Analysis of the virulence mechanisms and adaptation of *Pseudomonas aeruginosa* strains in patients with cystic fibrosis

Pseudomonas aeruginosa is an opportunistic microorganism capable of causing life-threatening, multiple clinical forms of acute and chronic infections. It is one of the most important etiologic agents of nosocomial infections, including ventilator-associated pneumonia. *P. aeruginosa* is the predominant pathogen of chronic respiratory tract infections in patients with cystic fibrosis, in whom it increases the incidence of exacerbations and accelerates disease progression. Cystic fibrosis is an autosomal recessive genetic disorder caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The mutation results in an abnormal production of CFTR protein and a consequent disturbance of ion transport in tissues of epithelial origin, located mainly in the respiratory, gastrointestinal and reproductive systems. Although cystic fibrosis is a multi-organ disease, the quality and length patients' life is determined primarily by bronchopulmonary disease. Patients with cystic fibrosis are predisposed to chronic respiratory infections caused by *P. aeruginosa*, due to changes in lung physiology, including reduced the periciliary layer, the accumulation of sticky and thick mucus secretions, and impaired mucociliary clearance. The frequency of chronic *P. aeruginosa* infections increases as patients age, affecting 60-70% of adult patients.

The key elements determining the severity of *P. aeruginosa* infections include cell-associated and extracellular virulence factors that can damage host cells, induce an enhanced immune response, and increase inflammation and lung tissue damage. Alkaline protease, elastase, pyocyanin, mucoid phenotype, motility, biofilm-forming capacity, and drug resistance of the tested strains play an important role in the pathomechanism of *P. aeruginosa* infections.

Alkaline protease and elastase produced by *P. aeruginosa* allow colonization and development of infection. They can act independently or synergistically to degrade host proteins, weaken immune defences, and destroy physical barriers that prevent microbial adherence and penetration under normal conditions.

One of the major virulence factors of *P. aeruginosa* is pyocyanin, which exhibits a wide range of activities, including damage to host tissues by generating reactive oxygen species. Protease, elastase and pyocyanin, together with *P. aeruginosa* motility, are believed to play an important role in acute infections and early stages of chronic infections, due to their involvement in colonization of host tissues. In contrast, the *P. aeruginosa* ability to produce biofilm is extremely problematic in

clinical settings, due to its effect on increased antibiotic tolerance, resistance to phagocytosis, and other components of the innate and acquired immune system.

In patients with cystic fibrosis, the presence of the mucoid phenotype of *P. aeruginosa* in the lungs is strongly associated with chronic stages of infection, accelerated decline in lung function, and increased risk of pulmonary exacerbations.

Long-term persistence of *P. aeruginosa* in the airways of patients is possible due to the high plasticity of the genome, allowing rapid adaptation to adverse environmental conditions. Furthermore, host-pathogen interactions in patients suffering from cystic fibrosis evolve over time and in anatomical space, with the balance fluctuating between activation of host defence mechanisms, and immune evasion and tolerance of the microorganism.

Understanding the epidemiology and virulence of *P. aeruginosa* in chronic infections of patients with cystic fibrosis is crucial for developing new diagnostic methods and treatment strategies.

The purpose of this study was to characterize the phenotypic features that enable *P. aeruginosa* to survive during chronic respiratory infection in cystic fibrosis patients. In this study, phenotypic features of clinical strains obtained from acute non-cystic fibrosis (non-CF) infections and chronic infections in cystic fibrosis (CF) were compared in order to determine pathoadaptive features.

An analysis of virulence factors conducted by comparing 639 strains, including 90 strains from patients with acute clinical infections and 549 strains of *P. aeruginosa* from chronic CF infections. Phenotypic characterization included assays to determine proteolytic activity, motility, biofilm-forming capacity, mucoid phenotype, pyocyanin production and drug resistance phenotype.

