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NEONATAL CYSTIC FIBROSIS SCREENING IN LATVIA: A PILOT PROJECT

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Cystic fibrosis (CF) is one of the most common severe autosomal recessive diseases in the Northern European population affecting 1:2000–4000 individuals worldwide and 1:3300 in Latvia. Every year in Latvia since 1994, the approximate birth rate is 20,000 newborns per year, so theoretically there should be 6–8 new cases of CF per year. In Latvia, since 1998, there have been 49 patients with clinically confirmed diagnosis of CF. Recognition of a person with cystic fibrosis has always been a challenging task for clinicians, because of the constellation of symptoms, which can easily be misleading and explained by other aetiology. Therefore, in several countries, neonatal screening programmes using immunoreactive trypsinogen (IRT) have been introduced. The aim of this study was to evaluate the possible introduction of a neonatal IRT and DNA screening programme for diagnostic confirmation of cystic fibrosis in Latvian infants. IRT was measured in dried blood spots from 7,040 newborns, and DNA analysis for mutations dF508 and 394delTT performed for all individuals with increased IRT. Two persons suspected to have CF were identified, which is in accordance with population studies of CF frequency in Latvia. The estimated frequency of CF in Latvia of 1:3,520 corresponds to the average frequency in Europe. A mutation panel examining 230 mutations by APEX was applied to all samples with an IRT level above 100 ng/ml and two patients with the diagnosis of CF were identified.

Key words: *cystic fibrosis, neonatal screening, IRT/DNA.*

INTRODUCTION

Cystic fibrosis (CF) is one of the most common severe autosomal recessive diseases in the Northern European population, affecting 1:2,000–4,000 individuals worldwide (Scriver *et al.,* 2001). CF frequency in Latvia was estimated based on previous studies by Krumina *et al*. who reported it as 1:3300. CF is a complex disease affecting a number of organ systems including the genitourinary tract, respiratory tract, and causing pancreatic insufficiency (Ferec *et al*., 2006).

Cystic fibrosis is caused by numerous mutations in the *CFTR* (*ABCC7*) gene. Thus far, approximately 1,640 mutations have been identified (Anonymous, 2009). Phenylalanine deletion in the 508 position or dF508 is the most common mutation with highest incidence in Northern Europe reaching 70% (*Karem et al*., 1989). The contribution of the dF508 mutation to all CF mutations in Latvia is 61% (Krumina *et al*., 2001). Every year, since 1994, in Latvia, the approximate birth rate is 20,000 newborns per year, so theoretically there should be 6–8 new cases of CF per year (Svabe, 2001). Thus far in Latvia, since 1998, there have been 49 patients with clinically confirmed diagnosis of CF (Svabe *et al*., 2001), 162 cases had been registered by archive studies on post-mortem data overall in Latvia (Znotina *et al*., 2006).

Recognition of a person with cystic fibrosis has always been a challenging task for clinicians, because of the constellation of symptoms, which can easily be misleading and explained by other aetiology. Therefore, in several countries, neonatal screening programmes using immunoreactive trypsinogen (IRT) have been introduced (Hammond *et al*., 1991).

Immunoreactive trypsinogen screening is highly specific, but five percent of cases produce false negative results. Therefore, several tests should be added to confirm diagnosis so that patients may be appropriately referred to a CF centre (Wilcken *et al*., 2003). IRT could be followed by repeated IRT after three weeks (IRT/IRT). Its sensitivity is

considered low at 80.2%, and considerably increased sensitivity (96%) has been observed for IRT complemented with DNA analysis (IRT/DNA) using a population-specific mutation panel with at least 24 mutations (Kloosterboer *et al*., 2009; Corbetta *et al*., 2002).

The immunoreactive trypsinogen test was developed to identify diseases affecting pancreatic function such as pancreatitis, pancreatic cancer, and CF. Trypsinogen is an enzyme secreted by the pancreas and is a precursor of trypsin. Trypsin breaks down protein in the duodenum (Scriver *et al*., 2001). Proposals for neonatal screening with IRT are often met with counter arguments about the cost–benefit of an expensive neonatal test followed by molecular analysis and failure to heal disease completely after its recognition. There have been several publications on long-term follow-up showing considerably better overall health of patients with cystic fibrosis, if they are treated early in comparison with delayed treatment, which may result for some in irreversible organ damage. It was concluded that CF patients identified in newborn screening programmes have better growth and reduced morbidity, with slower CF lung disease progression (Sims *et al*., 2007; Hammond *et al*., 1991).

