Cystic Fibrosis Newborn Screening: The importance of bloodspot sample quality

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Abstract

Objective: Wales has an IRT-DNA Cystic Fibrosis (CF) newborn screening (NBS) programme. Most CF NBS false negative cases are due to an immunoreactive trypsin (IRT) level below the screening threshold. The accuracy of IRT results is dependent on the quality of the dried bloodspot sample (DBS). The aim of this study was to determine the cause of false negative cases in CF NBS, and their relationship to DBS quality.

Design: Longitudinal birth cohort

Setting: Wales 1996 – 2016.

Patients: Children with CF

Interventions: Identification of all CF patients with triangulation of multiple data sources to detect false negative cases.

Main outcome measures: False negative cases.

Results: Over 20 years 673,952 infants were screened and 239 were diagnosed with CF (incidence 1:2819). The sensitivity of the programme was 0.958, and positive predictive value (PPV) was 0.476. Eighteen potential false negatives were identified, of whom 8 were excluded - four screened outside Wales; two had complex co-morbidities, no identified cystic fibrosis transmembrane conductance regulator (CFTR) variants on extended analysis and thus not considered to have CF; two were diagnosed after their 16th birthday. Of the 10 false negatives, 9 had a low DBS IRT and at least one common CFTR variant and thus should have received a sweat test under the programme. DBS cards were available for inspection for 5 of the 9 low IRT cases – all were considered poor quality samples.

Conclusions: The majority of false negatives had a low bloodspot IRT, and this was associated with poor quality DBS'. The optimal means to improve the sensitivity of our CF NBS programme would be to improve DBS sample quality.

Introduction

Cystic Fibrosis (CF) newborn screening (NBS) programmes are all predicated on the analysis of immunoreactive trypsin (IRT) in newborn dried bloodspot (DBS) samples. Those with concentrations above a predefined cut-off usually proceed to DNA analysis for a varying number of cystic fibrosis transmembrane conductance regulator (CFTR) variants (IRT-DNA), while some protocols employ a second IRT measurement (IRT-IRT or IRT-DNA-IRT)[1]. National CF NBS programmes should aim for a sensitivity (number of true positive results as a proportion of all infants born with CF) of at least 0.95 and a positive predictive value (PPV) (number of true positive CF detected as a proportion of the total number of positive tests) of at least 0.3[2]. The vast majority of false negatives cases are associated with an IRT concentration below the threshold[1]. Decreasing the cut-off will increase sensitivity, but at the cost of significantly decreased PPV. We have recently demonstrated that the accuracy of IRT screening results is dependent on the quality of the DBS sample received for analysis[3].

In IRT-DNA programmes increasing the number of screened CFTR variants will also increase sensitivity, but at the cost of increasing the number unaffected carriers detected and the number of infants with Cystic Fibrosis Screen Positive, Inconclusive Diagnosis (CFSPID)[4]. Identification of infants with CFSPID raises significant dilemmas on counseling families and monitoring children[5,6] and European guidance recommends that NBS protocols should aim to minimise CFSPID recognition[7].

CF NBS commenced in Wales in December 1996[8] with an IRT-DNA protocol, although in 2007 the rest of the UK adopted an IRT-DNA-IRT protocol. The Wales

IRT-DNA protocol has evolved, screening initially for 5 CFTR variants, proceeding to a commercial 32 variants kit, and latterly to a bespoke 8 variants. Evaluation of the Welsh CF NBS programme is helpful for a number of reasons. The NBS protocol has remained consistent through this period, and the programme is centralised with a single NBS laboratory stored DBS cards. The country has a relatively stable population with limited migration, while the combination of an established network of care and local and national databases makes case identification and reporting reliable.

The aim of this study was to identify factors associated with false negative cases and evaluate the impact of different CFTR variant panels on the performance of the programme, including the recognition of infants with CFSPID.

