may also help explain the common clinical observation why death by susceptible isolates occurs early. In this context, prompt intervention is needed when systemic infection by one susceptible isolate is suspected.

**Financial Disclosure and Conflicts of Interest**

All authors declared no conflicts of interest related to this work.

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