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ARTICLE

Newborn screening for cystic fibrosis: Polish 4 years' experience with *CFTR* sequencing strategy

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Newborn screening for cystic fibrosis (NBS CF) in Poland was started in September 2006. Summary from 4 years' experience is presented in this study. The immunoreactive trypsin/DNA sequencing strategy was implemented. The group of 1 212 487 newborns were screened for cystic fibrosis during the programme. We identified a total of 221 CF cases during this period, including, 4 CF cases were reported to be omitted by NBS CF. Disease incidence in Poland based on the programme results was estimated as 1/4394 and carrier frequency as 1/33. The frequency of the F508del was similar (62%) to population data previously reported. This strategy allowed us to identify 29 affected infants with rare genotypes. The frequency of some mutations (eg, 2184insA, K710X) was assessed in Poland for the first time. Thus, sequencing assay seems to be accurate method for screening programme using blood spots in the Polish population.
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INTRODUCTION

Cystic fibrosis (CF) is one of the most common life-shortening autosomal recessive diseases in Caucasian population, affecting about 1 person in every 2500–3500.[1] Previously, the incidence of CF in Poland equalled 1/2500 as calculated on the basis of the available epidemiological data.[2] However, only 1440 CF patients in Poland (of over 38 million inhabitants) were registered in Polish Cystic Fibrosis Registry (data from 30 September 2010).[3] According to the Wisconsin Cystic Fibrosis Neonatal Screening Study Group and others,[4,5] newborn screening for CF (NBS CF) provides an immediate and accurate diagnosis, often before the onset of clinical symptoms and is associated with significantly improved physical development and a lower prevalence of lung infections. Neonatal screening for CF has been widely implemented and accepted, however, ethical aspects with regard to possible benefits and risks are still a matter of debate.[6]

CF screening programmes are based on the immunoreactive trypsin (IRT) assay on dried blood spots.[7] The elevated levels of IRT have a relatively poor positive predictive value in the neonatal period, therefore earlier protocols relied on a second sample to confirm the initial screening result (IRT/IRT protocol),[8] thereby enhancing sensitivity of affected newborn detection during the NBS CF to 80%.[9] The identification of the *CFTR* gene and pathogenic mutations subsequently have opened a gateway for the genetic testing of the DNA from the original blood spot. A two-tier neonatal screening programme (IRT/DNA) was first performed in South Australia in 1989.[10] Furthermore, a reduction of molecular techniques costs has allowed the possibility of direct gene analysis as a second analytic stage in many national screening programmes. Unfortunately, in the *CFTR* gene (188 kb, 27 exons) nearly two thousand sequencing variants have been identified to date (precise count for 31 October 2011: 1893 according to the *CFTR* Mutation Database, http://www.genet.sickkids.on.ca/app). The relative frequencies of mutations vary in different ethnic groups and populations[11]. Only one mutation, the F508del accounts for ~70% of *CFTR* mutant alleles in Europe but this also differs between populations.[12,13] In Polish CF patients the frequency of the F508del mutation is estimated as 53–57%.[14,15]

According to the a panel of the most frequent mutations in Polish CF patients published in 2009[16] and our personal experience (unpublished data), mutations such as K710X and 2184insA have a frequency >0.45%. As these sequence variants, are not included in commercial assays (eg, InnoLipa Innogenetics, CF assay Abbott, Tepnel Diagnostics Elucigene), we decided to apply the direct sequencing of selected *CFTR* gene regions as part of the genetic protocol of NBS CF, in order to avoid the poor effectiveness of the IRT/DNA protocol using commercial kits.

The first pilot NBS CF study was introduced in Poland in 1999 and continued until 2002, covering one-quarter of the Polish population.[17] The current programme was implemented in 2006 and was expanded to cover whole country in summer 2009 (Figure 1a).

The aim of this summary is to evaluate the strategy of the NBS CF programme in Poland with respect to the usefulness of the DNA sequencing, as well as to revise the panel of most frequent *CFTR* mutations in the Polish population.

