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Original

Two years of newborn screening for cystic fibrosis in Turkey: Çukurova experience

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SUMMARY: Şaşihüseyinoğlu AŞ, Altıntaş DU, Bişgin A, Doğruel D, Yılmaz M, Serbes M. Two years of newborn screening for cystic fibrosis in Turkey: Çukurova experience. Turk J Pediatr 2019; 61: 505-512.

The severity of cystic fibrosis (CF) depends on the type of cystic fibrosis transmembrane conductance regulator (*CFTR*) mutation. The primary goal of newborn screening (NBS) is to decrease morbidity, mortality and associated disabilities. The National NBS for CF programme was initiated in Turkey since 01.01.2015. The aim of this study was to present two years of experience of our CF center which is located in the south of Turkey. The study population comprised of infants who were born in Adana between 1 January 2015 - 31 December 2016, referred to our CF center as part of NBS for CF and performed *CFTR* gene analysis. The infants were divided into three groups according to laboratory tests and symptoms as CF, CRMS (cystic fibrosis transmembrane conductance regulator-related metabolic syndrome) and false positive NBS. Between January 1, 2015 and December 31, 2016, NBS was performed in 77,437 newborns in Adana. Two hundred seven (0.26%) newborns screened were positive for CF. A total of 184 infants were included to the study. We reported 12 babies as CF with an incidence of 1:6,452. The babies diagnosed as CF constituted 6.5% of positive CF NBS. Rest of study group diagnosed with CRMS/CFSPID (54/184, 29.5%) and false positive (118/184, 64%). Positive predictive value (PPV) of NBS was 6.5%. The most common *CFTR* mutations were 508del, p.F1052L and p.L997 F. The implementation of CF-NBS program has been successful in Turkey. But it is too early to determine the specificity and sensitivity of the program.

Key words: child, cystic fibrosis, newborn screening.

Cystic fibrosis (CF) is caused by a mutation in the gene that codes for cystic fibrosis transmembrane conductance regulator (*CFTR*) protein, which is most commonly present in the epithelial membrane. CF is the most frequent autosomal recessive hereditary disease in Caucasians, with an incidence of 1:2,000 to 1:3,500 live born infants.[1] Although the frequency of cystic fibrosis in Turkey is not known clearly, it was found to be 1:3,000 by Gürson[2] and his colleagues in 1973. It is widely recognized that CF is a variable condition that may affect the respiratory tract, pancreas, intestine, male genital tract, hepatobiliary system and exocrine sweat glands, resulting in complex multisystem disease.[3] The severity of clinical manifestation depends on the type of *CFTR* mutation. But it is also affected by several other factors, including a complex interaction of infection and inflammation.[4] Although CF remains a multisystem disease, the chronic pulmonary disease is the cause of death in more than 90% of patients.[5] A diagnosis of CF initially relied on phenotype, with clinical recognition of characteristic signs and symptoms.[6] Early diagnosis could reduce morbidity and prolong life, especially if the patients are treated in specialized CF centers.[7]

The primary goal of newborn screening (NBS) is to decrease morbidity, mortality and associated disabilities in affected infants early in life.[1] All CF NBS programs begin with detection of an elevated immunoreactive trypsinogen (IRT) level in a dried blood specimen from the newborn. A positive IRT screen is triaged to second-tier testing, which is repeat enzyme testing, DNA mutation testing, or both. Despite the advent of NBS and improved knowledge about *CFTR* genetics, CF diagnosis remains complex for many reasons, such as inconclusive sweat chloride values, *CFTR* mutations of uncertain pathogenicity, and differential expression of *CFTR* or modifier effects.[8] Also CF NBS introduced a new complexity and diagnostic dilemma, namely infants with abnormal screening tests because of elevated immunoreactive IRT levels but inconclusive sweat tests and/or DNA results. Two different terms for infants with an inconclusive diagnosis have been proposed. In the US, these children are labeled CF-transmembrane conductance regulator-related metabolic syndrome (CRMS), according to the American CF Foundation, whereas "CF screen positive, inconclusive diagnosis" (CFSPID)[9] in Europe.

The National NBS for CF programme has been initiated in Turkey since 01.01.2015 and performed by The Public health institution of Turkey - Child and Adolescent Health Department. IRT/IRT protocol is used for NBS for CF in Turkey. The infants who are identified as positive in the NBS program are directed to CF centers for sweat testing. The algorithm for CF NBS in Turkey is shown in Figure 1.

