Review Article

Newborn Screening

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Abstract
Early detection of many disorders, mainly inherited, is feasible with population-wide analysis of newborn dried blood spot samples. Phenylketonuria was the prototype disorder for newborn screening (NBS) and early dietary treatment has resulted in vastly improved outcomes for this disorder. Testing for primary hypothyroidism and cystic fibrosis (CF) was later added to NBS programs following the development of robust immunoassays and molecular testing. Current CF testing usually relies on a combined immunoreactive trypsin/mutation detection strategy. Multiplex testing for approximately 25 inborn errors of metabolism using tandem mass spectrometry is a relatively recent addition to NBS. The simultaneous introduction of many disorders has caused some re-evaluation of the traditional guidelines for NBS, because very rare disorders or disorders without good treatments can be included with minimal effort. NBS tests for many other disorders have been developed, but these are less uniformly applied or are currently considered developmental. This review focuses on Australasian NBS practices.

Introduction

The goal of NBS is the pre-symptomatic detection of infants with congenital conditions so that treatment may be commenced as early as possible to prevent, or ameliorate, the long-term consequences of the condition. The foundations of NBS stem from the work of Robert Guthrie in the 1960s. Guthrie was a microbiologist with a disabled child, initially thought to have phenylketonuria (PKU). This spurred Guthrie to develop testing procedures for this disorder. Guthrie's child was ultimately shown not to have PKU but an intellectually impaired niece did.[1] The testing procedures developed by Guthrie allowed rapid and large-scale testing of many children suspected to have this condition. Previous work had shown that a low phenylalanine diet could be used to treat PKU[2,3] and this was most effective in preventing mental retardation if the diet was commenced soon after birth and before any clinical symptoms became apparent. This raised the possibility of testing all infants soon after birth to detect PKU. Starting in the 1960s, many countries recognised the benefits of NBS for PKU and commenced programs, and today virtually all countries in which PKU is prevalent provide NBS for this disorder.

With the development of robust immunoassays for thyroxine and thyroid stimulating hormone (TSH) in the 1970s it became feasible to add congenital hypothyroidism (CH) to the NBS panel. Other disorders have subsequently been added to NBS panels and the composition of the NBS panel can vary between regions, depending on local prevalence, amongst other factors. While many conditions are potential candidates for NBS, this is not practical for all. Guidelines for deciding whether a particular condition is a suitable candidate for screening were formulated by Wilson and Jungner in 1968[4] and are summarised in Table 1. Unfortunately, many aspects of these guidelines are subjective and there is not always agreement about which disorders should be part of the NBS panel.[5-7] There is almost universal consensus that NBS for PKU and CH results in greatly improved outcomes. There is also increasing evidence of improved pulmonary function, nutrition and long-term survival resulting from NBS for CF[8-10] and similar evidence is emerging for the disorders detected by tandem mass spectrometry (MSMS).[11,12] There is less evidence for other disorders. Comprehensive cost-benefit analyses are difficult to conduct for rare disorders and separate analyses often result in widely discrepant results. Furthermore, how does one place a monetary value on death if this is an outcome in unscreened babies? The introduction of multiplex testing, exemplified by MSMS testing for several inborn errors of metabolism (IEMs), has further confounded these issues because disorders which, on their own, would not be strong candidates for NBS, can be included with minimal extra effort or cost. This has led some to propose revised NBS guidelines. As a result, NBS panels vary, even for regions with a similar population. For example, within Australasia, testing for CH,

Table 1. Major NBS criteria. Summarised from Wilson and Jungner criteria. ref.[4]

1. The condition is an important health problem and early diagnosis benefits the baby.
2. An accepted treatment is available as well as facilities for confirmation, counselling and treatment.
3. A suitable test is available.
4. The natural history of the condition is understood and there is a recognisable latent or early symptomatic stage.
5. The costs of screening, confirmation and treatment should be balanced against the overall costs of not screening.