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a

Period of NBS CF	Method	The most frequent mutations in Polish population under analysis
September 2006 - December 2007	Estonia Asper Biotech assay	E60X, G85E, 394delTT, R117H, R117P, R117L, I148T, 621G>A, 711+1G>T, 711+5G>A, 1078delT, R334W, R347H, R347P, R347L, IVS8-T, A455E, I507del, F508del, 1717-1G>A, G542X, p.G551D, Q552X, R553X, R553G, R560T, R560K, 1898+1G>A, 1898+1G>T, 1898+1G>C, 2143delT, 2184delA, 2183AA>G, 2789+5G>A, 3120+1G>A, 3199del6, 3272-26A>G, R1162X, 3659delC, 3849+10kbC>T, 3905insT, S1235R, S1251N, W1282X, W1282C, N1303K, CFTRdele2,3
January 2007 - June 2009	Sanger sequencing of exons: 4, 7, 10, 11, 13, 21, fragment of intron 19	F508del, CFTRdele2,3, 3849+10kbC>T, R117H+IVS8-T*, R334W, R347P, 1717-1G>A, G542X, R553X, K710X, 2184insA, 2143delT, 2183AA>G, N1303K
July 2009 - currently	Sanger sequencing of exons: 7, 10, 11, 13, 17b, 20, 21, fragment of intron 19	F508del, CFTRdele2,3, 3849+10kbC>T, R334W, R347P, 1717-1G>A, G542X, R553X, K710X, 2184insA, 2143delT, 2183AA>G, N1303K, 3272-26A>G**, W1282X**

* removed from DNA analysis since July 2009 , **added into DNA analysis since July 2009

b

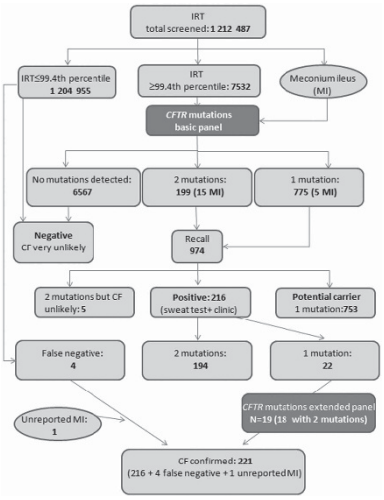

Figure 1 NBS CF in Poland. (**a**) Spectrum of mutations analysed; (**b**) strategy and summary of obtained results.

PATIENTS AND METHODS

Study population

The study group includes neonates born between September 2006 to December 2010. The screening programme was gradually introduced into Polish territory and since the summer of 2009, all neonates born in Poland have been included into NBS CF programme. A total of 1 212 487 newborns were screened for CF.

IRT analysis protocol

IRT was measured in dried blood spot samples from neonates (aged 3–5 days). To determine an IRT concentration (ng/ml), the IRT Neonatal Screening ELISA colorimetric assay (IBL International, Hamburg, Germany) was used. According to results from the pilot NBS CF programme completed in 2002, the IRT concentration cutoff was established as >99.4 percentile. In addition in all neonates with meconium ileus (MI), the DNA analysis was performed regardless of IRT value.

DNA analysis

Genomic DNA was eluted from two 3-mm diamater discs using the Extract Blood PCR Kit (Sigma Aldrich, St Louis, MO, USA). According to the Polish Society of Cystic Fibrosis recommendation,[16] for the purpose of NBS CF, a panel of the *CFTR* gene mutations showed in Figure 1a was implemented. This panel and the examined regions of the *CFTR* gene were slightly altered during the screening programme.

