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MICROBIOLOGICAL FEATURES OF *PSEUDOMONAS AERUGINOSA* IN CHILDREN WITH CYSTIC FIBROSIS

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Ключевые слова: *муковисцидоз, хроническая инфекция, Pseudomonas aeruginosa, микробиом дыхательных путей, резистентность*

Abstract. Microbiological features of *Pseudomonas aeruginosa* in children with Cystic Fibrosis. Ishchenko O.V., Stepaniuk D.O. The purpose of the study was to determine the frequency rate of *Pseudomonas aeruginosa* infection among children with cystic fibrosis (CF) in Dnipro region and to provide microbiological characteristics of the obtained isolates. The study was conducting from January 2019 to December 2020. Children with genetically confirmed CF diagnosis were enrolled. The main research method was bacteriological with identification of microorganisms by biochemical properties; antimicrobial susceptibility was determined by disk-diffusion method. Biological material: mucus from a deep smear from the posterior pharyngeal wall, sputum and tracheobronchial lavage waters. The Leeds criteria were used to define persons with chronic infection. The study involved 21 children. We collected 183 respiratory samples with 49 isolates of *P. aeruginosa*. The most important co-existing pathogens were *Staphylococcus aureus*, *Aspergillus spp.* and *Candida spp.* In our study, *P. aeruginosa* was associated with *Aspergillus spp.* ($\chi^2=20.952$; $df=1$; $p<0.001$). Mucoid isolates were found in 24.49% of cases. *P. aeruginosa* showed variable sensitivity to different groups of antimicrobial agents, but the highest resistance was to penicillins. Mucoid *P. aeruginosa* was more resistant to penicillins ($p<0.001$) and cephalosporins ($p=0.036$). Infection *P. aeruginosa* is frequent among children with CF; there were three children with chronic bronchopulmonary infection *P. aeruginosa* in Dnipro region in the end of 2020. The likelihood of *Aspergillus spp.* infection was higher in the case of current *P. aeruginosa* infection. *P. aeruginosa* showed variable susceptibility to different groups of antimicrobial agents, but mucoid isolates were more resistant.

Реферат. Мікробіологічні особливості *Pseudomonas aeruginosa* в дітей з муковісцидозом. Іщенко О.В., Степанський Д.О. Метою дослідження було з'ясувати частоту інфекції *Pseudomonas aeruginosa* серед дітей з муковісцидозом (МВ) у Дніпровському регіоні та надати мікробіологічну характеристику отриманим ізолятам. Дослідження проводилось із січня 2019 року до грудня 2020 року. Критерії включення – дитячий вік та генетично підтверджений діагноз МВ. Основний метод дослідження – бактеріологічний з ідентифікацією ізолятів за біохімічними показниками; чутливість до антибіотиків визначали диско-дифузійним методом. Матеріал – слиз із глибокого мазка із задньої стінки глотки, мокротиння, промивні води трахеобронхіального лаважу. Критерії Leeds – для стратифікації осіб з хронічною інфекцією. У дослідженні взяла участь 21 дитина. Відібрано 183 зразки з дихальних шляхів, отримано 49 ізолятів *P. aeruginosa*. Найважливішими співіснуючими патогенами були *Staphylococcus aureus*, *Aspergillus spp.* та *Candida spp.* У нашому дослідженні інфекція *P. aeruginosa* була асоційована з *Aspergillus spp.* ($\chi^2=20,952$; $df=1$; $p<0,001$). Колонії *P. aeruginosa* з мукоїдним фенотипом мали у 24,49% випадків. *P. aeruginosa* показала варіабельну чутливість до різних груп антимікробних засобів, але найбільшу резистентність зареєстрували до пеніцилінів. Мукоїдні ізоляти були більш стійкими до пеніцилінів ($p<0,001$) та цефалоспоринів ($p=0,036$). Інфекція *P. aeruginosa* є поширеною серед дітей з МВ, на кінець 2020 року три дитини в Дніпровському регіоні мають хронічну легеневу інфекцію *P. aeruginosa*. Ймовірність інфікування *Aspergillus spp.* вище серед пацієнтів з поточною інфекцією *P. aeruginosa*. Мукоїдні ізоляти показують більш високу резистентність.