CF, PKU and other disorders detected by MSMS is universal, but only New Zealand currently tests for congenital adrenal hyperplasia (CAH) and biotinidase deficiency. Testing for galactosaemia is performed in all states except Victoria.

This review considers NBS using biochemical testing and the overall strategies and practicalities for population-wide NBS testing, with an emphasis on Australasian practices. The interested reader is referred to recent reviews of PKU,[13-15] CF,[16,17] CH[18-20] and CAH[21,22] for more detail on the biochemistry, genetics and pathophysiology of these disorders. Non-biochemical NBS tests, such as newborn hearing testing, are outside the scope of the current review.

Models of Service Provision

NBS programs are well-established as the standard of care in most developed countries. In many regions, including Australasia, programs are state funded and testing is provided without charge to parents. Testing is voluntary in Australasia and programs report >97% coverage of newborns.[23,24] Other countries offer mandated NBS programs or fee-for-service NBS. A typical service model comprises a central laboratory performing all tests and screening a sufficient number of babies to allow economies of scale and also to make adequate numbers of diagnoses to maintain laboratory expertise. NBS cards are easily mailed and many NBS programs are heavily reliant on this mode of transport, however days on which there is no mail delivery may cause delayed turn around times and swings in laboratory workloads. Due to limitations in the way samples are collected and the inherent imprecision in dried blood spot (DBS) samples (below), it is important to emphasise that NBS is not diagnostic and any presumptive positive result therefore requires confirmation, preferably with an independent sample and test method. Referral and follow-up of abnormal results may be under the control of the NBS laboratory or through liaison with relevant clinical departments such as endocrinology, metabolic and respiratory medicine. It is important that close communication exists between these departments and the NBS laboratory.

False positive results are a concern for any NBS program because they cause parental anxiety and increased indirect costs associated with follow-up contact and testing. In some situations second-tier testing is used to improve the relatively poor positive predictive value (PPV) for some NBS tests. Samples with an abnormal result on the primary screen test are subjected to a second, more specific test. This approach has been successfully used for CF NBS and several MSMS second-tier tests have been developed. However, each of these second-tier tests adds to the complexity and expense of the NBS program and it remains to be seen how practical some of the more recently developed second-tier tests will be, particularly for disorders that require rapid confirmation. It is also recognised that some affected babies will be missed by NBS tests e.g. CF screening protocols will not detect ~5% of babies with CF.[25-27] It is important that false negative cases are referred back to the NBS laboratory for assessment and inclusion in audits of overall performance metrics.

There are increasing regulatory, legal and ethical issues surrounding the collection, storage and use of NBS cards. Most NBS programs have established advisory committees with broad representation to assist with these issues. The introduction of new NBS tests is another complex area with various lobby groups proposing new screening tests that then require evaluation by funding bodies. In the past this has sometimes happened in an *ad hoc* fashion. In most countries there is a move to greater uniformity of NBS panels and protocols and clear guidelines for evaluating proposals for new tests. The US is probably the most advanced in this regard with comprehensive policies published by the American College of Medical Genetics.[28] The Human Genetics Society of Australasia[29] and the Australian Health Ministers Advisory Group on Human Gene Patents and Genetic Testing[30] have also developed local guidelines for screening.

Sampling

Cord blood sampling is attractive in regions with early discharge after delivery. Maternal contamination is a problem and, while cord blood can be used for some NBS tests such as

CH testing,[31,32] it is recognised that better results are obtained from samples collected by heel prick at a later stage.[33] In particular, cord blood has been shown to be of limited value in detecting disorders by MSMS.[34]

Collection of blood onto an absorbent paper card, often referred to as a 'Guthrie card', is the commonest type of NBS sample. A few drops of blood from a heel prick are collected onto a high quality cotton fibre-based paper and allowed to dry in air for a few hours before being sent to the central NBS laboratory. Whatman™ 903 paper is widely used due to its well characterised properties and a few drops of blood are usually sufficient to complete most NBS panels. Some separation of blood components occurs during the spreading and drying of blood on the paper resulting in small concentration gradients across the blood spots. These effects are influenced by haematocrit and drying conditions and limit the overall imprecision of any DBS test to approximately 10%. Timing of the collection is important because some metabolite and hormone levels vary markedly in the neonatal period both in normal and affected babies.[35] Some markers decrease with age in affected babies while others increase. Consequently, sample timing is a compromise and most programs currently recommend sampling at 48 to 72 hours of age.

