Neonatal screening for cystic fibrosis using immunoreactive trypsinogen and direct gene analysis: four years' experience

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Abstract

***Objective*—To assess the performance and impact of a two tier neonatal screening programme for cystic fibrosis based on an initial estimation of immunoreactive trypsinogen followed by direct gene analysis.**

***Design*—Four year prospective study of two tier screening strategy. First tier: immunoreactive trypsinogen measured in dried blood spot samples from neonates aged 3-5 days. Second tier: direct gene analysis of cystic fibrosis mutations (ΔF_{508}, ΔI_{506}, $G_{551}D$, $G_{542}X$, and $R_{553}X$) in samples with immunoreactive trypsinogen concentrations in highest 1% and in all neonates with meconium ileus or family history of cystic fibrosis.**

***Setting*—South Australian Neonatal Screening Programme, Adelaide.**

***Subjects*—All 88 752 neonates born in South Australia between December 1989 and December 1993.**

***Interventions*—Neonates with two identifiable mutations were referred directly for clinical assessment and confirmatory sweat test; infants with only one identifiable mutation were recalled for sweat test at age 3-4 weeks. Parents of neonates identified as carriers of cystic fibrosis mutation were counselled and offered genetic testing.**

***Main outcome measures*—Identification of all children with cystic fibrosis in the screened population.**

***Results*—Of 1004 (1·13%) neonates with immunoreactive trypsinogen ≥ 99th centile, 912 (90·8%) had no identifiable mutation. 23 neonates were homozygotes or compound heterozygotes; 69 carried one identifiable mutation, of whom six had positive sweat tests. Median age at clinical assessment for the 29 neonates with cystic fibrosis was 3 weeks; six had meconium ileus and two had affected siblings. 63 neonates were identified as carriers of a cystic fibrosis mutation. Extra laboratory costs for measuring immunoreactive trypsinogen and direct gene analysis were $A1.50 per neonate screened.**

***Conclusion*—This strategy results in early and accurate diagnosis of cystic fibrosis and performs better than screening strategies based on immunoreactive trypsinogen measurement alone.**

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BMJ 1994;308:1469-72

Introduction

Neonatal screening for cystic fibrosis has not been widely accepted.[1-4] There is continuing debate as to whether diagnosis before symptoms appear and early treatment improve the long term outcome significantly,[5-7] and there have been difficulties in developing an acceptable screening strategy. Initial strategies, based on measurement of immunoreactive trypsinogen in neonatal blood spot samples, had high false positive rates, and there was uncertainty about the decision level to be applied.[8,9] To follow this initial measurement by a repeat immunoreactive trypsinogen test or a sweat test, it was necessary to recall large numbers of neonates for further testing, generating considerable anxiety within families.[10-12] If neonatal screening and early diagnosis of cystic fibrosis is to have an effect on the long term outcome of those affected then a screening strategy with improved sensitivity and specificity at an acceptable cost is required.

With the identification of the cystic fibrosis gene[13] it has become evident that direct gene analysis can be applied to screening for cystic fibrosis in certain populations. A successful two tier neonatal screening programme for cystic fibrosis has been operating in South Australia since December 1989. It consists of an initial immunoreactive trypsinogen measurement from blood spots, followed by direct gene analysis from the same sample.[14] We report our experience with this programme up to December 1993 and confirm the effectiveness of this strategy and its advantages in improved sensitivity and low rate of recall. We also outline our approach to the recall of the infants and counselling of their families.

Methods

TESTING

Blood spot specimens—Dried blood spots (Guthrie cards) were collected on Schleicher and Schuell 903 filter paper from neonates aged 3-5 days as part of routine neonatal screening for hypothyroidism, phenylketonuria, and galactosaemia in South Australia. The presence of meconium ileus or a family history of cystic fibrosis was additional information required by the screening laboratory. This information was either given by telephone to the screening laboratory or written on the Guthrie card.

Estimation of immunoreactive trypsinogen—Discs 3 mm in diameter punched from the dried blood spot samples were assayed for immunoreactive trypsinogen by using the AGEN immunoassay modified to use time resolved fluorescence, as previously described.[15] From June 1992, the method was changed to a commercial, time resolved, two site immunofluorometric assay which identifies both the cationic and anionic forms of trypsinogen (neonatal immunoreactive trypsinogen, DELFIA, Kabi-Pharmacia Australia, Sydney). For both assay systems trypsin blood spot calibrators (μg trypsin/l whole blood) were prepared, independently of the commercial reagents, on Schleicher and Schuell 903 filter paper.

