Bulgarian cystic fibrosis *Pseudomonas aeruginosa* isolates: antimicrobial susceptibility and neuraminidase-encoding gene distribution

Cystic fibrosis (CF) is an autosomal recessive disorder affecting multiple organ systems. The leading cause of morbidity and mortality is a progressive decline in pulmonary function resulting from airway damage caused by thickened secretions, complicated by chronic microbial infection. *Pseudomonas aeruginosa* is the most prevalent organism colonizing the lungs of CF patients (up to 80% of the colonizations of lungs of patients aged >18 years) (Ramsey, 1996; CFF, 2002). It is rarely eradicated after colonization, regardless of the antimicrobial chemotherapy employed (Govan & Deretic, 1996). An extracellular neuraminidase is thought to play an important role in *P. aeruginosa* implantation of CF respiratory epithelium (Davies *et al.*, 1999). Nevertheless, the bulk of clinical opinion supports the view that antibiotic therapy leads to improvement in lung function and improves survival of patients, particularly if aggressive treatment is begun on the first isolation of the organism.

The aim of this study was to determine the antimicrobial susceptibility and neuraminidase-encoding gene (*nan1*) distribution among *P. aeruginosa* isolates recovered from the sputum of CF patients from Bulgaria. A total of 102 *P. aeruginosa* isolates (including 87 mucoid and 15 non-mucoid strains) identified with the BBL enteric/nonfermenter ID system (Becton Dickinson) were studied. The strains were isolated between December 2005 and April 2008 from 50 patients with CF (30 females and 20 males) aged 6–27. The CF patients came from 17 towns and villages scattered across Bulgaria. The following mutations were found within the cystic fibrosis transmembrane conductance regulator-encoding gene (*cftr*) of the studied patients: $\Delta F508/\Delta F508$ ($n=20$), unknown/ $\Delta F508$ ($n=6$), N1303K/ $\Delta F508$ ($n=6$), unknown/N1303K ($n=2$), R1070Q/

218insA ($n=2$), $\Delta F508/R1070Q$ ($n=2$), $\Delta F508/306\text{delTAGA}$ ($n=2$), G1069R+L88X/ $\Delta F508$ ($n=2$), Q220X/2183G-A ($n=2$), unknown/2184ins108n ($n=2$) and unknown ($n=4$). The susceptibilities of the investigated *P. aeruginosa* isolates to 17 antimicrobial agents were determined by the agar dilution method on Mueller–Hinton II agar plates (Becton Dickinson) according to the Clinical and Laboratory Standards Institute (CLSI) 2005 recommendations (CLSI, 2005).

In an attempt to evaluate the evolution of pulmonary disease, we searched for *nan1* among the studied isolates of *P. aeruginosa* (Lanotte *et al.*, 2004) using PCR. Total DNA was extracted by boiling. PCR amplification was performed with specific primers *nan1*-F (5′-ATGAATACTTAT-TTTGATA-3′) and *nan1*-R (5′-CTAAATCCATGCTCTGACCC-3′) yielding a 1317 bp product (Strateva, 2008). The PCR was carried out with 2 μl template DNA, 0.25 μM each primer (Alpha DNA), 0.2 mM dNTPs, 1 $\times$ reaction buffer, 2 mM MgCl_2 and 1.5 U *Prime Taq* DNA polymerase (GeNet Bio) in a total volume of 25 μl . The following PCR protocol was used: an initial denaturation (95 °C, 4 min), followed by 25 cycles of denaturation (95 °C, 35 s), annealing (53 °C, 1 min) and extension (72 °C, 1 min 30 s), and a single final extension (72 °C, 7 min). Selected PCR products were purified with an ExoSAP-IT reagent (Amersham Biosciences). Sequencing reactions were performed using a sequencing primer, *nan1*-F-seq (5′-GGTATTTGGAGTGCGCCTA-3′) (Strateva, 2008), the primers for amplification and a BigDye terminator v.3.1. kit (Applied Biosystems) with an automated sequencer (ABI 310 genetic analyzer; Applied Biosystems). The nucleotide and deduced amino acid sequences were analysed with software available from the

National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The sequence analysis revealed total identity with the *P. aeruginosa* neuraminidase-encoding gene (GenBank accession no. AF236853). Statistical analysis was performed by Student's *t* test.