Methods

IRT protocol

We analysed the performance of the programme over two decades (1996 to 2016). The Wales CF NBS programme is an IRT-DNA protocol culminating in a sweat test for those identified with either one or two CF variants (Figure 1). DBSs are collected on day 5 to 8 of life and posted to the centralised laboratory. Immuno-reactive trypsin is analysed in the DBS sample using the AutoDELFIA Neonatal IRT kit (PerkinElmer, Turku, Finland). The top 0.5% of values are regarded as abnormally high; in practice this gives a cut-off of ~60 ng/ml. From 1997 to 2009, specimens with elevated concentrations of IRT were re-assayed, and blood spots where at least one of the two assay results was >70 ng/ml were referred for CFTR variant analysis. From 2010 onwards analysis of all DBSs were repeated and the mean of the duplicate used. In 2003, following a false negative case in an infant of Indian subcontinent ethnicity, we added in a safety net of a sweat test for all infants with an IRT >170ng/ml irrespective of identification of CFTR variants.

DNA analysis

Between 1996 and early 2000 DNA analysis comprised a CF4 kit (Phe508del, G542X, G551D, R553X) plus 1898+1G>A (a relatively common variant in this population). Between 2000 and August 2006 a commercial CF30/32 kit (which included R117H but not D1152H), and since September 2006 variant analysis has utilised a bespoke CF8 kit (F508del, 621+1G>T, G551D, G542X, R553X, G85E, R1283M and 1898+1G>A).

All CF NBS notifications in Wales are directed to the paediatric CF centre in Cardiff for dissemination to the local CF clinic, who are responsible for approaching the family to arrange the diagnostic assessment (including a sweat test)[8]. There is a longstanding network of care between local hospitals and the specialist paediatric CF centres in Cardiff and Liverpool. We were thus confident that all subjects diagnosed with CF in childhood in Wales would be captured, including all false negative cases. We applied to the adult CF centres in Cardiff and Liverpool for details of potential false negative cases born in Wales since 1996. We applied to the national UK CF Registry for details of all patients born in Wales since 1996, without meconium ileus (MI), not detected through NBS and not living in Wales. We then triangulated the list of subjects identified through NBS with the patient details held by the CF centre in Cardiff; by the paediatric CF centre in Liverpool; by the adult CF centres and the data from the CF Registry. We defined a false negative as a person with CF diagnosed before their 16th birthday, and not detected by NBS or a clinical presentation of meconium ileus (MI).

Meconium Ileus and family history of CF

Meconium ileus is a well described cause for false negative results as the IRT concentration is sometimes low[1]. Infants with MI were thus considered as CF until proven otherwise, and those with a family history of CF or who were detected antenatally were included in sensitivity calculations.

Case definition

We used the standard international definition for a diagnosis of CF[9]. Infants with a sweat chloride concentration >60mmol/l were treated as CF. Infants with

intermediate concentrations (30-59 mmol/L) were offered a repeat sweat test, and if persistently above normal (i.e. >30mmol/L), extended CFTR variant analysis was undertaken. Infants with normal sweat chloride concentrations were not routinely followed up. Infants with one CF variant and a normal sweat chloride (<30mmol/L) were offered cascade screening. Early in the NBS programme there was uncertainty over the management of infants identified with intermediate sweat tests and/or CFTR variants of unclear significance. Recent improved CFTR variant characterisation by the CFTR2 programme and consensus on the evaluation, designation and management of infants with an inconclusive diagnosis, has clarified the situation. We have retrospectively reassessed these infants, most notably those with the relatively common R117H variant.

Results

Between 1996 and 2016 673,952 infants were screened and 239 were diagnosed with CF (incidence 1:2819) (figure 1). Two CFTR variants were identified in 155 infants and one variant identified in 34 infants, with a CF diagnosis confirmed by sweat testing. 232 had a normal sweat test and were reported as carriers. There were 10 false negative cases and 19 with intermediate sweat chloride results. Over the 20 years the CF DNA referral rate varied between 0.40% and 0.76%, with a mean of 0.593%. Results on the performance of the CF programme in Wales is shown in table 1. The median age at referral has decreased over this period from 33 (1997-2000) to 21 days (2013-2016). There was a step change improvement in timeliness after the programme was overseen by Public Health Wales in 2011. Over the last 4 years of the programme only one child (30 days) was diagnosed after 28 days of age. Sensitivity (excluding MI) was 0.958, and PPV was 0.476.

