



***Pseudomonas aeruginosa* biofilms in the lungs of Cystic Fibrosis Patients**

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Please refer to the original scientific article published by ***Pediatric Pulmonology*** for all the results and references included in this present article.

Introduction

Recent research indicates that chronic bacterial infections are caused by the ability of bacteria to organize themselves in microcolonies also called biofilms. In this state the bacteria are imbedded in a self produced protective matrix, often with surrounding inflammatory cells. Bacteria living in biofilms are very well protected against antibiotics and the host defense.

In the past, CF patients suffered from severe lung infections caused by *Haemophilus influenzae*, *Streptococcus pneumoniae*, and especially *Staphylococcus aureus*, resulting in high mortality in the early childhood. These bacteria have been treatable the last 30 years, but other bacteria such as *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* have become a serious problem. Today up to 80% of young adults suffering from CF are chronically infected with *P. aeruginosa*.

***P. aeruginosa* in general**

P. aeruginosa is an "opportunistic human pathogen". This means that the bacterium only infects individuals with a compromised host defense such as people with CF. The bacterium is a low virulent pathogen because its disease causing properties in the initial infection are few and the infection has a slow progress. In contrast, highly virulent bacteria, such as haemolytic streptococci and pneumococci, cause an acute infection with fever and inflammation.

P. aeruginosa can be isolated from most environments: soil, marshes, the sea, plants and mammal tissue. In hospital environments *P. aeruginosa* grows in moist reservoirs such as food, cut flowers, sinks, toilets, floor mops, dialysis equipment, and even in disinfectants. *P. aeruginosa* has become synonym with the lung infection of people suffering from CF. In CF lungs, *P. aeruginosa* exists as free single bacteria (planktonic) or in enclaves of bacteria, in which the bacteria grow as biofilms.

P. aeruginosa may become extremely slimy, a form called mucoid *P. aeruginosa*. This is caused by a genetic change of the bacterium (mutation), resulting in the release of large quantities of alginate (a kind of sugar). The change to mucoid strengthens its defense capacity against antibiotics and the host defense even further and may provoke inflammation.

Why a scientific demonstration of biofilms in CF

Since 1976 great success in treating CF patients, suffering from chronic *P. aeruginosa* lung infection, has been obtained by intensive treatment using high concentrations of antibiotics at the Copenhagen CF center. This was initially done by routinely i.v. anti- *P. aeruginosa* treatment for 14 days, every third month. Since 1987 daily inhalation of antibiotics was added. Before 1976 only 50% of the CF patients would survive 5 years of chronic *P. aeruginosa* lung infection. Today most survive for decades with chronic infection.

Despite intensive treatment of chronic *P. aeruginosa* infections the bacteria remain. The intensive treatment postpones and reduces the damage caused by the chronic infection, but can not eradicate it. During the chronic *P. aeruginosa* infection the CF patients experience a continuous degradation of lung tissue. This is caused in part by the infection and part by the inflammatory processes (the body's reaction to the infection). The consequence is a decline in the lung function, which is the primary cause of death for CF patients.

The present study was based on the following assumption (hypothesis): The intensive usage of antibiotics delays the damage of the chronic *P. aeruginosa* infection, but does not stop the bacteria from forming biofilms in the lungs. To test our hypothesis we have compared the lungs from two different groups of CF patients. Group 1: CF patients suffering from chronic *P. aeruginosa* infection treated intensively with antibiotics. Group 2: CF patients suffering from chronic *P. aeruginosa* infection NOT treated intensively with antibiotics.

The scientific work – material

Lungs in group 1 were obtained from 3 CF patients, age range: 30-42 years, having had lung transplantation due to their chronic *P. aeruginosa* lung infection. The patients have had chronic *P. aeruginosa* infections on average for 27 years when transplanted. The lungs were collected with acceptance of the patients as approved by the Danish Scientific Ethical Board.