The aim of this study was to present two-years experience of our CF center where the proportion of consanguineous marriages is 23% .[10]

Methods

Study population

The study population comprised of infants who were born in Adana between 1 January 2015 - 31 December 2016 and were referred to our CF center as a part of NBS for CF and performed *CFTR* gene analysis.

Procedure of the Turkey CF-NBS

Blood samples were taken from newborns by heel prick and spotted on filter paper sampling cards (Guthrie cards) at 72 hour of life. Infants who were above the first IRT level (≥ 90 μg/L) were called for second IRT measurement on the heel blood in 7-14 day of life . If second IRT concentration was above ≥ 70 μg/L, the infant was directed to the nearest CF center for sweat testing by The Public health institution of Turkey - Child and Adolescent Health Department.

Sweat tests

The sweat test was performed for each infant who was a gestational age of 38 weeks or more and a minimum weight of 2,000 g. It was performed at the first day when infants applied to our center. Second sweat test was performed at a different day to the infants who had first sweat test that was borderline or abnormal and had at least one *CFTR* gene mutation or strongly suspected CF because of clinic symptoms. The sweat test was performed by conductivity method. Conductivity method was measured from the sweat sample collected with Macroduct coil system.[12] The conductivity is determined as mmol/L, and this unit represents the molar concentration of sodium chloride solution having the same conductivity as the same sweat sample at the same temperature. The procedure was performed by using NBS program-CF sweat testıng guidelines by three technicians trained in the clinical laboratory of our hospital.[13] The value was considered normal when it was lower than 50 mmol/L, borderline when it was between 50 mmol/L and 89 mmol/L, and abnormal when it was greater than 90 mmol/L.

CFTR gene mutation analysis

All gene sequence analysis was performed for *CFTR* by using a peripheral blood sample. Before periferal blood sampling informed parental consent was taken from all the participants. In all gene sequence analysis, all of the exonic regions and exon intron junctions that encode broadly with the new generation sequencer were analyzed. When mutation was detected, confirmation was made with the