The aim of this study was to evaluate possible introduction of a neonatal IRT/DNA screening and diagnostic confirmation programme for cystic fibrosis in Latvia.

MATERIALS AND METHODS

Immunoreactive trypsinogen was measured in the dried blood spots from 7,040 newborns (3,000 samples in 2007 and 4,040 samples in 2008).

IRT detection. Cut-off values for IRT in 2007 were 60 ng/ml and in 2008 this was increased to 80 ng/ml. Patients with increased IRT were invited to the Children's University Hospital Medical Genetics clinic to undergo blood sampling for further DNA analysis and a sweat test.

DNA analysis. To isolate DNA, dried blood samples were dissolved them in water, and subsequently incubated in methanol at room temperature for 15 min. Denaturing was performed using 5 mM NaOH for 10 min at 100 ^{o}C. Samples were chilled on ice and stored at –20 ^{o}C.

CFTR gene mutation analysis was performed first for the most common mutation, dF508, using PCR (polymerase chain reaction) and a heteroduplex method visualised by PAAG (polyacrylamide gel) electrophoresis. In cases when patients had increased IRT levels (100), but no dF508 mutation in either allele or only in one allele, APEX (Asper Biotech, Estonia) genotyping technology or an INNO-LiPA CFTR17+Tn test (Innogenetics, Belgium) was applied. INNO-LiPA CFTR17+Tn assays are line probe assays based on reverse hybridisation. This method can detect and identify 17 CF related mutations and their wild-type sequences (621+1G→T, 3849+10 kbC→T, 2183AA→G, 394delTT, 2789+5G→A, R1162X, 3659delC, R117H, R334W, R347P, G85E, 1078delT, A455E, 2143delT, E60X, 2184delA, 711+5G→A), as well as the congenital bilateral absence of the vas deferens (CBAVD) related to Tn polymorphism. APEX (Arrayed Primer Extension) is a genotyping technology that combines the efficiency of a microarray-based assay with the comparable accuracy of Sanger dideoxy sequencing. This method can detect 230 CF mutations. Both methods have standard protocols established by their manufacturer.

Research was approved by the Ethics Committee of the Republic of Latvia, No. 28.05.2003 A-10.

RESULTS

In 2007, there were 3,000 samples screened and 22 (0.7%) persons were identified with IRT values above the cut-off level of 60 ng/ml.

According to recent publications and kit manufacturer recommendations, in 2008, the cut-off level was increased to 80 ng/ml; 4,040 samples were screened and 13 (0.32%) neonates identified (Table 1).

Table 1

IRT* VALUES IN NEWBORNS

Total samples	Number of newborns with elevated IRT	IRT mean ng/ml	IRT max ng/ml	IRT min ng/ml
3,000	22	98.1 SD 33.9	184.3	59.9
4,040	13	187.4 SD 146.4	541.0	87.4
3,000**	14	114.9 SD 29.0	184.3	83.2

* IRt, immunoreactive trypsinogen; ** excluded samples with IRT values between 59.9–79.9. Cut-off value established as 80 ng/ml.

Thirty-five neonates identified from IRT screening were tested for the most common mutation dF508 and 394delTT, and two heterozygous individuals (dF508/?) were identified. Both of them performed a sweat test, and in one patient, this test was positive, thus confirming diagnosis of cystic fibrosis in the first patient in Latvia through neonatal CF screening. The IRT value of the CF patient was 282 ng/ml. Mutation screening performed for 230 mutations failed to identify the second mutation in the *CFTR* gene.

The second patient identified as heterozygous showed normal sweat test results. The IRT value was 87.4 ng/ml and it was concluded that the identification as heterozygous for dF508 mutation was a false positive.

The mutation del394delTT was not identified in patients and the most common 17 mutations from the INNO-LiPA panel were negative.

For the remaining persons with an elevated IRT 100 ng/ml, mutation screening is still in progress.

DISCUSSION

Cystic fibrosis is one of the most common life-threatening autosomal recessive disorders in the Western world. The estimated frequency of CF in Latvia of 1:3,520 corresponds to the previously calculated frequency in Latvia based on population studies, i.e., 1:3,300 (Krumina *et al*., 2001).

Worldwide, the dF508 mutation is responsible for approximately two-thirds (61%) of all CF chromosomal aberrations. This common mutation varies from a maximum of 100% in the isolated Faroe Islands of Denmark, to a minimum of about 20% in Turkey (Bobadilla *et al*., 2002). In Latvia, the dF508 mutation is responsible for 61% of cases (Krumina *et al*., 2001). Whilst there is mostly great mutational heterogeneity in the remaining one-third of all alleles globally, there are partially private mutations arising *de novo* in families, and therefore population-based mutation panels do not recognise them.