The selected *CFTR* gene regions (Figure 1a) were analysed by direct sequencing. PCR and sequencing primers were either made as described previously[18] or designed with the Primer 3 software (Geeknet Inc., Fairfax, VA, USA). The presence of the CFTRdele2,3 mutation was determined according to Dörk *et al*[19] PCR reaction mix and conditions are available on request. PCR products were purified using EXOSAP-IT (USB Corporation, Cleveland, OH, USA) according to the manufacturer's protocol. Standard sequencing reaction and capillary electrophoresis using ABI PRISM 3730 sequencer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) were performed. Fluorochromatograms were analysed using the Mutation Surveyor software (SoftGenetics, State College, PA, USA). Sequence NM_000492.3 was used as a reference. Novel mutations were analysed with prediction module of Alamut version 1.53 software (Interactive Biosoftware, Rouen, France). The *CFTR* mutations nomenclature is consistent with the *CFTR* Mutation Database legacy names (http://www.genet.sickkids.on.ca). Other databases used in the study were as follows: NCBI (http://www.ncbi.nlm.nih.gov), Ensembl (http://www.ensembl.org/index.html), 1000 Genomes (http://www.1000genomes.org) and HGMD Professional (https://portal.biobase-international.com/hgmd, http://www.hgmd.cf.ac.uk/ac/index.php).

Clinical confirmation of CF

Neonates with one or two identified mutations were referred directly for clinical assessment and a sweat test at the age of 3–4 weeks to CF Centres in Poland (eight centres). The sweat tests were performed by the classic Gibson and Cook method and Nanoduct method parallely.[20] The diagnosis of CF was established according to recommendations.[16] Parents of neonates that were identified as affected or carriers were suggested genetic counselling and were offered genetic testing.

RESULTS

During a screening project lasting over 4 years, more than one million newborns were screened (Figure 1b). For each positive IRT result (>99.4 percentile) direct *CFTR* gene analysis was performed by sequencing of selected regions. The complete analysis using this diagnostic panel allowed us to identify (1) nearly 80% of the mutated alleles in Poland and (2) mutations in one or both alleles in nearly 95% of screened CF patients (according to frequency data published by Bobadilla *et al*[15]). Blood trypsin values above the threshold were found in 7532 neonates. After biochemical, genetic and clinical evaluation, 216 affected subjects (194 genotyped and 22 with 1 mutation identified so far) were identified in the NBS CF programme. This group includes 20 cases of (MI; 9.3%, 20/216). In addition, one newborn with MI did not have any genetic analyses performed, because information about MI was not printed on the screening card and IRT was below the cutoff for IRT. This newborn had *CFTR* molecular analysis performed at the age of 2.5 years (genotype: [F508del];[F508del]) due to clinical symptoms. So far (until March 2012) we have had information about four cases of false-negative results in NBS CF (Table 1). Thus, the total number of CF cases identified in the period of NBS CF in Poland is 221 (216 from NBS CF, 4 omitted by NBS CF and 1 with unreported MI).

In five other children, despite two *CFTR* mutations detected, a clinical evaluation did not confirm the presence of CF. These newborns had the *CFTR* genotype as follows: [F508del];[D537N], [F508del];[P731L], [F508del];[T1053I] (two unrelated newborns) and [F508del];[L467F]. Mutations D537N and P731L have not been

Table 1 Characteristic of the cases omitted in the screening for CF programme owing to IRT values <99.4 percentile

Newborn	*Patients' genotype after first stage CFTR analysis*[a]	*Sweat test (pilocarpine ionthoforesis (mmol/l))*	*Clinical history*	*Patients' genotype after extended CFTR analysis (performed on physician's request; sequencing of entire coding region)*
1	[2183AA>G];[=]	116; 139	Recurrent diarrhoea, pneumonia, liver dysfunction	[2183AA>G];[E92K]
2	[F508del];[=]	80; 127; 136	Chronic diarrhoea, failure to thrive, pneumonia	[F508del];[4218insT]
3	[=];[=]	118;140	Pneumonia, liver dysfunction	[Q207X];[=]
4	[=];[=]	56	Diarrhoea, pneumonia	[L997F];[1210-12T[5] +1210-13G>T][b]

Abbreviations: CF, cystic fibrosis; IRT, immunoreactive trypsin; NBS CF, newborn screening for CF; =, no mutation identified.
[a]The analysis covered the same mutation panel as in the case of NBS CF.
[b]cs3G>T=IVS8-5T+(TG)13.