Use of urine as a NBS sample stems from its early use for PKU testing using ferric chloride reagent for the detection of phenylketones. Urine was collected onto an absorbent paper placed in the baby's nappy. Amino acid screening could also detect several other IEMs.[36] However, it was soon realised that urine testing was relatively insensitive for PKU and that blood phenylalanine levels were more effective in detecting it. The additional need for a blood sample for CH testing resulted in most programs dropping urine testing in favour of DBS testing. However, some NBS programs continued the practice of urine screening[37] and this has recently been given renewed impetus by the application of multiplex MSMS testing to detect some IEMs that would be difficult to detect with blood testing.

Prematurity, birth weight, neonatal jaundice, parenteral nutrition, transfusions and type of feeds can all potentially influence NBS results and need to be taken into account when establishing cut-off values and interpreting results. Ideally, this information should be recorded on the NBS card to aid in the interpretation of results. Transport of samples is also important because some markers are relatively unstable and heat, humidity and delays in transport can cause degradation and potential false negatives.

Individual Tests

PKU and Hyperphenylalaninaemia

PKU is caused by mutations in the phenylalanine hydroxylase (*PAH*) gene and results in excessive levels of phenylalanine which are detrimental to brain development. Birth prevalence is 1:14,000.[35] Treatment with a phenylalanine-restricted diet is effective in preventing the long-term consequences of PKU but needs to be commenced early in life. The early NBS programs for PKU measured phenylalanine in DBS using a bacterial inhibition assay which monitored the growth of a mutant strain of *Bacillus subtilis* with a requirement for exogenous phenylalanine for growth.[38] DBS samples were inoculated onto agar plates containing mutant bacteria and the size of the colonies assessed after incubation. The bacterial inhibition assay for phenylalanine has been largely replaced by other, more sensitive and specific assays such as enzymatic/colourimetric assays and most recently MSMS. MSMS testing also incorporates testing for many other metabolites (below).

Babies with PKU typically have DBS phenylalanine levels >200 µmol/L (a typical NBS cut-off is ~150 µmol/L), and an increased phenylalanine/tyrosine ratio. Follow-up testing involves formal measurement of plasma amino acid levels. Some cases of classical PKU are responsive to tetrahydrobiopterin,[39,40] the co-factor for phenylalanine hydroxylase, and follow-up may also involve assessment of this response. NBS also detects babies with mildly elevated levels of phenylalanine, termed hyperphenylalaninaemia, which may be caused by rarer defects in the biosynthesis or recycling of tetrahydrobiopterin. Assessment of urine pterin levels and response to phenylalanine and tetrahydrobiopterin loads are useful in classifying these babies.

Congenital Hypothyroidism

Primary CH has a birth prevalence of 1:2750.[35] Detection of CH by NBS relies on the immunoassay measurement of various combinations of TSH, thyroxine and thyroxine-binding globulin.[18,41,42] Many NBS programs, including those in Australasia, perform a single TSH test due to its simplicity and relatively low false positive rate compared to combined strategies. This strategy will not detect central hypothyroidism and low birth weight and premature babies are a potential source of false negative screens due to hypothalamic immaturity and thus require a second sample.[43,44] Iodine-containing disinfectants and contrast agents are a potential source of false positive results.[45] Positive screen results require follow-up with formal thyroid functions tests and thyroid scans. Worldwide, iodine deficiency is an important cause of hypothyroidism.[46] There is some evidence of mild iodine deficiency in Australasia, although the connection with hypothyroidism is uncertain.[47]