Cystic fibrosis (CF) is an inherited disease with an autosomal recessive type of inheritance. Approximately every 25-th European is a heterozygous carrier of the defective genes responsible for dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Functional failure of CFTR in airways leads to the formation of viscous secretions and impairment of mucociliary clearance that predispose to further bacterial colonization. The key factor in the pathogenesis of CF is chronic lung infection *Pseudomonas aeruginosa* which is considered as a leading contributor to morbidity and mortality [1, 8, 13].

According to the European Cystic Fibrosis Society Patient Registry (ECFSPR), there were 169 children and only 36 adults with CF in Ukraine at the end of 2018. In our country more than 40% of patients have chronic infection *P. aeruginosa* [8]. The life expectancy in people with CF in developed countries comprises up to 40.7 years old (with an impact of CFTR-modulators therapy); however,

domestic literature data are absent [13]. Without exaggeration, exactly *P. aeruginosa* determines the prognosis in patients with CF [6, 9, 13].

P. aeruginosa is the main contributor to the chronic lung disease are biofilm-forming properties with resistance to a wide range of antimicrobials. In the early stages of colonization, *P. aeruginosa* resembles wild-type isolates with typical microbiological properties but later develops an alternated mucoid phenotype that facilitates the increased growth of biofilms. This modification has been linked to difficulties involved in the eradication of *P. aeruginosa*, even under antimicrobial treatment with both parenteral and inhaled medications [9, 11]. Furthermore, several reports about dominant epidemic strains with poor clinical outcomes were published, so monitoring and surveillance of *P. aeruginosa* infection in individuals with CF is supposed to be necessary [7, 9].

The purpose of the study was to determine the frequency rate of *P. aeruginosa* infection among

children with CF in Dnipro region, to provide microbiological characteristics of the obtained isolates and to determine their sensitivity to antimicrobials.

MATERIALS AND METHODS OF RESEARCH

The study was conducted in the scientific laboratory of the department of microbiology, virology, immunology and epidemiology from January 2019 to December 2020. Inclusion criteria were: age from 0 to 17 years 11 months 29 days and genetically confirmed diagnosis of CF. Children without genetically conformation were not enrolled. Sputum and/or deep smear from the posterior pharyngeal wall were taken, and we did not rule out the opportunity to collect tracheobronchial lavage waters. The planned frequency of visits was 1 time per 3 months, unscheduled visit in a case of deterioration [4]. Parents or healthcare proxies signed the Patient Informed Consent Form. Previously, the work was approved by the Bioethics Committee of the SE «Dnipropetrovsk Medical Academy of the Ministry of Health of Ukraine» (No. 8, 17.10.2018). The research was conducted in accordance with the principles of bioethics set out in the WMA Declaration of Helsinki – “Ethical principles for medical research involving human subjects” and “Universal Declaration on Bioethics and Human Rights” (UNESCO).

The main method of the research was bacteriological. The samples were taken in the morning before hygiene practices. Sputum and lavage waters were collected in sterile containers; in a case, without expectoration, a swab from the posterior pharyngeal wall was taken [4]. The obtained biological material *extempore* was plated on a standard set of growth mediums. For selective isolation of *Pseudomonas spp.* we used Cetrimide agar with glycerol (BioLife, Italy) [5, 9, 12]. Salt agar (Farmaktiv, Ukraine) with egg-yolk emulsion (Hi-Media, India) was used for selective isolation of *Staphylococcus aureus* [13, 15]. Sabouraud dextrose agar with chloramphenicol (Farmaktiv, Ukraine) was used for isolation of fungi [5, 12]. Columbia agar with 5% sheep blood (bioMerieux, France) was used for non-selective cultivation and assessment of hemolytic activity [12]. The plated Petri dishes were incubated in a thermostat for 24-72 hours. at 37°C [15]. The Petri dishes with salt agar and blood-containing medium were left at indoor temperature for up to 5 days for selection of small colony variants (SCVs) of *S. aureus* [11]. The number of microorganisms was evaluated in CFU/ml. The yielded cultures were stained by Gram [12].

