REFERENCES


Стаття надійшла до редакції 17.05.2021
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 colonisation. 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 (BioloLife, 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,  beta-glucuronidase, N-acetyl-β-D-glucosaminidase, mannitol, xylose, cellobiose and galactose, nitrites, nitrates, 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, ceftizime 30 mcg, cefazidime 10 mcg, impenem 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 biota, and monoculture was found only in 7.10% of cheobronchial lavage (\( n=3 \)).

**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. fluorescense, and P. putida. It should be noted, that the isolation of P. fluorescense and P. putida was episodic in some children in non-etiologically significant 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 colonisation of P. aeruginosa. Suppressing 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 compassion 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 – small with an irregular shape. Mucoid colonies were formed four main types of colonies – small with an irregular shape. Mucoid colonies were usually 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*.

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. 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

**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
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 susceptibility 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)

<table>
<thead>
<tr>
<th>Disk with antimicrobial agent</th>
<th>Proportion of sensitive isolates, %</th>
<th>Diameter of growth delay zone – Me (25%; 75%), mm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin 30 mcg</td>
<td>28.57</td>
<td>12 (8; 18)</td>
</tr>
<tr>
<td>Piperacillin/ tazobactam 30/6 mcg</td>
<td>34.69</td>
<td>10 (10; 22)</td>
</tr>
<tr>
<td>Ticarcillin 75 mcg</td>
<td>32.65</td>
<td>18 (12; 23)</td>
</tr>
<tr>
<td>Ticarcillin/ clavulanic acid 75/10 mcg</td>
<td>32.66</td>
<td>22 (20; 24)</td>
</tr>
<tr>
<td>Cefepime 30 mcg</td>
<td>85.71</td>
<td>26 (23; 26)</td>
</tr>
<tr>
<td>Ceftazidim 10 mcg</td>
<td>93.88</td>
<td>21 (21; 25)</td>
</tr>
<tr>
<td>Imipenem 10 mcg</td>
<td>91.84</td>
<td>22 (20; 26)</td>
</tr>
<tr>
<td>Meropenem 10 mcg</td>
<td>89.80</td>
<td>24 (22; 24)</td>
</tr>
<tr>
<td>Aztreonam 30 mcg</td>
<td>100.00</td>
<td>27 (18; 30)</td>
</tr>
</tbody>
</table>

Phenotype of resistance to fluoroquinolones

<table>
<thead>
<tr>
<th>Disk with antimicrobial agent</th>
<th>Proportion of sensitive isolates, %</th>
<th>Diameter of growth delay zone – Me (25%; 75%), mm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin 5 mcg</td>
<td>89.80</td>
<td>28 (24; 32)</td>
</tr>
<tr>
<td>Levofloxacin 5 mcg</td>
<td>91.84</td>
<td>29 (24; 34)</td>
</tr>
</tbody>
</table>

Phenotype of resistance to aminoglycosides

<table>
<thead>
<tr>
<th>Disk with antimicrobial agent</th>
<th>Proportion of sensitive isolates, %</th>
<th>Diameter of growth delay zone – Me (25%; 75%), mm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobramycin 10 mcg</td>
<td>100.00</td>
<td>24 (22; 25)</td>
</tr>
<tr>
<td>Amikacin 30 mcg</td>
<td>100.00</td>
<td>24 (23; 28)</td>
</tr>
</tbody>
</table>

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 \( P. \text{aeruginosa} \) were detected in 24.49%.

4. Sensitivity of \( P. \text{aeruginosa} \) 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 \( P. \text{aeruginosa} \) acquired resistance to penicillins and ceftazidime in a few children.

5. Therefore, \( P. \text{aeruginosa} \) 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.

REFERENCES


СПИСОК ЛІТЕРАТУРИ

1. Ільченко С. І. Клініко-мікробіологічні особливості перебігу муковісцидозу у дітей великого промислового міста. Патологія. 2014. № 3. С. 73-77. URL: http://nbuv.gov.ua/UJRN/pathology_2014_3_17

Стаття надійшла до редакції 29.01.2021