Galactosaemia

Galactosaemia due to galactose-1-phosphate uridyl transferase (GALT) deficiency can result in on-going jaundice and *E. coli* sepsis in newborns. A galactose-restricted diet is effective in minimising these symptoms. Birth prevalence is about 1:50,000.[35] Most programs detect galactosaemia by measuring 'galactose metabolites' i.e. galactose and galactose-1-phosphate using enzymatic assays.[48-50] This protocol may also detect deficiencies of galactokinase, which can result in juvenile cataracts, and galactose epimerase, usually a benign condition. Milder GALT variants, such as the Duarte variant with some residual activity, are also detected but these do not generally require treatment.[51] Measurement of GALT activity[52] is usually used as a second-tier test to help distinguish between the different forms of galactosaemia. Its use as a first-tier test is sub-optimal because it can result in both false positives (due to instability of the enzyme activity in DBS or use of EDTA blood) and false negatives (due to exogenous addition of activity from transfusions).[53]

Early detection of galactosaemia due to GALT deficiency can prevent short-term morbidity and occasional infant deaths. However, many cases present with typical symptoms in the neonatal period and are clinically diagnosed[54] regardless of NBS. There is also uncertainty about the overall benefit of NBS for galactosaemia because the long-term benefits of earlier diagnosis and the need for dietary treatment are unclear. Despite early NBS diagnosis, many patients have intellectual and developmental deficits and many adult females are infertile due to ovarian failure.[55-57]

Cystic Fibrosis

CF is caused by mutations in the *CFTR* gene and causes altered properties of secretions as a result of altered chloride transport. Lung and pancreatic function are affected and long term prognosis is significantly improved by early commencement of physiotherapy and antibiotic treatment. Several blood biomarkers are available but immunoreactive trypsin (IRT) measurement with immunoassay is most widely used in NBS.[58-61] IRT is somewhat unstable so NBS testing is unreliable if there is a delay in analysis or DBS samples have been poorly stored. Elevated IRT has relatively poor PPV in the neonatal period and early programs relied on a second DBS sample to confirm the initial screening result. The identification of the *CFTR* gene and disease-causing mutations opened the way to second-tier mutation testing of the original blood spot, thus eliminating the need for the second sample[62] and also providing superior performance.[63] Most NBS programs have now adopted this IRT/*CFTR* mutation protocol. A typical protocol is outlined in the Figure. Pancreatitis-associated protein is also increased in CF and an interesting alternative strategy is the measurement of both IRT and pancreatitis-associated protein.[64] This strategy has similar performance to IRT followed by second-tier *CFTR* mutation analysis and avoids the problems of carrier detection and the need for a genetic test, viewed as controversial by some sections of the community.

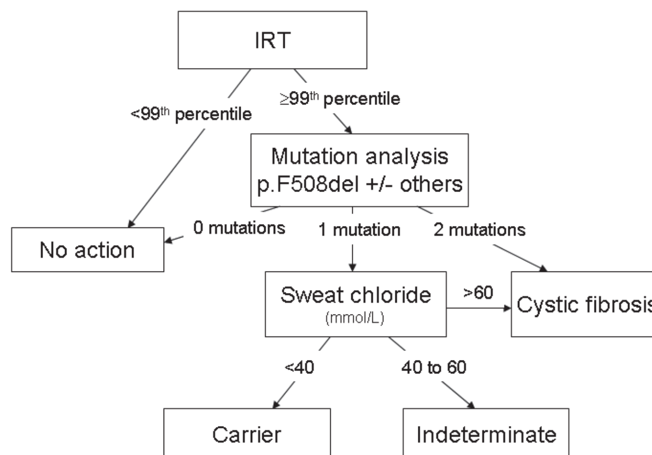

Figure. A typical screening protocol for CF. IRT, immunoreactive trypsin.