Identification of microorganisms was performed considering the morphological, tinctorial, cultural and biochemical properties according to the Bergey's Manual of Systematic Bacteriology (2004). Identification

of *Pseudomonas spp.* was done using a commercial test kit NEFERM test 24 (Erba Lachema, Czech Republic). The kit contains 24 biochemical tests (arginine, indole, urease, glucose, lysine, fructose, sucrose, inositol, beta-galactosidase, phosphatase, β -glucuronidase, N-acetyl- β -D-glucosaminase, mannitol, xylose, cellobiose and galactose, nitrates, nitrites, esculin, γ -glutamyltransferase, lactose, maltose, trehalose, Simon's citrate), located in a 3-row plate, that were supplemented by a test for determination of cytochrome oxidase activity. For evaluation of pigments production, we used a qualitative observational method. For identification of *S. aureus* STAFI test 16 (Erba Lachema, Czech Republic) was used.

The susceptibility of isolates to antimicrobials was determined on Mueller-Hinton agar (SRE IFR NAAS, Ukraine) by disc-diffusion method according to recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). However, here we describe data according to EUCAST 2020, version 10.0. We used the following disks with antimicrobials (Hi-Media, India): piperacillin 30 mcg, piperacillin/tazobactam 30/6 mcg, ticarcillin 75 mcg, ticarcillin/clavulanic acid 75/10 mcg, cefepime 30 mcg, ceftazidime 10 mcg, imipenem 10 mcg, meropenem 10 mcg, aztreonam 30 mcg, ciprofloxacin 5 mcg, levofloxacin 5 mcg, amikacin 30 mcg, tobramycin 10 mcg [14]. The obtained data were entered into the table of results.

Based on the results of the bacteriological study, the *Leeds* criteria were used for stratification of the patients. Chronic infection refers to patients if more than 50% of respiratory samples during the explored year were positive for *P. aeruginosa*. Intermittent infection refers to patients if less than 50% of respiratory samples were positive for *P. aeruginosa*. Patients with positive *P. aeruginosa* infection in the past but not during the last 12 months. Obviously, never infected were patients without respiratory samples of *P. aeruginosa* [10].

The Microsoft Office Excel 2010[®] with customizations was used for statistical data processing (version 14.0.7265.5000, license No. 02278-581-0218935-38456). Normality data distribution was evaluated with the Shapiro-Wilk test, the hypothesis of normality distribution was rejected at $p < 0.05$. We used the frequency rate for 100 and determinations and extensive indicator (proportion, %) to describe our data. For quantitative data with non-normality distribution Median, % and its interquartile range – Me (25%; 75%) were used. The Pearson's chi-square test, ϕ -coefficient, and odds ratio (OR) with its 95% confidence interval (95% CI) was used for comparison of independent samples by qualitative data. The Kruskal-Wallis test was used for multiplying

comparisons of quantitative data; it was completed by the Mann-Whitney U-test. The results of the experiments were statistically significant at $p < 0.05$ [15].

RESULTS AND DISCUSSION

The study involved 21 children (10 boys and 11 girls) aged 2 years 8 months to 16 years 10 months, the mean age at the beginning of the study was 10.1 (3.7) years ($p=0.558$). All children finished the study with no exceptions. For 24 months, 183 airway samples were taken. We had 168 planned and 15 unscheduled samples. The samples included sputum ($n=90$), mucus from a deep smear from the posterior pharyngeal wall ($n=75$), and lavage water after tracheobronchial lavage ($n=3$).

All samples were positive for pathogenic microbiota, and monoculture was found only in 7.10% of cases ($n=13$). Culture of *Pseudomonas spp.* was obtained from 29.51% of samples ($n=54$). The species composition of the genus *Pseudomonas* was represented by *P. aeruginosa*, *P. fluorescence*, and *P. putida*. It should be noted, that the isolation of *P. fluorescence* and *P. putida* was episodic in some children in non-etiological quantities.

Concerning *P. aeruginosa* isolates, the situation was the opposite. Within 24 months the culture was obtained from 10 children (47.62%), the average age of children at the beginning of the study was 11.2 (3.7) years ($p=0.816$).

According to the Leeds criteria, there were three children with chronic bronchopulmonary infection *P. aeruginosa* in Dnipro region at the end of 2020. They had at least three positive consecutive cultures of *P. aeruginosa* during the year. We found two children with detectable *P. aeruginosa* in 2019, so nowadays they are patients with "infection in the past". Therefore, at the end of 2020, there are five children in Dnipro region with intermittent infection *P. aeruginosa*. Consequently, there were only 11 children with CF never infected.