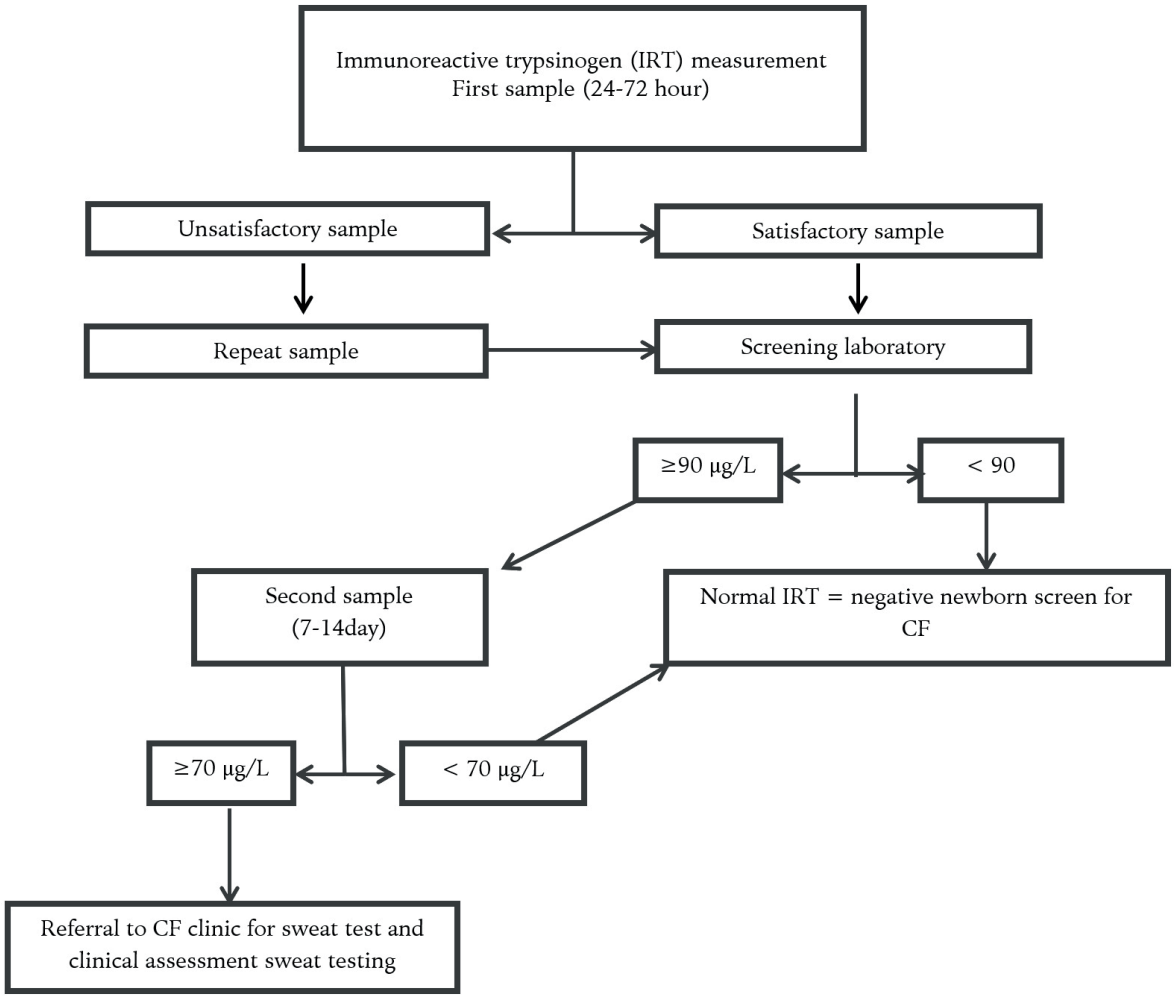

Fig. 1. Cystic fibrosis (CF) newborn screening protocol in Turkey.

Sanger method. The identified mutations were investigated in the *CFTR*2 database or Human Gene Mutation Database (HGMD) database for their clinical significance.

Clinical evaluation

The infants who were referred to our CF center because of positive CF NBS programme had been evaluated at the Cukurova University CF Center at least once per 3 months. The infants were divided to three groups according to laboratory tests and symptoms as CF, CRMS/CFSPID and false positive NBS. CF was diagnosed in infants based on characteristic symptoms in addition to evidence of *CFTR* dysfunction.[14] The designation CRMS/CFSPID was established to address asymptomatic CF NBS positive infants if they presented a positive CF NBS test plus: (1) sweat chloride <30 mmol/L and 2 *CFTR* mutations with 0-1 CF-causing *CFTR* mutations or (2) sweat chloride 30-59 mmol/L and <2 CF-causing *CFTR* mutations.[15] The asymptomatic CF NBS positive infant with presence of no *CFTR* mutation plus a negative sweat test, referred as false positive NBS.

Ethics statement

The research was reviewed and approved by the Institutional Ethics Committee of Çukurova University Balcalı Hospital (08.09.2017-project no: 6/68) in compliance with Declaration Helsinki[11] and informed parental consent was taken from all the participants before inclusion.

Statistical analysis

Quantitative variables were expressed as mean

± standard deviation. Frequencies were used for categorical variables. Non-parametric Kruskal–Wallis and Mann–Whitney tests were used to compare continuous variables. Pearson's and Spearman's correlations were used to examine relationships between continuous variables, as appropriate.

Results

Demographic features

Between January 1, 2015 and December 31, 2016, 77,437 newborns were performed NBS in Adana. Of these, 1,496 (1.9 %) had high initial IRT and second IRT was measured at 7-14 days of life. Three hundred sixty seven (0.47 %) children were evaluated in our CF center because of at least one high IRT level. In the first two years of the CF NBS in Adana, two hundred seven (0.26 %) newborns screened were positive for CF. Twenty three parents of the infant refused to perform genetic analysis. A total of 184 infants were included to the study. The median age of evaluating in CF center was 47 days (range: 27-297). There was no statistically significant difference in age of reference day between the groups. Demographic and laboratory features of study population is shown at Table I.

Laboratory results

The median level of first IRT was 101.8 μg/L (range: 90-368) and 80.1 μg/L (range: 70-259) for second IRT in the study population. Although there was no statistically significant difference in 1. IRT between the groups, 2. IRT values (median 117 ng/ ml; $p \leq 0.001$) were higher in CF group. First sweat analysis was normal in 163 infant (89 %), borderline in 13 infant (7 %) and abnormal in 8 infant (4 %). One hundred sixty four infants (89.3 %) had normal and 14 infants (7.6 %) had borderline sweat analysis. Only seven infants (4 %) had a positive sweat test analysis. Individuals with CF had significantly ($p \leq 0.001$) higher first sweat test values (median 95.1 mmol/L) than subjects classified as CRMS/CFSPID (median 35.2 mmol/L) or false positive (median 29 mmol/L). Ninety three infants (50.5 %) were performed a second sweat test. Only 19 % of second sweat tests was abnormal. As suspected the second sweat test values were higher in the CF group ($p \leq 0.001$). Among the CF patients three babies had normal and one baby had borderline sweat test analysis. Seventy three *CFTR* mutations were found in 64 samples, representing 34.8 % of 184 infants. There were 6 homozygous, 10 compound heterozygote and 47 heterozygote mutations. The most common *CFTR* mutation worldwide p.508del, was found in 6 reported infants, of which three were homozygous. p.F1052L (in six infants), p.L997 F (in 5 infants) were the other common mutations in our study. The features of the groups are shown at Table II.