Table 2 Genotypes of CF newborns with mutations not included into common commercial kits applied in Poland and European countries*

Genotype	*Number of cases*
[F508del]; [1767-8T>A*]	1
[F508del];[2184insA*]	6
[F508del];[E33X*]	1
[F508del];[F1286C*]	1
[F508del];[G314R*]	1
[F508del];[K710X*]	1
[F508del];[W1282R*]	1
[F508del];[1898+1G>C*]	1
[F508del];[3600+2insT*]	1
[F508del];[F1052V*]	1
[F508del];[V1240G*]	1
[F508del];[T582I*]	1
[2143delT];[R1102X*]	1
[2143delT];[2721del11*]	1
[3272-26A>G];[K967S*]	1
[CFTRdele2,3];[Y1092X*]	1
[K710X*];[K710X*]	1
[L732X*];[3600+2insT*]	1
[N1303K];[2184insA*]	1
[N1303L];[T1036I*]	1
[R553X];[3182ins8*]	1
[2143delT];[V1240G*]	1
[R553X];[Trp356X*]	1
[L997F*];[1210-12T[5];1210-13G>T]	1
Total	29

Table 3 Frequency of *CFTR* mutations in Polish CF patients from newborns screening programme

CFTR mutations		*Frequency according to Bobadilla et al*[15] *%*	*Frequency according to NBS CF results (all=442 CF alleles) %*
Name	*Position*		
F508del	Exon11	57.1	62.4
3849+10kbC>T	Intron 22	2.7	3.0
G542X	Exon 12	2.6	1.6
1717-1G>A	Intron 11	2.4	1.4
R553X	Exon 12	1.9	2.5
CFTRdele2,3	Exons 2 and 3	1.8	6.2
N1303K	Exon 24	1.8	2.1
2143delT	Exon 14	No data	2.8
2184insA	Exon 14	No data	1.8
2183AA>G	Exon 14	No data	1.6
W1282X	Exon 23	0.7	1.5
R334W	Exon 8	No data	0.7
R347P	Exon 8	No data	0.5
G551D	Exon 12	0.5	0.0
K710X	Exon 14	No data	0.7
3272-26A>G	Intron 19	No data	0.7
3600+2insT	Intron 21	No data	0.5
1898+1G>C	Intron 13	No data	0.5
V1240G	Exon 23	No data	0.5
Others[a]	—	No data	10.0

Abbreviations: CF, cystic fibrosis; NBS CF, newborn screening for CF.
[a]Mutations not included into common commercial kits.

reported to the *CFTR* Mutation Database so far. Beside these two, several other new defects not registered in the *CFTR* Mutation Database were detected as well as rare mutations that were not included into commercial assays (Tables 2 and 3).

The strategy based on sequencing of *CFTR* regions implemented into NBS CF allowed us to identify a full genotype for 29 newborns (13% of all identified CF cases), which could not be detected by other routinely used molecular methods (Table 2). In addition, in 22 subjects, extended *CFTR* genetic analysis was suggested owing to abnormal or borderline sweat tests results and clinical suspicion of CF.

The pathogenicy status of novel and rare missense variants was assessed by bioinformatic analysis using the Alamut software. This programme evaluates effect of particular sequence variations using PolyPhen, Align-GVGD and SIFT alghoritms. The D537N variant in exon 11 was designated as possibly pathogenic and P731L as possibly tolerated. Of note, in the case of some other changes, the results were unclear – for example, in the case of already known mutations published in the CFTR Mutation Database: T1053I (according to Align-GVGD: 'less likely to be pathogenic', score C0, PolyPhen: 'possible damaging', score 0.816 and SIFT: 'deleterious', score 0.01), similarly for L467F (Align-GVGD: 'less likely', class C0, PolyPhen: 'probably damaging', score 0.994 and SIFT: 'deleterious', score 0.03).