Babies with a single detected *CFTR* mutation require follow-up sweat testing because they may carry a rare *CFTR* mutation that is not part of the NBS panel.[17,61] A sweat chloride measurement >60 mmol/L is considered diagnostic for CF and remains the 'gold standard' for diagnosis. It should be noted that sweat chloride reference intervals vary somewhat with age which should be taken into account if older individuals are tested.[17] Babies with a single detected mutation and sweat chloride <40 mmol/L are assumed to be simple carriers with a consequent need for genetic counselling of the parents and possible carrier testing in relatives. A small number of babies will fall into the 'grey zone' i.e. a single detected mutation and a sweat chloride between 40 and 60 mmol/L. *CFTR* sequencing may help in this situation to find a second, rare mutation.[65] The p.F508del mutation accounts for approximately 70% of mutant *CFTR* alleles in populations with a European background[66,67] and is the main target for mutation detection. More than 1500 other *CFTR* mutations are known[16] and some of these may also be included in the NBS panel. Inclusion of additional, successively less prevalent mutations does not have a major impact on the overall performance of CF NBS and can result in somewhat fewer false negatives but more referrals for sweat testing and detection of more carriers.[68] Because of the small, incremental nature of adding additional mutations, there is no widely agreed panel of mutations and the actual number of mutations provided by different programs varies.

Multiplex Testing for IEMs Using MSMS

The early work of Millington *et al.*[69] demonstrated the potential of MSMS to simultaneously measure amino acids and acyl carnitines in a DBS sample and diagnose many IEMs. Elimination of accumulating acyl-CoA esters in the form of acyl carnitines is a common detoxification mechanism and provides convenient markers for several IEMs involving fatty acid or organic acid metabolism. This early work relied on fast atom bombardment to produce ionisation but this process was difficult to automate in a robust fashion. This limitation was overcome with the development of electrospray ionisation in the late 1980s and high throughput testing suitable for NBS was later demonstrated.[70] Metabolites are usually extracted from DBS samples using methanol and quantification is achieved by incorporating stable isotope internal standards. Formation of butyl derivatives is commonly employed to improve sensitivity but analysis of underivatised samples[71] using current generation MSMS instruments with increased sensitivity is gaining in popularity because it simplifies the assay procedure and eliminates the toxic and corrosive reagent used for butylation. Samples are directly introduced into the electrospray ionisation source without chromatography with typical run times of less than two minutes. The MSMS is usually operated in multiple reaction monitoring mode in which specifically programmed metabolites are measured, but it can also be operated in scanning mode to produce full amino acid and acyl carnitine profiles. In practice, not all metabolites detected in scanning mode are particularly useful NBS targets. For example, glycine has limited value in diagnosing non-ketotic hyperglycinaemia.[72] Phenylalanine is easily measured using MSMS and the introduction of MSMS testing therefore replaces the need for a separate discrete test for PKU while at the same time introducing testing for many other disorders. The most important of these is medium chain acyl-CoA dehydrogenase deficiency, a disorder of fatty acid oxidation that can result in sudden, unexpected infant death if not diagnosed. Other IEMs detectable by MSMS are summarised in Table 2. Several other IEMs, such as deficiencies of ornithine transcarbamylase and carbamoyl phosphate synthetase, are not usually detectable by MSMS because the metabolic abnormalities are not reliably detected in DBS using current technology.

The ability of MSMS to simultaneously measure many metabolites changed the previous focus of NBS from 'one test for one disorder' to 'one test for many disorders' i.e. a multiplex test. Doing so has changed the previous views of the traditional Wilson and Jungner guidelines. Very rare disorders, or disorders without effective treatments, can be incorporated into MSMS testing with minimal extra effort for the laboratory, merely by including the MSMS parameters for the appropriate metabolite markers. It can be argued that while detection of such disorders may not be of great benefit to the baby, early diagnosis can be helpful to the family in avoiding a long search for a diagnosis and in planning future pregnancies.[5] On the negative side, every additional metabolite is associated with a small false positive rate. Collectively, these can potentially result in an overall significant false positive rate but, with appropriate planning and selection of cut-offs, overall false positive rates of $<0.5\%$ are achievable. Several metabolite ratios can also be used to improve the PPV of MSMS NBS. For example, C3 carnitine has a relatively poor PPV for methylmalonic and propionic acidaemias, partly due to the fact that babies with increased free carnitine have secondary increases in C2 carnitine and C3 carnitine. A ratio of C3 carnitine to C2 carnitine and other combinations of metabolites can be used to improve the PPV.[73]