Follow up and diagnosis

We reported 12 babies as CF with an incidence of 1:6,452. The babies diagnosed as CF

Table I. Demographic and Laboratory Features of Study Population (n=184).

Female, n (%)	97 (52.4%)
Age of reference, days	47.7
Consanguinity, n (%)	41 (22.5%)
First IRT, mean± SD (min-max)	117.8 ± 44.6 (90-368)
Second IRT, mean± SD (min-max)	91.65 ± 28.6 (70-259)
First sweat analysis, mean± SD (min-max)	37.4 ± 24 (10-158)
Second sweat analysis, mean± SD (min-max)	36.4 ± 24 (12-160)
Diagnosis, n (%)	
Cystic fibrosis	12 (6.5%)
CRMS	54 (29.5%)
False positive	118 (64.0%)

IRT: immunoreactive trypsinogen

Table II. Features of Groups.

Features	Cystic fibrosis (n:12)	CRMS (n:54)	False positive (n:118)	p
Age of reference, days*	42.8 (22-72)	44.8 (21-154)	49.5 (20-297)	0.70
Consanguinity, n (%)	3 (25.0%)	16 (29.4%)	16 (13.6%)	0.44
First IRT, μg/L*	139 (22-72)	109.1 (90-242)	119.7 (90-368)	0.17
Second IRT, μg/L*	134.8 (74-259)	87.4 (70-157)	88.3 (70-169)	0.001
First sweat analysis, mmol/L*	95.1 (17-158)	35.2 (13-89)	32.6 (10-75)	0.001
Second sweat analysis, mmol/L*	74.5 (74-110)	32.7 (12-110)	26.9 (14-48)	0.001

*: results are presented as median (minimum-maximum)
CRMS: cystic fibrosis transmembrane conductance regulator-related metabolic syndrome, IRT: immunoreactive trypsinogen

constituted 6.5 % of positive CF NBS. The rest of the study group was diagnosed with CRMS/CFSPID (54/184, 29.5 %) and false positive (118/184, 64.0 %). Positive predictive value (PPV) of NBS was 6.5%.

All babies who were diagnosed as CF had respiratory symptoms during study period. There were two cases presented with meconium ileus. The first case (case 67) was referred because of positive NBS when she was 58 days old and p.508del homozygous mutation was found. The second case (case 142) was diagnosed before NBS because of presenting as meconium ileus at the age of 4 days. Then this baby also referred to the clinic because of positive NBS (1.IRT: 183 μg/L, 2. IRT: 163 μg/L, sweat test: 38 mmmol/L and p.Q353X homozygous mutation). It was learned that she was hospitalized because of mecomiun ileus when she was seven days old. Besides that during the study period one infant with false negative NBS was diagnosed as CF. The reasons for initiating the diagnostic process were recurrent infections of the respiratory tract, body mass insufficiency and the CF positive diagnosis in his elder sister. The features of patients with CF are shown at Table III. One infant with CF (p.508del homozygous) died because of sepsis in another hospital.

Discussion

All CF NBS programs begin with detection of a high IRT level in a dried blood specimen from the newborn.[15] Elevated IRT is thought to be related to pancreatic damage often present in infants with CF. But high IRT levels may be associated with intrapartum asphyxia, neonatal infection or respiratory distress.[16] Besides that it is known that infants with meconium ileus may have a normal IRT value even they have CF.[17] To limit the number of false positives and achieve an acceptable combination of sensitivity and specificity second-tier tests are used in infants with raised initial IRT. Second tier tests vary from programme to programme. In Turkey it is a repeat IRT measurement from a second sample taken at day 10-21 of life (IRT-2). The advantages of IRT/IRT algorithm is its low cost and its non-detection of carriers, whereas the disadvantages are the large number of children called for consultation visits, which is connected with the parent's stress and the large number of sweat tests performed.[18] But it is known that IRT/IRT algorithm has lower sensitivity, delayed completion, and higher false-negative rates compared with IRT/DNA NBS algorithms.[19-21] Reports from other NBS programs using the IRT/IRT algorithm had similar PPV with our study.[22,23] Once a positive CF NBS result has been found, sweat chloride testing must be performed to establish a CF diagnosis. Newborns greater than 36 weeks' gestation and >2 kg body weight with a positive CF newborn screen, or positive prenatal genetic test, should have sweat chloride testing performed as soon as possible after 10 days of age, ideally by the end of the neonatal period (4 weeks of age).[17] In our study first sweat test was performed at the inital visit on average, on the 47th day of life. Measurement of sweat chloride concentration by the quantitative pilocarpine iontophoresis test has been accepted as

Table II. Features of Groups.