Direct gene analysis—The method of analysis for the cystic fibrosis mutations has been described previously.[16] Genomic DNA, eluted from discs 3 mm in diameter, was amplified and the mutations within exon 10 (ΔF_{508} and ΔI_{506}) and, after November 1991, exon 11 ($G_{542}X$, $G_{551}D$, and $R_{553}X$) detected by standard techniques.[17] Results of the mutation analysis are available one to two weeks after receipt of sample.

Sweat testing—Most sweat tests were performed by a limited number of trained staff at the Women's and Children's Hospital by using pilocarpine iontophoresis.[18] The normal reference interval for sweat sodium was < 50 mmol/l and for chloride, < 50 mmol/l.

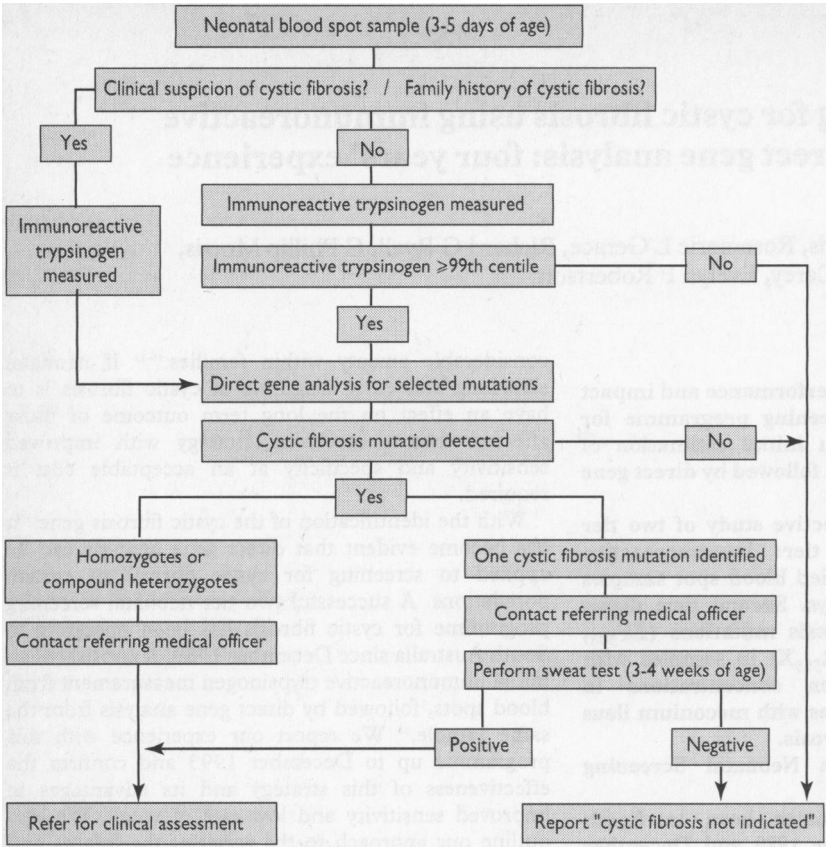

FIG 1—*Neonatal screening strategy for cystic fibrosis in South Australia*

SCREENING STRATEGY

The same screening strategy was used throughout the study (fig 1). Immunoreactive trypsinogen concentrations of 78 μg and 142 μg trypsin/l whole blood were selected as the decision levels for the AGEN and DELFIA immunoreactive trypsinogen methods respectively, being equivalent to the 99th centile for the unpartitioned neonatal population as seen by each assay. Those blood spot samples with concentrations at or above the decision level were subjected to direct gene analysis. The prevalence of the ΔF_{508}, ΔI_{506}, $G_{551}D$, $G_{542}X$, and $R_{553}X$ mutations within the South Australian cystic fibrosis population is 71·2%, 1·6%, 4·3%, 1·3%, and 1·1%, respectively (determined from 372 cystic fibrosis chromosomes). Consequently, 95·8% of patients with cystic fibrosis in our screened population would be expected to carry at least one of these mutations.

Seminars were held to explain the nature of the screening programme to health care workers, and information leaflets were provided to parents of neonates being screened.