For group 2 we used material from 11 CF patients who died from 1974 to 1978. Nine of the CF patients, age range: 3-20 years, died due to chronic *P. aeruginosa* lung infection. The duration of the chronic lung infection was on average 3.5 years (1 month – 8 years). At this time the mortality of CF patients was high at Rigshospitalet (University Hospital of Copenhagen). *P. aeruginosa* was not yet recognized as an important CF pathogen and the infection was only treated in case of severe clinical conditions. No regular maintenance therapy was used. Lungs for control were obtained from 2 CF patients who died at 15 and 18 years and did not suffer from *P. aeruginosa* infection. Lungs belonging to group 2 were collected at Rigshospitalet before the intensive antibiotic treatment regime was initiated in 1976 by one of the authors (NH), who conducted a medical research project involving lung autopsies for immune pathology and bacteriology.

Additionally, 77 recent sputum samples from chronically infected, present CF patients, age range: 1.5-49 years, was examined and compared to the biofilms produced by *P. aeruginosa* in the lower part of the lungs.

The scientific work – method

We used a species specific staining of the bacteria to identify and localize the bacteria in the lungs and sputum. In addition, we used a traditional non-specific stain, the Gram stain. In all the lungs, we examined all the relevant areas such as the bronchi, alveoli, inflamed tissue and healthy tissue.

The scientific work – Results

Sputum

P. aeruginosa was the only bacteria that could be isolated from the sputum samples. The mucoid *P. aeruginosa* dominated the samples though the non-mucoid was present as well. *P. aeruginosa* appeared both as single cells (planktonic) and as aggregates in the sputum (figure 1). The single bacteria were unevenly distributed and separated from the microcolonies. The microcolonies were of different sizes and shapes, and randomly distributed throughout the samples. Most of the microcolonies were surrounded by PMNs (white blood cells), but in a few cases PMNs were detected inside the aggregates (figure 1c). The aggregates bear resemblance to Gram-stained, mucoid *P. aeruginosa* colonies grown in the laboratory.

Lungs from the intensively treated chronically infected CF patients – group 1

The lung function of the explanted lungs was reduced to an extent that requires lung transplantation for the patient to survive. The bacteria were mainly localized in the conductive zone (the upper part of the lung with large and small bronchi, which function as pipes transporting air back and forth to the alveoli) (figure 2). All of the bacteria were imbedded in mucus plugs. The mucus plugs containing bacteria varied much in size and spatial orientation. The bacteria within the mucus were dominated by aggregates and only few planktonic bacteria were detected. Vast amount of PMNs surrounded the aggregates. The bacteria were not found adhering to the epithelial wall, demonstrating that the bacteria grew within the mucus and not in the lung

tissue. Using specific antibodies against alginate we observed a majority of mucoid *P. aeruginosa* aggregates intraluminally in the conductive zone together with few possible non-mucoid planktonic bacteria. *P. aeruginosa* was the only detected bacteria in this study.

In the respiratory part of the lungs (the lower part – the alveoli – where the gas-exchange with the blood occur) we saw both dead alveoli and air filled healthy alveoli (figure 2 G-I). Relatively few bacteria were detected in the respiratory part of the lungs as compared to the conductive part. We found both a few single planktonic cells and small aggregates in the respiratory zone, however the planktonic bacteria were always phagocytosed (eaten) by PMNs and the aggregated bacteria were closely surrounded by PMNs (figure 2 I).

Lungs from the non-intensively treated chronically infected CF patients – group 2

In group 2, the bacteria in the conductive zone were also found inside the mucus surrounded by an abundance of PMNs, with no bacteria adhering to the airway epithelia (figure 3).

In striking contrast to the explanted group 1 lungs, the alveoli of the respiratory zone were filled with aggregating bacteria and PMNs (figure 3 G and H). Most of the lung tissue was extensively destroyed probably caused by the heavy infection and inflammation.

Using the specific stain and antibodies against alginate we detected abundance of mucoid *P. aeruginosa* aggregates in these non-intensively treated chronically infected patients.

What can we learn from our work?

The present work underlines that the lung infection in CF patients is a biofilm based infection. We found *P. aeruginosa* as microcolonies embedded in alginate. Since alginate embedded, aggregated bacteria are considered a strong evidence for the presence of bacterial biofilms the aggregates detected in this study should be considered as biofilms.