The incidence of CF decreased from 1:2466 between 1996 and 2005 to 1:3241 between 2006 and 2016, while over the same time periods the sensitivity increased from 0.946 to 0.972 and the PPV decreased from 0.508 to 0.444.

<u>False negatives</u>

We identified 18 potential false negatives of which 8 were excluded. Four were screened outside Wales. Two had complex co-morbidities and when investigated later in life had sweat chloride >60mmol/L, but no variants detected on extended CFTR gene analysis, and neither were considered to have CF. Two were diagnosed after their 16th birthday – one (phe508del/R117H (5T/9T)) presented with

bronchiectasis at age 18 years, and one (phe508del/D1152H) had no symptoms and was diagnosed at age 19 years through cascade screening.

The details of the 10 false negatives are shown in table 2. With the exception of case 7 (who was of Indian sub-continent ethnicity and precipitated the safety net), 9 had CFTR variants that would have precipitated a sweat test if their IRT had been elevated, although one case (621+1G>T/D1152H) subsequently had a normal sweat chloride concentration. DBS cards were available for review for 5 of the remaining 9 false negative cases and all 5 were considered poor quality (analysed independently).

CF SPID

We identified 19 infants consistent with a label of CFSPID (figure 1). Seven were identified between 1996 and 2006 and all were heterozygous for R117H (table 3). Twelve infants were identified between 2006 and 2016 - 7 and subsequently had normal sweat test results and were discharged, and 3 were diagnosed as CF. One has persistently equivocal sweat chloride concentrations and 1 was lost to follow up – thus 2 CFSPIDs. No infants were heterozygous for R117H - since 2006 the only infant identified was the sibling of an affected child (table 2, case 9). We consider there were 7 CFSPIDS in the 116 non-MI infants diagnosed pre-2006 (CF/CFSPID 1:17), and since 2006 2 CFSPIDs and 86 non-MI CF cases (CF/CFSPID 1:43).

Safety net

The safety net referred 20 infants with very high IRT for sweat tests, of which one was diagnosed with CF. One infant detected through NBS pre-2006 (S549N/R117H 5T; IRT = 63/79) would not have been detected.

Discussion

Cystic Fibrosis NBS in Wales has evolved over 20 years but remains an IRT-DNA programme with commendable sensitivity and PPV. Although our population is relatively small, we have triangulated a number of data sources to provide a high level of confidence that we have fully captured all outcomes, especially infants with a false negative NBS result. Where DBS samples were available to inspect, the vast majority of false negatives had poor quality DBS samples. In contrast, increasing the number of CFTR mutations screened for had little effect on sensitivity but did decrease the CF/CFSPID ratio. Our findings suggest that in our population the intervention that would most improve sensitivity is an improvement in DBS quality.

We have recently demonstrated that small and poor-quality DBS samples cause falsely low IRT concentrations[3]. Of our false negatives cases, low IRT results contributed to 9 of the10 cases. All 9 cases had at least one identified CF variant and thus would have received a sweat test. We were unable to access the DBS of the first 4 subjects, but we speculate that if the other 5 subjects DBS had been of acceptable quality the sensitivity of the programme would be 0.979. .

Following false negative case 7, we instituted a safety net of performing a sweat test on all subjects with an IRT>170ng/ml irrespective of the number of CF variants identified. Our safety net IRT concentration is higher than other programmes, and thus fewer infants proceed to a sweat test. Although numbers are small our ratio of 1 case per 20 referrals compares very favourably with other programmes[10].

In 2006 we decreased the number of screened CF variants from 32 to 8, and concurrently the incidence of CF decreased from 1:2466 to 1:3241. We do not believe the decrease in the incidence is wholly due to the decrease in the number of mutations, although we did identify fewer CFSPIDs. However with the exception of one case all infants diagnosed pre-2006 would have been detected by the CF8 panel used post-2006, and our incidence of 1:3241 is comparable with the incidence in England which lies between 1:3780 and 1:3168 infants[11].