Neonates detected as homozygotes or compound heterozygotes for any of the mutations were referred directly for clinical assessment and a confirmatory sweat test. Those neonates with only one identifiable mutation were recalled for a sweat test at age 3-4 weeks and their parents were counselled as to the significance of the results and offered genetic testing for themselves and their relatives. These parents were again contacted two weeks later by both telephone and letter to summarise the genetic results and to reiterate the counselled information. All neonates with meconium ileus were given a sweat test regardless of the result of direct gene analysis.

Results

A total of 88 752 neonates were screened with the two tier strategy between December 1989 and December 1993. The table summarises the results of the programme. Of the neonates screened, 87 748 had an immunoreactive trypsinogen concentration below the decision level and were reported as "cystic fibrosis not indicated." In 1004 neonates (1·13%) the immunoreactive trypsinogen was at or above the highest 1% of the unpartitioned population and direct gene analysis was performed. Of these, 912 (90·8%) had no identifiable cystic fibrosis mutations and were reported as "cystic fibrosis not indicated." This left 92 (0·1%) neonates who carried at least one identifiable cystic fibrosis mutation. Twenty three of these were homozygotes or compound heterozygotes for the mutations sought. Of the 69 neonates who carried only one identifiable mutation, six were ultimately diagnosed as having cystic fibrosis after being recalled for a sweat test. Further mutation analysis (11 other cystic fibrosis mutations tested) in these six neonates was able to identify the second mutation in only one case. Sixty three neonates who had normal results on sweat tests were carriers of a cystic fibrosis mutation (60 with ΔF_{508}, two with $G_{542}X$, and one $G_{551}D$). The frequency of carrying a cystic fibrosis mutation in neonates with an increased immunoreactive trypsinogen was 1:15·5 (95% confidence interval 12 to 20), which is higher than the general population frequency of 1:30 ($P < 0·0001$).

Twenty nine neonates were detected with cystic fibrosis, giving an incidence of the disease in the screened population of 1:3060 (3·27/10 000). They were referred for clinical assessment at the cystic fibrosis clinic at the Women's and Children's Hospital at a median age of 3 weeks. Before that assessment, clinical complications had already been noted in several of these infants: six had meconium ileus, one had a small bowel atresia, three were failing to thrive (one associated with persistent cough, one with jaundice, and one with prematurity), and one neonate was being investigated for persistent vomiting. Two had siblings with cystic fibrosis. One neonate with meconium ileus had an immunoreactive trypsinogen concentration below the 99th centile but on direct gene analysis was homozygous for the ΔF_{508} mutation. One neonate who carried one copy of the ΔF_{508} mutation had three equivocal sweat test results (average sweat sodium 36 mmol/l and chloride 59 mmol/l). By age 5 months he was clinically suspected of having cystic fibrosis because of recurrent chest infections and pseudomonas colonisation of his bronchial tract. He was ultimately shown to be a $\Delta F_{508}/R_{117}H$ compound heterozygote.

The mean sweat sodium concentration for the neonates with cystic fibrosis was 85 mmol/l (range 36-100 mmol/l) and chloride was 96 mmol/l (59-120 mmol/l). The immunoreactive trypsinogen results for this group are plotted in figure 2 against their populations.

The positive predictive value of the two tier screening strategy was 31·5% (29/92), with 3·2 neonates recalled for each case of cystic fibrosis detected. To date, no

Results of two tier screening for cystic fibrosis in neonates

	No (%)
Total population of neonates	88 752
Immunoreactive trypsinogen < 99th centile	87 748
Selected for mutation analysis	1 004 (1·13)
No identifiable mutation	912 (90·8)
One identifiable mutation	69 (6·9)
Sweat test positive	6 (0·6)
Sweat test negative	63
Two identifiable mutations	23 (2·3)
Neonates with cystic fibrosis	29 (0·03)
Genotype detected:	
$\Delta F_{508}/\Delta F_{508}$	18 (62)
$\Delta F_{508}/G_{551}D$	3 (10)
$\Delta F_{508}/G_{542}X$	2 (7)
$\Delta F_{508}/R_{117}H$	1 (4)
$\Delta F_{508}/X$	5 (17)