The tissue damage during the chronic infection is due to an extensive, ongoing inflammatory response, which does not eradicate the bacteria, but instead persistently degrades the lung. In addition, though antibiotics fail to clear the bacteria completely, the survival in the Copenhagen CF Centre has significantly increased due to intensive anti- *P. aeruginosa* therapy. This intensive therapy builds on monthly examination including identification and susceptibility testing to antibiotics of all the isolated bacteria and fungi from sputum or lower respiratory tract secretions obtained by endolaryngeal suction. Until 1975, chronic *P. aeruginosa* infection was only treated during acute exacerbations (on an average one 2-week course per year). But after 1976 all chronically infected patients received elective 2-week courses of i.v. anti *Pseudomonas* antibiotics every 3 months. This treatment was supplemented with daily inhaled colistin since 1987 and daily oral azithromycin since 2001.

As an example one of the intensively treated patients has been treated with 114 two-week intravenous courses of all together 1 kg tobramycin, 10 kg beta-lactams and 1 kg of nebulized colistin before lung transplantation, but in spite of this, the biofilm growing bacteria survived.

This intensive antibiotic treatment at the Copenhagen CF center, may explain the nearly exclusive detection of *P. aeruginosa* in the samples, from in the conductive airways, investigated in this study.

Before the intensive antibiotic therapy, *P. aeruginosa* infected and destroyed the CF lungs faster. Our demonstration of the many massively infected alveoli of the non-intensively treated CF lungs suggests fast lung deterioration caused by to rapid bacterial spreading into the respiratory zone. Thus, the intensive antibiotic regime may suppress the bacteria, but does not eradicate them in the conductive zone, whereas the remaining respiratory zone is protected from massive biofilm infection for prolonged time.

These results suggest that the present intensive maintenance antibiotic therapy offers a better protection of the lungs of the CF patients than could be achieved before the aggressive maintenance therapy was initiated. The daily inhalation of antibiotics may suppress the bacteria in the conductive zone. The remaining relatively healthy respiratory zone may be protected from massive biofilm infection for prolonged time due to the intravenous antibiotics, administered repeatedly every 3 month. We are currently expanding these studies on all the explanted CF patients in Copenhagen. Similar studies in CF centers with less intensive antibiotic protocols would be interesting.