In most European countries, neonatal CF screening has been recognised as important cost-effective health care provision based on CF frequency (Loeber, 2007).

The frequency of most *CFTR* mutations is highly variable and is often a function of the ethnic or geographic origin of the parents and grandparents of the affected child. Firstly, every country needs to determine their main population mutation panel. With the introduction of new technology such as APEX, which allows for the implementation of large-scale, multi-mutation screening programmes, it is essential to have a complete understanding of which populations are carriers for selected mutations.

In a project using molecular methods, PCR, heteroduplex, reverse hybridisation, and APEX were not universal. For CF mutation detection, methods that are more complex are required. For example, PCR and heteroduplex methods are inexpensive, fast, specific, easy to analyse, and do not require expensive equipment. However, this method can detect only a few CF mutations. Reverse hybridisation and APEX methods are expensive and time-consuming, and, for APEX methods, require technical skill, but they can detect a comparatively large spectrum of mutations. The INNO-LiPA CFTR kit does not include mutations that are frequent in the Latvian population and demonstrates that a different DNA analysis method to complement CF neonatal screening is required in Latvia.

IRT cut-off values should not be lowered below 80 ng/ml. None of the individuals with lower IRT have been identified as CF patients. Lowering IRT cut-off values will only serve to increase the number of false positive results, thus aggravating more families with the concern of having children born with CF and increasing the total cost of neonatal CF screening programmes.

CF screening methodologies vary within and between countries worldwide as health care is a national undertaking. In Latvia between the years 2007–2008, 7,040 newborns period were tested to immunoreactive trypsinogen; in Austria, in 2004, 79,022 were screened for CF; in Germany 45,822, in Scotland 54,612 and in France 784,663 infants (Loeber, 2007).

There are different CF screening strategies in Europe: IRT, IRT/IRT, IRT/DNA (single (common) mutation), IRT/DNA (multiple mutations), IRT/DNA/IRT, IRT/DNA/failsafe step (sweat test), IRT/DNA/DNA (single (common) mutation/multiple mutations in second tier test). All current screening programmes use a measurement of immunoreactive trypsin as a primary screening test, and in most, a second tier test involves analysing DNA mutations (Wilcken *et al*., 2007). Different methods are used for mutation detection, for example, PCR (polymerase chain reaction), RFLP (restriction fragment length polymorphism), DGGE (denaturing gradient electrophoresis), APEX (Arrayed Primer Extension), reverse hybridisation and etc. The choice of DNA mutations and method depends on the genetic background in the region, and considerations of cost. No method or strategy will suit all regions. The IRT/DNA/sweat test model in Latvia was chosen mainly by unsatisfactory and aggravating response of parents of children with elevated IRT. The sweat test as a secondary step in CF neonatal screening can confirm CF diagnosis where mutation panel is limited.

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JAUNDZIMUŠO CISTISKĀS FIBROZES SKRĪNINGA PILOTPROJEKTS LATVIJĀ

Cistiskā fibroze (CF) ir viena no biežākajām autosomāli recesīvajām slimībām, kas Ziemeļeiropas populācijā sastopama 1:2000–4000 personām, Latvijā tā sastopama 1:3300 personām. Kopš 1994. gada Latvijā katru gadu piedzimst apmēram 20 000 jaundzimušo, teorētiski sagaidāmais CF pacientu skaits ir 6–8, bet no 1998. gada Latvijā klīniski diagnosticēti 49 CF pacienti. CF nav raksturīgi specifiski simptomi, tāpēc slimības diagnostika sagādā grūtības. Vairākās valstīs ir ieviests jaundzimušo CF skrīnings. Projekta mērķis bija ieviest Latvijā CF jaundzimušo skrīningu, izmantojot imunoreaktīvā tripsinogēna (IRT) noteikšanu, ko papildina DNS diagnostika. IRT tika noteikts 7040 jaundzimušajiem, visiem ar paaugstinātu IRT līmeni veica dF508 un 394delTT mutāciju analīzi. Projekta rezultātā tika identificēti divi jaundzimušie dF508 heterozigoti, kas atbilst teorētiski sagaidāmai CF sastopamībai Latvijā un Eiropā 1: 3520. Pacientiem, kuriem IRT līmenis bija augstāks par 100 ng/ml, veica 230 mutāciju paneļa analīzi, izmantojot APEX tehnoloģiju, un CF diagnoze tika apstiprināta diviem pacientiem.