Our NBS programme is well established, and we confined false negative cases to those diagnosed before their 16th birthday. We did not include 2 patients diagnosed in adulthood - NBS should identify infants with treatable conditions before they present clinically or suffer irreversible damage, and it is important to distinguish between screening that is predictive of disease in childhood versus screening that is predispositional of disease in adulthood[12]. The median age of diagnosis for children presenting clinically (pre NBS and excluding MI) was 2.4 years in South East England[13] and 3.8 years in Quebec[14]. It has been suggested that NBS should aim to diagnose conditions that present in the first 5 years of life[12].

This is particularly relevant for variants of varying clinical consequence (VVCC), particularly R117H (poly7T) and D1152H. Although R117H (7T) heterozygotes can have CF related symptoms in adulthood, the penetrance is likely to be less than 1%[15,16], and the vast majority of R117H (7T) detected through NBS are asymptomatic in childhood[16]. Although our numbers are small, all subjects with R117H (7T) were identified pre-2006, and all are well with normal pulmonary function and growth receiving no or minimal therapy. As yet, since 2006 no cases with R117H

(7T) have been diagnosed, either through NBS or presenting clinically. Only 2 cases were identified with D1152H, and arguably neither would have benefited from detection through NBS. Although D1152H is recognised in adults with CF, it's penetrance is likely to be very low. We would suggest that VVCC should not be included in the initial DNA analysis step for NBS for CF.

The detection of CFSPID is considered a negative consequence of NBS. The prognosis is uncertain, it is difficult to appropriately counsel families and the psychological burden is likely to be considerable[5,6]. The ratio of CF to CFSPID is primarily dependent on the number of screened CFTR variants. In California where screening comprises 40 CFTR variants with extensive gene sequencing the ratio is 2 CF:3CFSPID, while in the Netherlands the ratio is 1:4[17] and in France 1:9[18]. We have a low rate of CFSPID, and this was decreased when we reduced the number of screened variants (particularly R117H). This accords with the experience in France[18] and the Netherlands[17]. In the description of CF NBS in the South East of England[19], of the 11 infants labelled as CFSPID, 9 were heterozygous for VVCCs (5 with R117H 7T and 4 with D1152H). All would have been detected under our protocol, but because their sweat chloride concentrations were normal they would have been labelled as unaffected carriers. Furthermore we only proceed to extended mutational analysis in those with persistently abnormal sweat chloride results, possibly further decreasing CFSPID cases.

The Wales NBS programme differs from the rest of the UK which uses an IRT-DNA-IRT protocol. One aim of the IRT-DNA-IRT protocol was to minimize the detection of unaffected carriers. This was at the cost of an increased complexity, a period of

uncertainty for families following a second IRT, and a slightly later diagnosis in those positive through the second IRT route. The Welsh IRT-DNA programme offers a shorter period of uncertainty for families (no second DBS sample collection), earlier diagnosis for those with one identified CF variant, and decreased probability of CFSPID. The detection of CFSPID is a negative consequence of CF NBS. Screening for VVCCs is predispositional and carries a psychological burden[6], and we suggest they should not be included in NBS DNA analysis.

In summary, this study highlights the vulnerability of NBS for CF to seemingly small factors that can have a profound influence on performance, most notably the importance of high-quality DBS sampling to prevent the risk of a falsely low IRT measurement. The performance of our programme has not deteriorated with the use of a smaller bespoke DNA panel and recognition of CFSPID has reduced.

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ID was the lead author of this paper and undertook data collection, data processing and data analysis. He is the guarantor for the data.

CC assisted with data collection and data processing and contributed significantly to the writing of the paper.

RH assisted with data collection and data processing and contributed significantly to the writing of the paper

KS offered guidance and significant input into the writing of the paper.

JF contributed to the writing of this paper and provided significant clinical input.

LT contributed to the writing of this paper and provided significant clinical input.

SM assisted in facilitating the data collection, offered guidance and contributed significantly to the writing of the paper.

What is known on this topic:

All Cystic Fibrosis (CF) newborn screening (NBS) programmes are predicated on the analysis of immunoreactive trypsin (IRT) in newborn dried bloodspot (DBS) samples.

The vast majority of false negatives cases are due to IRT concentration below the screening threshold.