The antimicrobial susceptibility testing results are presented in Table 1. A total of 68 of the 102 (66.7%) CF *P. aeruginosa* isolates studied manifested susceptibilities to all antipseudomonal agents ('wild-type' phenotype). There were slight resistance rates to the following antibiotics: imipenem (5.9%), meropenem (3.9%), amikacin (3.9%), gentamicin (7.8%), tobramycin (5.9%), netilmicin (13.7%) and ciprofloxacin (13.7%). All strains were susceptible to colistin (MICs ranged from 0.03 to 2 mg l⁻¹). Colistin-resistant CF isolates of *P. aeruginosa* have been found in some European countries, such as Spain (2.9%) (Morosini *et al.*, 2005) and the UK (3.1%) (Pitt *et al.*, 2003). Recently, Johansen *et al.* (2008) reported two outbreaks and a prolonged spread of colistin-resistant non-mucoid *P. aeruginosa* among chronically infected Danish CF patients. The CF *P. aeruginosa* isolates from Europe were more susceptible than those isolates from the USA from the same time period. For example, the antibiotic resistance rates in Spain (2003–2004), the UK (2000) and the USA (2000) were, respectively: piperacillin – 20.9, 29.7 and 48.2%; ceftazidime – 14.6, 39.6 and 49.9%; tobramycin – 13.1, 10.1 and 22.2%; gentamicin – 33.0, 47.0 and 52.4%; ciprofloxacin – 28.6, 29.7 and 37.4% (Morosini *et al.*, 2005; Pitt *et al.*, 2003; Burns *et al.*, 2000). The antimicrobial susceptibility of our CF *P. aeruginosa* strains was higher than that of the analogous strains from western Europe and the USA during the last few years, which was related to the predominant stable clinical status of patients

Table 1. Antimicrobial susceptibility of 102 isolates of *P. aeruginosa* from Bulgarian CF patients

Antimicrobial agent	No. (%) of isolates		
	Susceptible	Intermediate	Resistant
Carbenicillin	98 (96.1)	2 (2)	2 (2)
Azlocillin	102 (100)	0 (0)	0 (0)
Piperacillin	102 (100)	0 (0)	0 (0)
Piperacillin + tazobactam	102 (100)	0 (0)	0 (0)
Ceftazidime	102 (100)	0 (0)	0 (0)
Cefoperazone	102 (100)	0 (0)	0 (0)
Cefepime	98 (96.1)	2 (2)	2 (2)
Cefpirome	98 (96.1)	2 (2)	2 (2)
Aztreonam	98 (96.1)	2 (2)	2 (2)
Imipenem	94 (92.1)	2 (2)	6 (5.9)
Meropenem	98 (96.1)	0 (0)	4 (3.9)
Amikacin	98 (96.1)	0 (0)	4 (3.9)
Gentamicin	90 (88.2)	4 (3.9)	8 (7.8)
Tobramycin	96 (94.1)	0 (0)	6 (5.9)
Netilmicin	86 (84.3)	2 (2)	14 (13.7)
Ciprofloxacin	84 (82.3)	4 (3.9)	14 (13.7)
Colistin	102 (100)	0 (0)	0 (0)

Table 2. Characteristics of the CF patients ($n=14$) who had *P. aeruginosa* isolates expressing a neuraminidase-encoding gene