The evaluation of the most frequent *CFTR* mutations in the Polish population in examined gene regions showed that three additional mutations reached our adopted cutoff (0.45% of frequency): 3600+2insT, 1898+1G>C and V1240G (Table 3). We assumed that during the period of the NBS CF programme, we detected all F508del homozygotes (90/221 cases, 41%). Based on this assumption, the previously published data of the frequency of this mutation in the Polish population (57%,[15]), the data from the Polish Cystic Fibrosis Patients Registry[3] (56–62%) and the results of the clinical follow-up

ending in December 2011, we calculated the CF incidence in our country as 1/4394 and carrier frequency as 1/33. The incidence and carrier frequency are lower (1/5249:5486 and 1/36:37, respectively) when results from NBS CF are taken into account.

DISCUSSION

The screening programme for CF has been implemented for many years in the Europe, USA and Australia despite the discussion over its benefits and risks.[21] Here, we present the first summary of the NBS CF in Poland based on the IRT and sequencing of selected *CFTR* fragments.

Our NBS CF strategy lead to the diagnosis of CF in 216 neonates. The DNA analysis protocol implemented here focused on regions containing the most frequent mutations in the Polish population covering almost 80% of mutated alleles. The 90 F508del homozygotes (41%) were identified during the period of the NBS CF programme and this percentage is in a large convergence with data from the Polish Cystic Fibrosis Registry (38%, according to Stężowska-Kubiak[3]).

In recent years, the population analysis data are often used for the recalculation of the CF incidence and *CFTR* mutation carriership. However, the significance of these values is questionable due to the extent of false-negative results, and the incompleteness of data. This particulary important within the first years of a screening programme, when clinical follow-ups are continued. The incidence of the CF in Poland calculated on the number of F508del homozygotes and the previously reported F508del allele frequency[15] would be 1/4394. The incidence of disease estimated directly on basis of the NBS CF programme results is 1 per 5248–5486. In past, in the 1970s this parameter has been generally defined in Poland as 1/2500, however, in study of Bozkowa *et al*[2] selection of examined groups was not accurately matched to disease prevalence calculation. CF prevalence is more difficult to ascertain for a number of reasons, such as different medical and scientific data from published literature and from patient registries. The results of other screening programmes also show a lower incidence of the disease, than traditionally adopted 1/2500.[22] Data from European countries, published in 2008 point an interregional variation as well (Table 4).[23] Similarly, The American College of Medical Genetics with American College of Obstetricians and Gynaecologists published summary from 8 years of CF testing and estimated carrier frequency as 1/37.6 in the tested panethnic population, which allows to calculate the CF frequency as 1/5655.[24]

It is probable that the incidence of the disease in Poland is lower than the value declared in the 70s, however, its precise calculation can only be possible when the results of the NBS CF programme are summarized with the addition of clinical follow-up. We also cannot rule out that more patients have been omitted by NBS CF, like the four cases reported in this study. According to Fritz formula,[9] the predicted false-negative rate for the Polish population is 6–7 cases per year, which gives 1–2 omitted CF cases per 100 000 live births. After a longer follow-up, some children that are currently defined as carriers would turn out to be affected with rather mild CF or *CFTR*related diseases. In addition, according to the tendency of a smaller family model (2 + 1) in Europe[25] and prenatal screening,[26] a lower prevalence of the disease will probably be observed in the future. Such tendencies have already been observed.[27,28]

Beside analytical and statistical limitations, another possible reason is the under representation of children with MI. In our study, the newborns with MI only represent 9.3%, while the literature data shows about 10–20%. In neonates with MI, normal values of the IRT are often observed.[21] Such newborns could have obtained a false-negative result of IRT and had not been redirected for genetic testing.

Table 4 Incidence of CF in selected European country from the literature, national CF registries and personal information from CF leaders in Europe (according to Farrell,[23] modified)

Country	*CF incidence*
Austria	1:3500
Belgium	1:2850
Bulgaria	1:2500
Czech Republic	1:2833
Denmark	1:4700
Estonia	1:4500
France	1:4700
Germany	1:3300
Greece	1:3500
Italy	1:4238
Netherlands	1:4750
Slovakia	1:1800
Slovenia	1:3000
Sweden	1:5600
Poland	1:5000
	1:4394 (from this study)

Abbreviation: CF, cystic fibrosis.