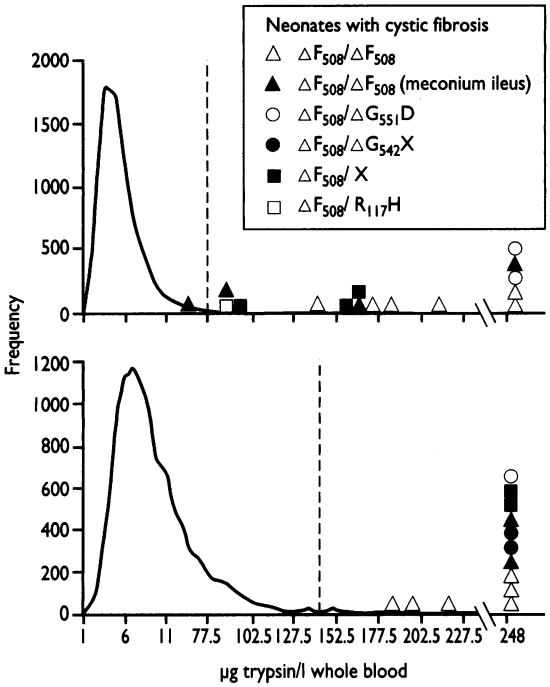

FIG 2—*Immunoreactive trypsinogen concentrations in dried blood spot specimens from 3-5 day old screened neonates. Decision value equivalent to 99th centile (dotted line) for AGEN method (top) is 78 µg/l whole blood and for DELFIA method (bottom) 142 µg/l*

child with cystic fibrosis has been identified as being missed by the screening programme.

The parents of the 63 neonates identified as carriers of a cystic fibrosis mutation were counselled and offered genetic testing. Of these, 61% (34 couples, nine mothers only, and one father only) elected to be tested. Identification of each of these carrier infants resulted, on average, in a further 1·8 family members requesting genetic testing.

The extra estimated laboratory cost of adding this two tier screening strategy to an existing neonatal screening programme is $A1.50 per neonate screened. This is based on costs of reagents, equipment, and laboratory labour.

Discussion

A two tier, neonatal screening strategy for cystic fibrosis, using immnoreactive trypsinogen quantification followed by direct gene analysis in blood spots from neonates aged 3-5 days, has been operating in South Australia since December 1989. Consistent with initial predictions,[14] this two tier strategy has functioned at high sensitivity, achieved by the use of a lower immunoreactive trypsinogen decision level (highest 1% of the unpartitioned population), and high specificity, achieved by direct gene analysis in the second tier. In the first four years of its operation this strategy has been highly effective, requiring the recall of only 3·2 families for every neonate detected with cystic fibrosis. Of the total neonates screened, only 0·08% (69/88 752) were recalled for a sweat test, which is an improvement on 0·18-0·66% in those strategies recalling neonates on the basis of the estimation of immunoreactive trypsinogen alone.[8 19-22] This reduction minimises the extent of anxiety generated in the population screened.

TWO TIER STRATEGY

The incidence of cystic fibrosis within the screened population was 1 in 3060. During the four year screening period there were six prenatal diagnoses of cystic fibrosis (four singleton pregnancies and one twin pregnancy), which resulted in termination of the pregnancies. This gives a total incidence of cystic fibrosis of 1 in 2535, which compares well with a population incidence in Australia of 1:2500.

As most sweat tests are performed at one hospital and all children with cystic fibrosis are reviewed at some stage in one cystic fibrosis clinic, the outcome of all neonates screened could be monitored. We are not aware of any child with cystic fibrosis who was missed by the screening programme. However, from the results of other workers the expected theoretical false negative rate in the first tier of the screening strategy will be about 3-4% when immunoreactive trypsinogen alone is measured.[4 5 8 21 23] For this reason we have incorporated clinical and familial information into our screening strategy. In such cases direct gene analysis is performed regardless of the immunoreactive trypsinogen result. One affected neonate, presenting with meconium ileus, was found through this branch of the strategy.

In the second tier of the screening strategy, direct gene analysis of the five common cystic fibrosis mutations within our population will theoretically fail to identify a further 5% of neonates with cystic fibrosis who carry mutations that cannot be identified at present. Overall, the two tier screening strategy operating in South Australia will fail to detect one child with cystic fibrosis every two years. However, if a fifth of children with cystic fibrosis do not present clinically until after the age of 5 years[1 24 25] (presumably these may be carriers of rarer mutations with mild phenotype or patients with low immunoreactive trypsinogen) these children might not be seen for several more years. An extreme example of this may be congenital bilateral absence of the vas deferens, in which mutations in the cystic fibrosis gene cause male infertility.[26 27]

COMMUNICATING RESULTS

The inclusion of four of the more common mutations, in addition to ΔF_{508}, in our screening strategy allows additional cases of cystic fibrosis to be detected at minimal extra cost. Furthermore, the additional information gained determines the way in which the recall process is handled. In 23 neonates the diagnosis of cystic fibrosis was made because they were either homozygotes or compound heterozygotes for the mutations sought. In this group the referring medical officer was contacted by telephone and informed of the result of the direct gene analysis as soon as this was available. The medical officer contacted the family of the child and discussed the screening results with them. Many of these neonates had clinically important complications noted before the screening result was available. It is important therefore to communicate the results of the direct gene analysis rapidly.