There is some confusion regarding the actual number of disorders detected by MSMS screening with laboratories typically listing between 20 and 30 disorders. This results from different metabolite panels used by different laboratories and varying performance metrics for metabolites. For example, tyrosine is a marker for tyrosinaemia types I and II. Levels of tyrosine are consistently quite high in tyrosinaemia type II and this disorder is readily detected. In contrast, NBS levels of tyrosine are quite modest in tyrosinaemia type I and may even be below the cut-offs used by many NBS laboratories.[74] Consequently, a substantial number of cases of tyrosinaemia type I are missed, which has led some laboratories to omit this disorder from their listing. Several other disorders have relatively poor diagnostic sensitivity and thus the decision as to whether or not to include these disorders is somewhat subjective.

Some of the disorders detected by MSMS NBS can be relatively mild or benign. This can be due to a wide range of phenotypic expressions of the disorders e.g. citrullinaemia type I can present in the newborn period with hyperammonaemic encephalopathy, but some patients detected by NBS appear to have a milder form of the disorder and remain relatively free of symptoms.[75] NBS levels of citrulline are not helpful in distinguishing these milder phenotypes. This phenomenon is not new and has been observed previously when new disorders were introduced to the NBS panel e.g. milder forms of CF are detected by NBS that would not have been detected clinically prior to the introduction of NBS. Short chain acyl-CoA dehydrogenase deficiency was initially included in NBS MSMS panels on the basis of a small number of clinically diagnosed cases but prospective studies of diagnosed cases have shown that most are without symptoms[76] which has led some programs to cease screening for this disorder. Some metabolites represent a diagnostic dilemma because they are markers for several disorders, some of them benign. For example, hydroxy-C5 carnitine is a marker

Table 2. Disorders and enzyme deficiencies detected by NBS using MSMS.

Amino Acid Disorders
Argininaemia
Argininosuccinic aciduria
Citrin deficiency[a]
Citrullinaemia type I
Homocystinuria[b]
Maple syrup urine disease[b]
Phenylketonuria and pterin defects
Tyrosinaemia type I[a]
Tyrosinaemia type II

Organic Acid Disorders
2-methylbutyryl-CoA dehydrogenase deficiency
3-hydroxy-3-methylglutaryl-CoA lyase deficiency
3-methylcrotonyl-CoA carboxylase[c] deficiency
3-methylglutaconic aciduria type I deficiency
beta-ketothiolase deficiency
Biotinidase[b]
Cobalamin C disease
Glutaric aciduria type I deficiency
Holocarboxylase deficiency
Isobutyryl-CoA dehydrogenase[c]
Isovaleric acidaemia
Methylmalonic acidaemias
Propionic acidaemia

Fatty Acid Oxidation Disorders
Carnitine:acyl carnitine translocase deficiency
Carnitine palmitoyl transferase I deficiency
Carnitine palmitoyl transferase II deficiency
Carnitine uptake defect
Long chain hydroxy acyl-CoA dehydrogenase/trifunctional protein deficiency
Medium chain acyl-CoA dehydrogenase deficiency
Multiple acyl-CoA dehydrogenase deficiency
Short chain acyl-CoA dehydrogenase deficiency
Short chain hydroxy acyl-CoA dehydrogenase[c] deficiency
Very long chain acyl-CoA dehydrogenase deficiency

[a]Missed cases are likely; [b]milder forms may be missed; [c]uncertain clinical significance, not included in some programs.

for several disorders including holocarboxylase synthase deficiency, which can cause profound metabolic acidosis, and 3-methylcrotonyl-CoA carboxylase deficiency, which is now generally considered a benign condition.[77,78] In general, the co-incidental detection of these mild or benign disorders is not a significant problem for NBS programs provided suitable follow-up testing and counselling is provided.