There was a total of 49 isolates of *P. aeruginosa* in our study, monoculture was obtained only 3 times. The leading co-existing pathogen was *S. aureus* (85.71% of cases *P. aeruginosa*). Also, except *S. aureus* with wild-phenotype and typical morphological properties, so-called SCVs were isolated from the samples. The paradigm which describes the microbial landscape of the airways in CF is in the sequential progression of the leading pathogens *S. aureus* and *Haemophilus influenzae* to *P. aeruginosa* and other non-fermenting gram-negative microorganisms [4, 8, 14]. In addition to changes caused by direct mutations in CFTR, the microorganisms themselves contribute to this pathological process. Most significantly, *S. aureus* can create specific conditions for further colonization of

P. aeruginosa. Suppressive effect of *P. aeruginosa* on *S. aureus* promotes the selection of auxotrophic variants, consequently, creates a base for the impossibility of the exogenous and endogenous elimination [6, 10, 11].

It stands to mention that in our study infection *P. aeruginosa* was associated with fungi. Both impaired physiology of airways and long-term and recurrent antibacterial therapy promotes the activation of fungi. Simultaneously, *P. aeruginosa* exerts an inhibitory effect on these microorganisms through siderophores and of iron to *Aspergillus spp.* However, the fungi have their mechanisms to avoid the inhibitory effect of the bacteria [5]. In our study, the frequency rate of detection of *Aspergillus spp.* in combination with *P. aeruginosa* was 20.41% ($n=10$) and statistically significantly higher than in the group without the bacteria ($\chi^2=20.952$; $df=1$; $p < 0.001$). Associative link of infection *P. aeruginosa* and *Aspergillus spp.* was moderate, $\phi=0.338$. The OR was 16.92 (95%CI 3.56-80.51), so a chance to have an infection *Aspergillus spp.* was higher almost by 17 times in the presence of *P. aeruginosa* than in case of its absence. Usually, the isolates of *Aspergillus spp.* were isolated in association with mucoid *P. aeruginosa* in children with chronic infection. These results are debatable and require further research, including immunological, but it is worth noting.

As for *Candida spp.* the situation was opposite and we did not find statistically significant differences between groups with and without *P. aeruginosa*. The frequency rate of *Candida spp.* among all cases of *P. aeruginosa* was 67.35% but there were no differences in comparison to the group without *P. aeruginosa* ($\chi^2=1.462$; $df=1$; $p=0.227$). Associative link between infections *P. aeruginosa* and *Candida spp.* was nonessential, $\phi=0.089$. OR=1.527 (95%CI 0.767-3.039).

Isolates of *Pseudomonas spp.* demonstrated typical biochemical properties, with inertness to most sugars; *P. aeruginosa* exhibited an acylamidase activity. On Cetrimide agar with glycerol, the microorganism formed four main types of colonies – mucoid (Fig. 1), large convex shiny, folded, and small with an irregular shape. Mucoid colonies were detected in a proportion of 24.49% of cases ($n=12$). Isolates of *P. aeruginosa* with the properties of SCVs were not found. The specific smell from the Petri dishes was always noted.

In our study there were several isolates of *P. aeruginosa* in some children with individual characteristics (shape of colonies and character of growth, pigments and sensitivity to antibiotics). First, *P. aeruginosa* with mucoid phenotype usually

was isolated from children with chronic and intermittent infection. An important feature of the four isolates of *P. aeruginosa*, sequentially obtained from a girl (age 7 years 4 months old girl at the beginning) was the appearance of the phenomenon of «rainbow lysis» which was manifested by the appearance of a film shimmering in reflected light with different colors of the rainbow on the surface of colonies. This phenomenon is due to the spontaneous action of the bacteriophage and is the feature characteristic of *P. aeruginosa*.