Our cystic fibrosis clinic offers to assess these neonates and counsel the families within 48 hours of the initial contact by the referring medical officer. At the time of the assessment, arrangements are made for a sweat test to confirm the direct gene analysis result to exclude the small possibility of error (such as an incorrectly labelled initial blood spot sample). The clinic offers review of the neonate by a team including a paediatric pulmonologist, gastroenterologist, nutritionist, physiotherapist, and social worker, and genetic counsellor. The median age of the neonates detected with cystic fibrosis at the time of initial clinical assessment is 3 weeks.

FURTHER TESTING

For those neonates carrying only one identifiable mutation a sweat test is arranged at age 3-4 weeks. A telephone call is made to the referring medical officer stating that the child is at risk of cystic fibrosis. For this group it is emphasised that the risk is relatively low, with only 1 in 12 of such neonates recalled having cystic fibrosis. It has been found that the quality of information and its presentation to parents at this point is critical to establishing their favourable response to the resolution of the diagnosis in their infant. In an expert centre there are few problems associated with

performing a sweat test on a 3-4 week old infant.

At the time of the sweat test the family is counselled for about 45 minutes; the genetics of cystic fibrosis inheritance, the implications of being a cystic fibrosis carrier, and the risks to future pregnancies, parental siblings, and for the infant in future years are discussed. The parents and referring medical officer are then contacted by telephone within 24 hours with the result of the sweat test. For most (91% in this study) the normal sweat test result confirms their child as a carrier of a cystic fibrosis mutation. These families are further contacted by telephone about two weeks later, when the results of the parents' genetic tests, if requested, are available. A summary of all the family results is sent to them in a letter that also reiterates what was discussed in the counselling session and offers further counselling and testing for themselves and other family members if desired.

The higher incidence of cystic fibrosis mutations (63/975, 1:15·5) among the population of neonates with increaed immunoreactive trypsinogen has been confirmed. This finding has been reported by several other workers[28 29] and has meant that twice as many sweat tests than theoretically expected have been performed to exclude cystic fibrosis.

CONCLUSION

The two tier screening strategy we implemented in 1989 has proved to be a highly sensitive and specific screen for cystic fibrosis. Deficiencies encountered in earlier strategies have been avoided.[8 9 22] Our current false negative rate is less than the theoretical rate of about 8%; this is acceptable given that the definition of cystic fibrosis is now changing in the light of genotype-phenotype studies.[29-31] In practice the resources involved in counselling of recalled families are offset by the reduced number of neonates requiring follow up. For this reason alone the strategy used here is an improvement on other screening programmes, and the difficulties associated with the genetic information generated by the programme can be overcome as long as a standardised protocol for counselling and monitoring recalled families is followed.

We believe this screening strategy is an effective, practical, and specific approach for the neonatal screening of cystic fibrosis. If future clinical studies confirm the benefit of presymptomatic diagnosis of cystic fibrosis[31 32] then this strategy will enhance neonatal screening for the disorder.

We thank Dr E Edkins from the Princess Margaret Hospital for Children in Perth, Western Australia, for determining the extra 11 cystic fibrosis mutations; staff of the cystic fibrosis unit at the Women and Children's Hospital; and families, medical officers and other allied health care workers for their support of the programme.

Clinical implications

- Neonatal screening for cystic fibrosis has not been widely accepted, mainly because of difficulties associated with screening strategies based on measurement of immunoreactive trypsinogen alone
- The value of presymptomatic and early treatment in improving long term outcome is becoming evident
- A two tier screening strategy incorporating immunoreactive trypsinogen measurement and direct gene analysis has overcome some of the difficulties associated with initial screening programmes
- This study, over a four year period, confirms the effectiveness of this strategy and its advantages in improved sensitivity and low rate of recall of only 0·08%
- This screening strategy is highly effective, with a positive predictive value of 31·5%, and required recall of 3·2 families for each case of cystic fibrosis detected

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(Accepted 13 May 1994)