Another complicating factor in MSMS NBS is the detection of maternal deficiencies from the transfer of metabolites to the baby (either *in utero* or through breast feeding). Such cases can readily be ascertained by follow-up testing of the mother. Maternal 3-methylcrotonyl-CoA carboxylase deficiency is one of the more common maternal deficiencies reported by NBS programs.[79] Most maternal deficiencies appear to be without major symptoms as would be expected from the fact that they have reached adulthood and been through pregnancy without coming to medical attention. Of greater importance is the detection of maternal nutritional vitamin B12 deficiency.[80] This may be recognised through the

detection of a profile similar to some of the genetic cobalamin defects (i.e. increased C3 carnitine). Although only the more extreme B12 deficiencies are recognised using this approach, early treatment can prevent long-term neurological damage to the baby.

The relatively poor PPV of some MSMS metabolites has led to the development of a number of second-tier tests. For example, succinyl acetone is a more specific marker than tyrosine for tyrosinaemia type I and second-tier liquid chromatography (LC)-MSMS tests suitable for DBS have been developed.[74,81] Other second-tier DBS tests include *allo*-isoleucine for maple syrup urine disease[82] and total homocysteine and methylmalonic acid for the differential diagnosis of homocystinuria, methylmalonic acidurias and cobalamin defects.[83] However, each of these second-tier tests currently requires a separate testing protocol and rapid turn around of results is usually required. It remains to be seen how effectively these second-tier tests can be incorporated into most NBS programs.

Congenital Adrenal Hyperplasia

CAH is caused by defects in steroid metabolism and has a birth prevalence of ~1:18,000.[22,84] It results in deficient cortisol and mineralocorticoid production with concomitant increases in androgen production. Undiagnosed infants can present with potentially lethal salt-wasting crises and masculinisation of females, while growth failure and masculinisation are longer-term consequences of untreated cases. NBS testing for classical CAH relies on detection of increased levels of 17-hydroxyprogesterone (17-OHP) using immunoassay. Milder, non-classical forms of CAH usually have normal 17-OHP levels in the newborn period and are consequently not detected by NBS.[22] NBS for classical CAH can prevent deaths from salt-wasting crises in neonates and older children. However, the value of NBS is diminished somewhat by the fact that most females with classical CAH are clinically obvious at birth due to masculinisation of their genitalia. Most CAH cases are due to steroid 21-hydroxylase deficiency, but CAH can also be due to steroid 11β-hydroxylase deficiency which also causes an increased 17-OHP level. Confirming and distinguishing between these two disorders is readily achieved by profiling 17-OHP and other steroids in serum or with second-tier steroid profiling of the original DBS.[85] Cases with equivocal results may require stimulation of the adrenal cortex with cosyntropin or synacthen to confirm the diagnosis.[86]

Existing immunoassays for 17-OHP are somewhat non-specific and antibodies cross-react with a number of steroids of foetal origin. As a consequence of these interfering steroids, premature and low birth weight babies have apparently high values for 17-OHP by immunoassay, confounding the interpretation of results in these babies and resulting in a poor PPV for some NBS programs. Use of age- and weight-related reference intervals can improve this situation. Removal of the interfering steroids by solvent extraction has also been used to improve testing.[87] More specific testing for 17-OHP using LC-MSMS has also been developed and has been shown to significantly improve PPV when used as a second-tier test for samples with an increased 17-OHP immunoassay level.[88-90]

Other NBS Tests

NBS programs for sickle cell anaemia and other haemoglobinopathies have been established using HPLC or isoelectric focusing.[91-93] NBS for glucose-6-phosphate dehydrogenase deficiency[94,95] is carried out in several Asian countries. There is currently insufficient justification to screen for these disorders in Australasia but this situation could change in the future with changes to the ethnic mix of the population. Biotinidase deficiency can cause seizures and developmental delay and NBS is carried out in several countries[96-98] including New Zealand. Testing for severe combined immunodeficiency has been developed[99] and was recently added to the mandatory NBS panel in the US.[100] Many NBS tests for other disorders have been developed over the years, but large numbers of these have been disbanded as they were impractical or the long term benefits of incorporating them into NBS were not established. Several new NBS tests have recently been developed but are currently regarded as developmental (see below).