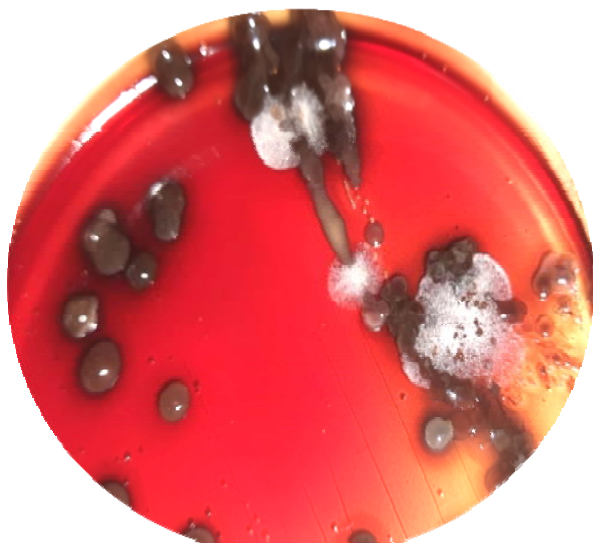


Fig. 1. Mixed culture of mucoid *P. aeruginosa* and *Aspergillus spp.* is presented on Columbia agar, 18 h. Culture was obtained from a child with chronic infection

The next particularity of *P. aeruginosa* was an ability to produce pigments that are one of the virulence factors [3]. Colonies that had a fluorescent green, yellow-green or brown color were considered as producers of pyoverdine, dark green or blue colonies were supposed to produce pyocyanin (Fig. 2). Isolates obtained from children with CF in our study secreted pyoverdine in 69.39% of cases and the proportion of pyocyanin-producers was 22.45%, both pigments were secreted in 4.08% of cases, none of the pigments – in 4.08%.

Antimicrobial susceptibility of *P. aeruginosa* isolates was determined in 100% of cases. The results of the chemotherapeutic sensitivity of *P. aeruginosa* are shown in the Table. It should be noted, that for the most antibiotics growth retardation zone of *P. aeruginosa* is well-defined for resistance detection, but an off-scale level in 50 mm makes it impossible to report the microorganism as sensitive to a particular antimicrobial agent. The concept of «sensitive, increased exposure» means a high probability of

therapeutic success when an influence of antimicrobial agent in the site of infection is increased [14].

In our study, *P. aeruginosa* showed the most variable sensitivity to β -lactam agents. It ranged from high resistance to absolute susceptibility. Therefore, the resistance of the microorganism to penicillins (piperacillin, piperacillin/tazobactam, ticarcillin, ticarcillin/clavulanic acid) was the highest. Only 28.57% of isolates (n=14) obtained from children with intermittent infection *P. aeruginosa* showed sensitivity to all tested penicillins. Sensitive in increased exposures were those isolates that showed sensitivity to an antibacterial agent at more than 18 mm.

93.88% of isolates (n=46) showed sensitivity in increased exposure to ceftazidime and resistant isolates were obtained from a child with chronic infection. Similarly, the sensitivity to cefepime in increased exposure was also quite high – 85.71% (n=42).

Susceptibility in increased exposure to aztreonam was absolute.

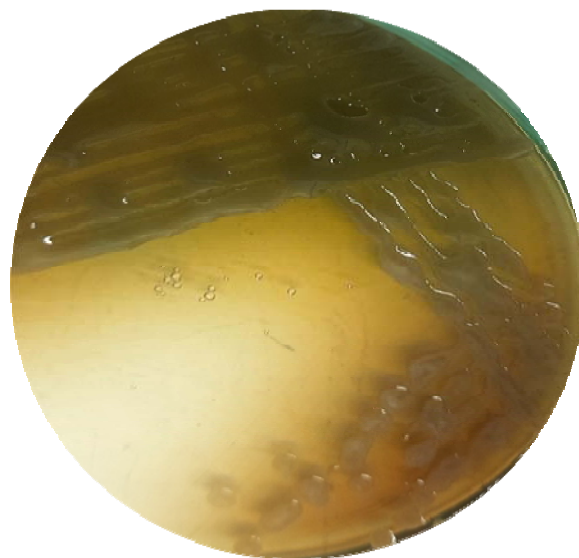


Fig. 2. Culture of *P. aeruginosa*. Mucoid producer of pyoverdine on Cetrimide agar with glycerol, 48 h. Culture obtained from a child with chronic infection

It should be noted, that in our study *P. aeruginosa* has shown an ability to be resistant to fluoroquinolones, both ciprofloxacin and levofloxacin. However, the overall susceptibility with increased exposure was quite high, 90.82%. The isolates with mucoid phenotype, obtained from a child with the chronic infection, showed constant resistance to levofloxacin, and sensitivity to ciprofloxacin was boundary – 25.2 (2.6) mm.