It was demonstrated that strains from patients with chronic CF infections produced the tested virulence factors significantly less frequently compared to strains isolated from non-CF patients ($p<0,001$), with the exception of elastase activity ($p=0,116$). When comparing the two patient groups, the *P. aeruginosa* strains with a mucoid phenotype, a marker of the transition from acute to chronic phase of infection, were identified significantly more often ($p<0,001$) in cystic fibrosis patients.

Analysis of strains obtained from chronic infections in CF patients in three age categories showed that the biofilm-forming capacity was significantly higher in the children's group, compared to strains isolated from patients in the adolescent and adult groups. In addition,

protease, pyocyanin, and swarming motility showed the lowest activity in the adult CF group. Frequency analysis of virulence factors showed that the percentage of strains capable of producing protease ($p<0,001$), pyocyanin ($p<0,001$), as well as swimming ($p=0,015$), swarming ($p<0,001$), and twitching ($p<0,001$) motility significantly decreases with the age of CF patients. Strains with a mucosal phenotype were identified most frequently in adults – at 57,2% – with the incidence of 12,5% in children and 43,5% in adolescents ($p=0,010$). It was found that the adaptation of *P. aeruginosa* strains in the respiratory tract of patients is characterized by loss of motility, alkaline protease secretion, conversion to mucoid phenotype, and increasing antibiotic resistance. Strains isolated from early-stage infections of cystic fibrosis patients, showed phenotypical similarity to strains isolated from acute infections. The loss of virulence factors was most pronounced in adult patients with cystic fibrosis.

One hundred twenty strains isolated from 10 adult CF patients chronically infected with *P. aeruginosa* were analysed in order to determine whether chronic infection is caused by a strain with the same phenotype and restriction pattern. The test distinguished 28 different genotypes and demonstrated strain transmission between patients.

Three transmission clusters were identified, including clusters IG1 and IG2 comprising 9 *P. aeruginosa* strains each, obtained from two patients, and cluster IG3 comprising 6 *P. aeruginosa* strains isolated from 3 patients. All patients had a history of shared hospitalizations in the hospital departments of the Institute of Tuberculosis and Lung Diseases in Warsaw, Poland, and it is likely that transmission occurred during these stays. However, since no environmental studies were conducted during the period analysed, it cannot be ruled out that the environment was the reservoir of transmission strains.

Analysing the activity of key virulence factors between transmissible and non-transmissible strains showed that twitching motility ($p=0,023$) and pyocyanin production ($p<0,001$) were statistically significantly higher for transmissible strains. Similar correlations were observed when studying the incidence of protease ($p=0,014$), elastase ($p=0,035$), pyocyanin ($p=0,013$) and twitching motion ($p<0,001$).

The results showed that the adaptation of *P. aeruginosa* in the lungs of cystic fibrosis patients is complicated. In some patients, it has been noted that several unrelated strains of *P. aeruginosa* may transiently or continuously infect the respiratory tracts of patients. Nevertheless, significant phenotypic diversity of *P. aeruginosa* strains has been demonstrated even among patients persistently infected with a strain of the same restriction pattern.

The number of hospitalization days was twice as high in patients with genotypic diversity when compared with patients with genotypic stability – at 489 *vs* 240 days. Number of cycles of intravenous therapy showed a similar relationship at 26 *vs* 16.

This study demonstrates that *P. aeruginosa* gradually changes from acutely virulent in the early stages of infection to adapted to the host airway in the late stages of infection over the course of chronic infection development in cystic fibrosis patients.

In addition, phenotypic and molecular analysis of strains in adult patients revealed significant genotypic diversity and phenotypic heterogeneity over time. Analysis of the *P. aeruginosa* adaptation to the lungs of cystic fibrosis patients is highly beneficial from both clinical and evolutionary perspectives. A comprehensive understanding of the *P. aeruginosa* adaptation process may help develop more effective antimicrobial therapies and identify new targets for future drugs to prevent progression of the infection to chronic stages.

Moreover, as showed in the study, microbiological diagnostics should be expanded to include virulence factor testing, as the methods used to date may not reflect the diversity of *P. aeruginosa* in individual clinical specimens in chronically infected patients.