By preparing a newborn screening programme in Poland, we collected information about the *CFTR* mutations detected in Polish CF patients that are recognized on the basis of the clinical outcome (data from The Polish Cystic Fibrosis Registry and Bobadilla *et al*[15]). According to these data, the F508del frequency was estimated as ;56–62%. The frequency of the F508del allele in this group is similar to the value calculated from the NBS CF programme (62%) and previously published data[15] (57%). Data from the Polish registry were used to designate the *CFTR* gene mutations and to prepare a 'Polish assay' with mutations such as K710X and 2184insA. The exonic 2184insA mutation in addition to others (eg, CFTRdele2,3, 3849 + 10kbC > T) occurs more frequently in Central and Eastern Europe.[29] Thus, the main reason for the application of our strategy was the diversity of mutations in the Polish population, for which commercial tests are not suited (Tables 2 and 3). An extended molecular analysis revealed that 3600 + 2insT, mutations in *loci* 1898 + 1 (G > C or G > A) were detected in two and three cases of CF children with extended analysis (thus > cut off 0.45% of mutated alleles detected in NBS CF). These variants, together with V1240G (two alleles, frequency > 0.45%) may therefore be candidates for a first line NBS CF molecular test panel in future. However, these results should be confirmed on a larger sample in subsequent years of NBS CF.

A selective *CFTR* gene scanning strategy was also used in other populations.[30] An additional advantage of such diagnostic procedure is its ability to discover new or ultra-rare *CFTR* alleles. Indeed, we discovered about 30 sequence variants unreported previously.

We realize that the discovery of new defects is not always beneficial owing to interpretational controversies. In example, the detection of a sporadic missense variants (eg, D537N, P731L), still do not provide a clear answer to the biological and clinical significance of the defect. The D537N variant was designated as possibly pathogenic by bioinformatic analysis, whereas the P731L was designated as 'tolerated'. Both variants were detected in the

newborns (current ages: 3 years 2 months and 1 year 5 months, respectively), that had the F508del mutation identified in the second allele, and sweat chloride concentration 14.9 and 31.2 mmol/l, respectively. Until now, the disease based on clinical data has not been confirmed in these children. However, at this time, we cannot exclude the possible development of an atypical CF or *CFTR*-related disorders (eg, CBAVD – both children are males).

A similar problem was observed while detecting rare known mutations published in the *CFTR* Mutation Database. For example, the diagnosis of CF has not been confirmed following the identification of the T1053I and L467F mutations. As this is common problem in the NBS CF programmes, the term '*CFTR*-related metabolic syndrome' was proposed.[31]

Dorfman *et al*[32] in his work concluded that none of the current generation of *in silico* software accurately predicted the clinical severity of the *CFTR* mutations in a reliable way. Therefore, each bioinformatic analysis performed by us only served as an indicator rather than a conclusive diagnostic marker. However, new genotype–phenotype relations are still being discovered that may lead to the requalification of a mutational status. For example, the I148T variant no longer has the qualification of a pathogenic mutation but is still included in the commercial kits (eg, InnoLipa, Innogenetics).[33] Prudence is therefore always essential in molecular genetics.

Our experience shows that DNA protocol based on sequencing method is an efficient screening strategy in Poland. In addition to issues described above, this approach seems to be highly cost-effective considering the fact that, there is no need to repeat the analysis of exons examined during NBS CF programme, if extension of the *CFTR* gene analysis is necessary. However, the limitations of the IRT/DNA test should not be forgotten and each patient with signs or symptoms of CF should be referred to the CF centre for standard diagnostic procedure.

In summary, to our knowledge, this is the world's first full description of a CF newborn screening strategy based on IRT/DNA-extended genetic analysis method, recently recommended by the Clinical and Laboratory Standards Institute.[34]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX

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