The sensitivity of *P. aeruginosa* to aminoglycosides was high. However, it should be empha-

sized, even in such case aminoglycosides must be used in a combination with another active therapy to support the activity of the agent or to expand the spectrum of therapy [14].

It is impossible to miss the fact that there was a difference in the sensitivity of mucoid and non-mucoid isolates. Comparison of differences in su-

ceptibility to β -lactam antibiotics showed that mucoid isolates were more resistant than non-mucoid isolates. According to the Kruskal-Wallis test, mucoid isolates were less susceptible to penicillins ($p<0.001$) and cephalosporins ($p=0.036$). Mucoid *P. aeruginosa* showed resistance to meropenem and imipenem in children with chronic infection.

Results of chemotherapeutic susceptibility testing of *Pseudomonas aeruginosa* isolates obtained from children with cystic fibrosis (disc-diffusion method; n=49)

Disk with antimicrobial agent	Proportion of sensitive isolates, %	Diameter of growth delay zone – Me (25%; 75%), mm*
Phenotype of resistance to β-lactam agents		
Piperacillin 30 mcg	28.57	12 (8; 18)
Piperacillin/ tazobactam 30/6 mcg	34.69	10 (10; 22)
Ticarcillin 75 mcg	32.65	18 (12; 23)
Ticarcillin/ clavulanic acid 75/10 mcg	32.66	22 (20; 24)
Cefepime 30 mcg	85.71	26 (23; 26)
Ceftazidim 10 mcg	93.88	21 (21; 25)
Imipenem 10 mcg	91.84	22 (20; 26)
Meropenem 10 mcg	89.80	24 (22; 24)
Aztreonam 30 mcg	100.00	27 (18; 30)
Phenotype of resistance to fluoroquinolones		
Ciprofloxacin 5 mcg	89.80	28 (24; 32)
Levofloxacin 5 mcg	91.84	29 (24; 34)
Phenotype of resistance to aminoglycosides		
Tobramycin 10 mcg	100.00	24 (22; 25)
Amikacin 30 mcg	100.00	24 (23; 28)

Note: * – $p<0.05$

When comparing the median of growth retardation zones and the frequency rate of isolates sensitive to aminoglycosides and fluoroquinolones, statistically significant difference was not found ($p>0.05$); however, it might be due to the small size of the mucoid sample.

It should be noted that during the study, two children acquired resistance to penicillins; a child with chronic infection positive to mucoid isolates became resistant to ceftazidime. Thus, the fact is prognostic for treatment outcomes and will determine the prognosis and life expectancy of patients with CF.

CONCLUSIONS

1. Respiratory microbiome in children with CF is altered and harbors such dangerous pathogens as *P. aeruginosa*, *S. aureus* and *Aspergillus spp.* The frequency rate of *P. aeruginosa* infection among children with CF in Dnipro region was 47.62%.

2. A chance to detect *Aspergillus spp.* was much higher in *P. aeruginosa* group in comparison to the group without the bacteria. Usually, the isolates of *Aspergillus spp.* were isolated in association with mucoid *P. aeruginosa*.

3. There were isolates of *P. aeruginosa* with individual characteristics (shape of colonies and



character of growth, pigments, and sensitivity to antibiotics). But one more specific feature was reflected in the appearance of the «rainbow lysis» in isolates sequentially obtained from one girl. Mucoid isolates of *P. aeruginosa* were detected in 24.49%.

4. Sensitivity of *P. aeruginosa* to antibiotics was variable. The microorganism showed increased resistance to penicillins, so only 28.57% of isolates were sensitive to piperacillin, piperacillin/tazobactam, ticarcillin, ticarcillin/clavulanic acid. Absolute sensitivity was reported to aztreonam and aminoglycosides. Mucoid isolates exhibited increased resistance to penicillins and cephalosporins than non-mucoid ($p < 0.001$ and $p = 0.036$, respectively).

The emergence of isolates resistant to cephalosporins, fluoroquinolones and carbapenems warns. Within 24 months *P. aeruginosa* acquired resistance to penicillins and ceftazidime in a few children.

5. Therefore, *P. aeruginosa* is a significant burden for patients with CF and our findings emphasize the importance of communication between clinical microbiologists and clinicians. The cooperation will provide beneficials for selecting the optimal treatment strategy and prevention of further development of antimicrobial resistance.

Conflict of interests. The authors declare no conflict of interest.

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