The accuracy of IRT screening results is dependent on the quality of the DBS sample received for analysis.

What this study adds:

The majority of false negatives had a low bloodspot IRT and were associated with poor quality samples.

Improving the quality of DBS will improve the sensitivity of CF NBS programmes.

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Conflicts of Interest

None declared

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Table 1: Age (days) of infants at initial bloodspot sampling, timely receipt of screening samples into the laboratory, timely analysis of CF mutation testing, and the age of the infant at the time of referral into clinical care by the Wales Newborn Screening Laboratory 1997-2016

	Age at initial blood spot sampling		Timely receipt of screening cards into laboratory		Timeliness of CF mutation analysis		Age at referral into clinical care		babies referred within 25 days of sample receipt
Time period	**Mean (SD)**	**Median (range)**	**Mean (SD)**	**Median (range)**	**Mean (SD)**	**Median (range)**	**Mean (SD)**	**Median (range)**	
1997-2000 (n=83)	7.3 (3.9)	6.0 (5-31)	5.3 (3.8)	5 (1-20)	8.1 (6.0)	7 (1-32)	32.2 (8.4)	33 (14-55)	24.4%
2001-2004 (n=111)	7.0 (2.7)	6.0 (5-22)	4.3 (2.5)	4 (1-15)	10.8 (7.6)	9 (1-44)	30.0 (9.5)	28 (14-58)	35.2%
2005-2008 (n=100)	6.5 (1.5)	6.0 (5-15)	4.0 (2.5)	3 (1-16)	6.5 (5.8)	5 (1-40)	22.8 (8.0)	21 (12-52)	69.0%
2009-2012 (n=114)	6.0 (1.3)	6.0 (5-16)	4.5 (2.8)	4 (1-16)	7.9 (5.2)	7 (1-29)	24.7 (6.6)	24 (14-49)	57.5%
2013-2016 (n=103)	5.5 (0.9)	5.0 (5-11)	3.8 (1.6)	4 (1-11)	7.9 (5.1)	7 (1-34)	22.5 (5.4)	21 (12-45)	83.0%

Table 2: False negative cases

Year	Age at diagnosis	Variant 1	Variant 2	Comment
1997	6 months	Phe508del	3272-26A>G	
1997	4 years	Phe508del	Phe508del	
1998	18 months	Phe508del	Phe508del	
2000	13 years	621+1G>T	D1152H	Chronic pancreatitis, sweat chloride 28+33
2000	6 months*	Phe508del	3659delC	
2001	12 years*	Phe508del	3272-26A>G	
2003	1 year	R709X	365dupT	Non-Caucasian, precipitated safety net
2002	2 years*	Phe508del	3849+10C>T	Complex co-morbidities
2011	Birth*	Phe508del	R117H (5T/9T)	Sibling of child with CF
2013	18 months*	Phe508del	Phe508del	

*Poor quality newborn bloodspot sample

Table 3: CFSPIDs and R117H

Year	Variant 1	Variant 2	PolyT*	Sweat Cl	Clinical course
2000	F508	R117H	7T/9T	27+27	Well, normal PFTs, CXR and growth. Had intravenous antibiotics. Asthma
2001	621+1	R117H	7T/9T		No medical input 13 years. Well, normal PFTs, CT scan and growth
2001	F508	R117H	7T/9T	19+20	Well, normal PFTs, normal growth, normal CXR
2003	621+1	R117H	7T/9T	36	Well, normal PFTs and CXR. Overweight
2003	R553X	R117H	5T/7T	56+63	Well, normal PFTs and growth, mild bronchiectasis on CT, had IVs
2004	F508	R117H	7T/9T	38	Well, normal PFTs, growth and CXR
2005	1898+1G>T	R117H	7T/7T	34+39	Well, normal PFTs, growth and CXR
2007	F508del	R117C		48+49	Became symptomatic and diagnosed as CF
2009	F508del	3849+10kbC>T		41+36	Elder sibling false negative case 9
2011	G551D			41	Lost to follow up
2014	G551D	5T		40+39	
2016	F508del	3353C>T		58+61	

*Intron 8 poly-T tract