	Case no	Clinical details	First IRT (μg/L)	Second IRT (μg/L)	Sweat tests (mmol/L)	Mutation analysis
1	17	Recurrent pneumonia	201	114	114-90	p.508del homozygous
2	40	Recurrent pneumonia	238	191	132-100	p.508del homozygous
3	67	Meconium ileus + recurrent pneumonia	104	124	90-110	p.508del homozygous
4	74	Recurrent pneumonia	95	80	110-82	p.A120T heterozygous + p.508del heterozygous
5	80	Recurrent pneumonia	140	118	158-92	IVS15-1G>C heterozygous + p.G241R heterozygous
6	87	Recurrent pneumonia, died at two months of age	282	259	92-*	p.508del homozygous
7	90	Recurrent pneumonia	95	80	110-48	p.G241R heterozygous+ p.T1057R heterozygous
8	105	Recurrent pneumonia	95	118	119-109	p.E528E heterozygous
9	142	Meconium ileus + recurrent pneumonia	183	163	38-58	p.Q353X homozygous
10	192	Recurrent pneumonia	91	74	17-24	p.I148T heterozygous + p.S95A heterozygous
11	261	Recurrent pneumonia	149	76	68-92	p.D1152H homozygous
12	277	Recurrent pneumonia	90	116	94-41	p.I807M heterozygous + p.508del heterozygous

*The patient died.
IRT: immunoreactive trypsinogen

the standard for sweat testing.[24] Also there are many studies that have shown that the conductivity results are well matched with the chloride concentrations.[11,25] It requires less sweat and more frequently yields a result.[26] But the National Committee for Clinical Laboratory Standards (NCCLS) does not accept it as a definitive diagnostic tool, and the Cystic Fibrosis Foundation accepts it only as a screening method.[24] There were CF patients with normal or border line sweat analysis in our study (Table II). Approximately 2% of patients who meet diagnostic criteria, even in individuals with clinical CF, sweat chloride values can be normal or borderline.[27] For this reason additionally *CFTR* gene sequence analysis was performed as a confirmatory test. Due to the spectrum of clinical heterogeneity the recent categorization scheme identifying *CFTR* mutations as 'CF causing' or of 'variable clinical significance' has limited use in actual clinical decision processes.[8,28] Close monitoring of the patients over time is warranted to determine whether they eventually develop CF or maintain milder clinical phenotypes. Additionally, classes I–III *CFTR* mutations that typically lead to classic cases of CF may not cause symptoms in infants and young children.[8,29] For this reason the length of the follow-up period for screening test–positive individuals must be extended.

The increased implementation of NBS has led to a new and complex diagnostic dilemma of infants with abnormal NBS tests but inconclusive sweat tests and/or DNA test results. The CF foundation published guidelines for CF diagnosis.[15] An expert panel used the Delphi method and created a new diagnostic term, *CFTR*-related metabolic syndrome (CRMS).[29] A similar term, CF screen positive, inconclusive diagnosis (CFSPID), was developed in a Delphi process by the European CF Society (ECFS) Neonatal Screening Working Group and introduced recently in Europe as an alternative to CRMS.[30,31] CFSPID reached high levels of agreement in the subsequent round

of the Delphi exercise, creating a category for infants who are asymptomatic, with hypertrypsinogenemia at birth and have either: (1) 0 or 1 *CFTR* mutations, plus intermediate sweat chloride (30-59 mmol/L); or (2) 2 *CFTR* mutations, at least 1 of which has unclear phenotypic consequences, plus a normal sweat chloride (<30 mmol/L).[25]

Several recent studies have provided information about CRMS/CFSPID prevalence and outcomes and longitudinal studies show that these infants do have a small risk of developing CF over time.[32-34] CRMS/CFSPID must be followed by a specialized CF care physician because some will develop manifestations of CF disease. In our study there was no patient who developed CF from other groups during two years, but due to this risk they continue to be followed up.

In conclusion, the implementation of CF-NBS program has been successful in Turkey. It is too early to determine the specity and sensitivity of the program. Continual tracking of outcomes through the CF newborn screening program is required to determine, in the long term, whether the individuals in CRMS/CFSPID and false positive NBS develop manifestations of CF.

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