Secondary Uses for NBS Samples

DBS cards are usually stored for quality assurance purposes e.g. follow-up of NBS false negative cases. Protocols vary between laboratories and storage periods typically range from two years (i.e. until any NBS false negatives should be clinically diagnosed) to indefinitely. Stored cards are a valuable resource as they represent a complete population and also may allow historical comparisons to be made when samples have been stored for long periods. Stored DBS cards have also been used for diagnostic purposes other than those for which they were originally collected e.g. retrospective genetic diagnosis when the proband has died and there is no other DNA-containing material available. Detection of cytomegalovirus DNA in NBS DBS samples is also useful in retrospectively establishing congenital cytomegalovirus infection as a cause of deafness in older children[101,102] because the infection has typically cleared at the time of diagnosis. Stored DBS cards have been used for the forensic identification of human remains in accidents[103] or natural disasters such as the Victorian bushfires of February 2009.

Researchers have also realised the potential of DBS cards e.g. establishing reference intervals or carrier frequencies in the

general population. Such research requires ethics approval and parental consent for projects which require identified DBS samples. De-identified DBS samples can be used for research without consent. Some sections of the community have concerns regarding the long-term storage of DBS cards and the potential privacy issues and use of genetic information. These concerns have led to the destruction of stored DBS samples in some programs.[104] However, it is important to emphasise that the vast majority of stored DBS samples are never accessed once the initial NBS testing has been completed and NBS programs hold minimal genetic information on any individuals, which is usually limited to specific genes e.g. *CFTR*. NBS programs have protocols in place to ensure privacy is maintained and that any requests for access to samples are carefully evaluated. It is to be hoped that these protocols and on-going education will maintain the public's confidence in the NBS process and prevent a vocal minority dictating NBS policies.

Future Trends

Advances in computerisation, automation and sensitivity of analytical instruments have resulted in the proliferation of potential new NBS tests in recent years. Furthermore, improvements in treatments such as bone marrow transplantation and enzyme replacement and newer pharmacological approaches such as chaperone therapy[105] and read-through of premature stop codons,[106,107] have raised the status of some disorders as candidates for NBS. NBS tests have been proposed for lysosomal storage disorders,[108-110] Duchenne muscular dystrophy[111] and Wilson's disease[112,113] to name a few. While pilot projects have demonstrated the potential of these tests, it remains to be seen how effectively they can be applied in most NBS centres and how effective the newer treatments are. Each new test adds an additional layer of complexity to the NBS program and some of the treatments are both expensive and long-term.

Multiplexed protein assays may be one way to simplify some laboratory aspects of NBS. This approach has been demonstrated for lysosomal storage disorders using coded microbead immunoassay technology,[110] but it can be applied to a wide range of proteins[114] and could be used to combine existing tests such as IRT, TSH and 17-OHP. Increased use of molecular testing in NBS is attractive because it has the potential to increase the performance metrics of testing and target disorders that may not be amenable to biochemical testing. An NBS chip with a large array of targeted mutations could detect a far greater range of disorders than is currently tested.[115] Pilot studies have shown the feasibility of genome-wide scans of DBS[116,117] and it has even been suggested that complete genome sequencing of newborns will occur in the near future.[118] These ideas are clearly controversial. Apart from the issues of accuracy, the detection of carriers and sequence changes of unknown significance, it is unclear if such large-scale genetic testing will be acceptable to parents and the general public. However, well-targeted molecular testing has obvious technical benefits for many disorders and will become increasingly attractive as the cost of molecular testing falls in comparison to biochemical testing.

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