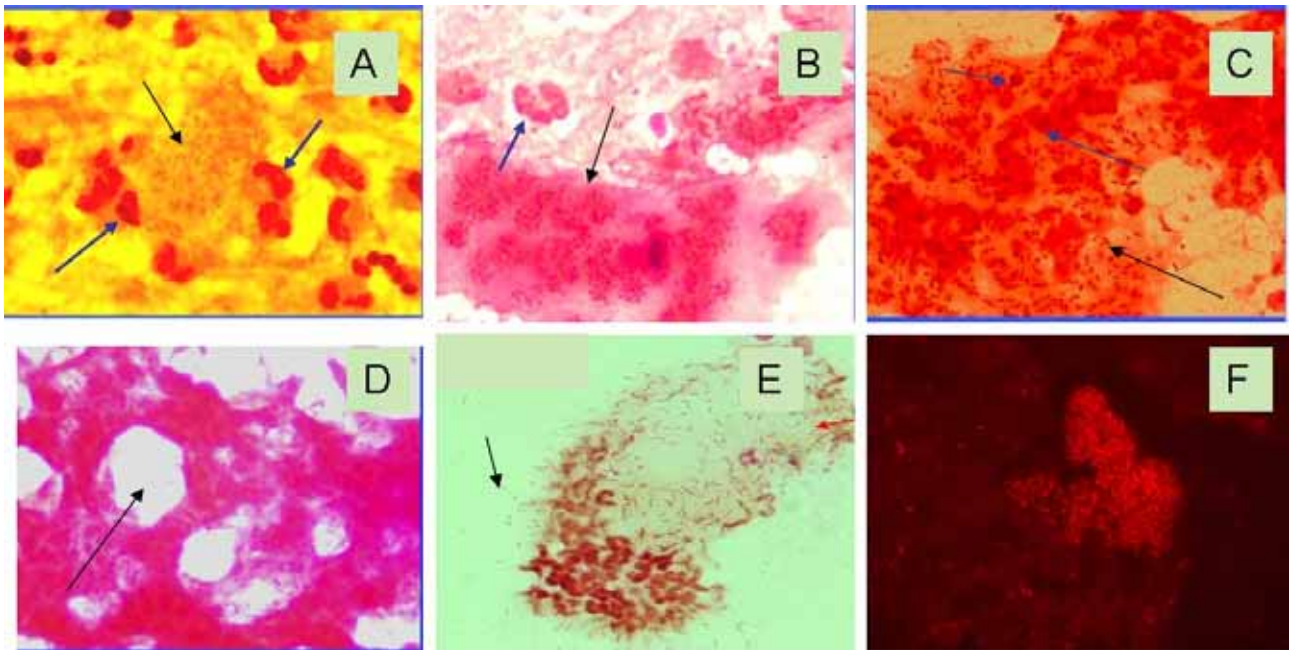


Figure 1. The diversity of *P. aeruginosa* biofilms in sputum. A-E). Visualization of *P. aeruginosa* biofilms and PMNs in sputum by Gram-staining. Blue arrows point to PMNs and black arrows point to bacteria. F). Specific staining of a *P. aeruginosa* microcolonies.