Characteristic	Value
Age	
Mean	13.4 years
Range	6–17 years
Sex	
Male	4 patients
Female	10 patients
Patient symptom	
Increased cough	14 patients
Increased sputum production	14 patients
Dyspnoea	6 patients
Weight loss	9 patients (loss in range 1–4 kg)
Temperature range	37.6–39.4 °C
New auscultation findings	In all patients
Sputum cytology	
PNL range	29–68 %
Metaplasia range	0–70 %
Complete blood count	
WBC range	$7.10\text{--}19.34 \times 10^9 \text{ cells l}^{-1}$
Neu range	51.40–83.16 %
ESR range	17–50 mm h ⁻¹
CRP serum concentration range	3.9–7.2 mg l ⁻¹
X-ray*	
Pneumonia	12 patients
Emphysema	11 patients
Spirometry	
Mean FEV ₁ at baseline as percentage of predicted value	66 % (range 49–82 %)
Mean FEV ₁ on day of exacerbation as percentage of predicted value	33 % (range 21–55 %)

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Neu, neutrophil granulocytes; PNL, polynuclear leukocytes; WBC, white blood cells.

*Here are noted only newly found changes, compared to the previous X-ray of the patient; we didn't count the old changes from previous exacerbations.

investigated, and thus the lack of prolonged antimicrobial chemotherapy. On auscultation, most patients had a clear vesicular murmur (with bronchial character in the places where bronchiectasis was formed) without any crackles (rales) or rhonchi. All data from complete blood counts were within normal limits or on the upper limit. There were no additional new changes on X-ray findings. The spirometry showed steady levels of forced vital capacity and forced expiratory volume in 1 s (FEV₁) slightly decreasing with the age of the patient. The sputum cytology manifested steady levels of polynuclear cells and the rate of metaplasia was not increasing significantly with check-ups. The patients in good clinical condition usually needed an antibiotic added to their regular treatment once or a maximum of twice yearly.

As has been described elsewhere, the prevalence of *nan1* in CF isolates has tended to increase as the clinical status worsened (Lanotte *et al.*, 2004). In this research a total of 39 of the 102 CF *P. aeruginosa* isolates expressed a *nan1* gene. The *nan1*-positive strains of *P. aeruginosa* (38.2%) were isolated from 14 patients with recurrent exacerbations of pulmonary disease and a weak clinical status. The characteristics of these patients are summarized in Table 2. Exacerbations were characterized by acute changes in respiratory signs and symptoms, such as increased cough frequency and sputum production, weight loss, new auscultation findings (crackles, wheezing, rales or rhonchi), inflammation changes according to the complete blood count (elevated leukocytes, erythrocyte sedimentation rate and C-reactive protein), new changes on X-ray and elevated levels of polynuclear cells and metaplasia on sputum cytology. In addition, the drop in forced vital capacity and FEV₁ was drastic, and hardly came back to the baseline after the treatment.

The frequency of *nan1* in our study (38.2%) was significantly lower than that among recent French CF isolates of *P. aeruginosa* (61.7%) ($P < 0.001$) (Lanotte *et al.*, 2004). The different frequencies of *nan1* among Bulgarian and French CF patients resulted from the clinical status of the patients.

There were also some features of the antibiotic policy in Bulgaria that contributed to the high antimicrobial susceptibility in CF *P. aeruginosa* isolates, such as an empirical therapy of *P. aeruginosa* colonization/infection predominantly with oral ciprofloxacin, difficulties in the supply of inhalatory therapy with tobramycin, because of the high price and the necessity of prolonged use of the drug (28 day courses), and a lack of a licence for the clinical use of colistin in Bulgaria.

The predominant portion (85.3%) of our CF strains were mucoid. Traditionally mucoid *P. aeruginosa* isolates have higher resistance and long-term colonization, but there are enough scientific reports describing more susceptible CF mucoid *P. aeruginosa* strains compared with non-mucoid strains (Ciofu *et al.*, 2001; Srifuengfung *et al.*, 2004).

In conclusion, the antimicrobial resistance of Bulgarian CF *P. aeruginosa* isolates was unusually low, which was related to the following causes: mostly intermittent and chronic colonization/infection without leaning towards recurrent exacerbations in the studied patients, and thus a lack of prolonged antimicrobial chemotherapy; and the antibiotic policy in Bulgaria. Also, the frequency of *nan1* was relatively low, and correlated with the predominant good clinical status of the patients investigated. The molecular-genetic detection of this gene may be used as an indirect measure of CF pulmonary disease evolution.

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