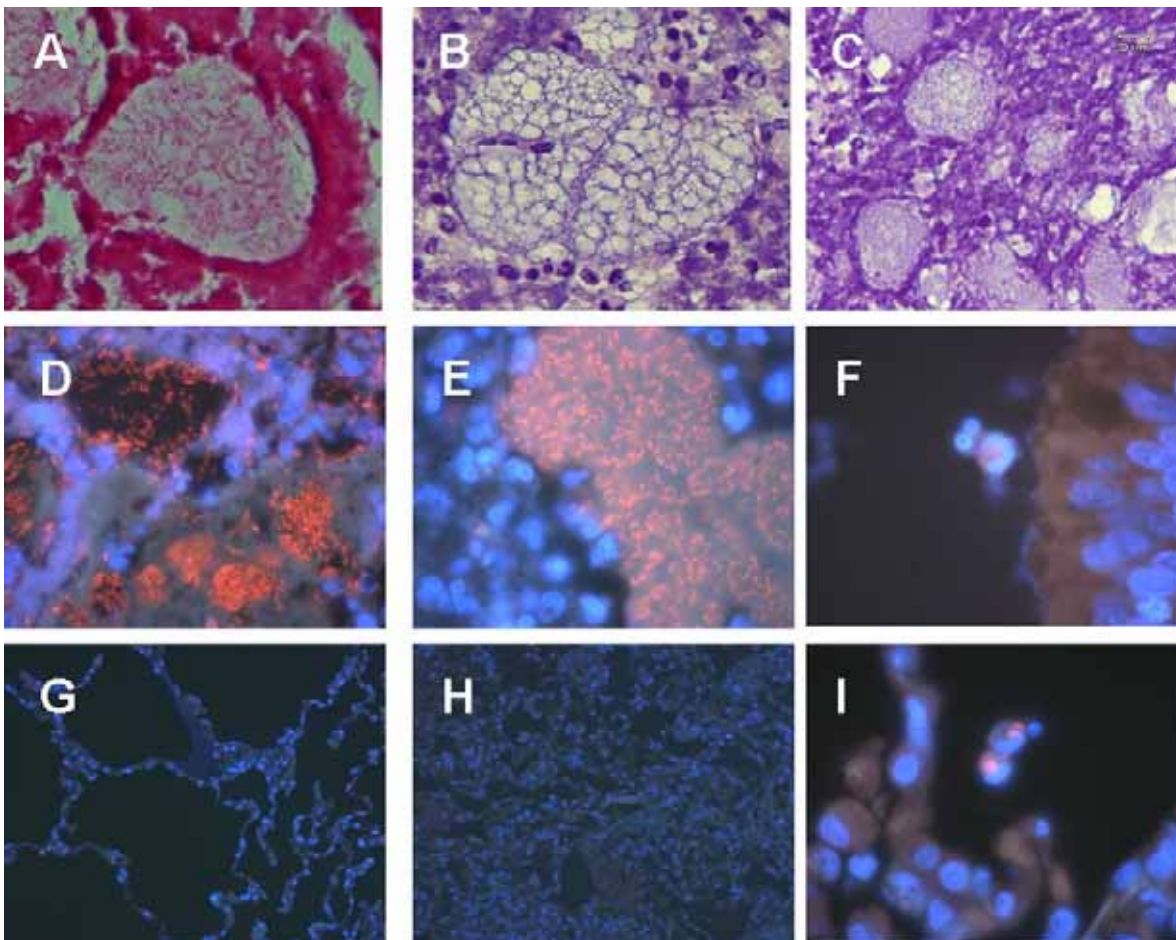


Figure 2. Microscopic investigation of lungs from group (1) *P. aeruginosa* biofilms are contained in the conductive zone, only very few bacteria are detected in the respiratory zone and here they are phagocytosed. A). Bacterial biofilm

in a bronchia visualized with Gram stain. B, C). HE stain of bacteria filled bronchiole. D, E). Intraluminal *P. aeruginosa* biofilms surrounded by PMNs. F) Intact bronchi wall. G, H). Increasing consolidation of alveoli. I). Single phagocytosed *P. aeruginosa* in the respiratory zone.

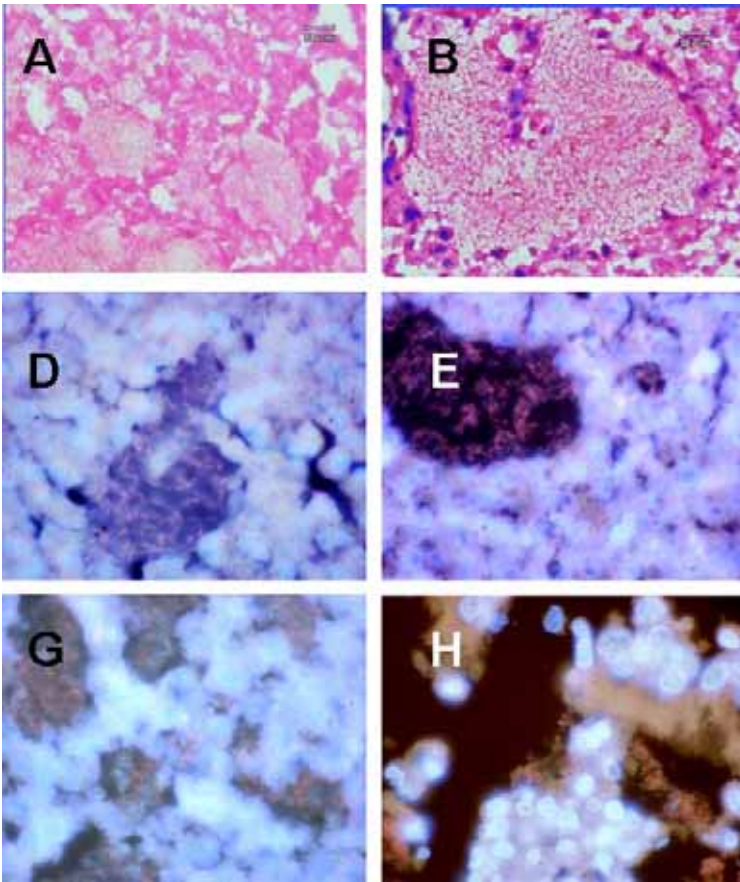


Figure 3. Microscopic investigation of the lungs from group 2. Massive bacterial biofilms detected by Gram stain of the conductive (A) and respiratory zone (B). Intraluminal *P. aeruginosa* biofilms surrounded by PMNs (D, E) and *P. aeruginosa* biofilms in the respiratory zone (G, H) visualized by specific *P. aeruginosa* stain. Most of the tissue in both zones is extensively destroyed.