Evidence Based Path to Newborn Screening for Duchenne Muscular Dystrophy

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Abstract

Background: Creatine kinase (CK) levels are increased on dried blood spots in newborns related to the birthing process. As a marker for newborn screening, CK in Duchenne muscular dystrophy (DMD) results in false-positive testing. In this report we introduced a two-tier system using the dried blood spot to first assess CK with follow up DMD gene testing.

Methods: A fluorometric assay based upon the enzymatic transphosphorylation of ADP to ATP was used to measure CK activity. Preliminary studies established a population-based range of CK in newborns using 30,547 de-identified anonymous dried blood spot samples. Mutation analysis used genomic DNA extracted from the dried blood spot followed by whole genome amplification with assessment of single/multi-exon deletions/duplications in the DMD gene using multiplex ligation-dependent probe amplification.

Results: DMD gene mutations (all exonic deletions) were found in six of 37,649 newborn male subjects, all of whom had CK levels > 2000 U/L. In three newborns with CK >2000 U/L in whom DMD gene abnormalities were not found, we identified limb-girdle muscular dystrophy gene mutations affecting DYSF, SGCB, and FKRP.

Conclusions: A two-tier system of analysis for newborn screening for DMD has been established. This path for newborn screening fits our health care system, minimizes false-positive testing, and uses predetermined levels of CK on dried blood spots to predict DMD gene mutations.
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Introduction

Over the past three decades, creatine kinase (CK) testing on dried blood spots has been attempted as a method for newborn screening (NBS) for Duchenne muscular dystrophy (DMD)\(^1-9\) since CK is elevated at birth in individuals with this condition\(^10-12\). CK elevation is then validated by re-testing of venous blood at 4-6 weeks of age with subsequent DMD gene analysis employed to establish a definitive diagnosis. Presently, this approach survives only in Antwerp, Belgium\(^9\) (NBS stopped in Wales November 30, 2011). It has been difficult for programs to justify NBS for DMD because of the lack of evidence that early treatment improves the outcome of affected newborns\(^13,14\). In addition, the Wales/Antwerp DMD NBS model requiring extensive follow-up through re-testing of venous blood several weeks post-birth for CK with subsequent DNA testing is impractical to implement in the United States.

Nevertheless, recent advances in diagnostic testing methods and promising molecular-based therapies for DMD have re-kindled interest in establishing a pathway for NBS for DMD\(^15-19\). Especially relevant is clinical improvement following the systemic administration of antisense oligonucleotide (AON) PRO051 to induce skipping of exon 51 during pre-messenger RNA splicing of the \(DMD\) gene\(^15\). Patients enrolled in this exon skipping trial upregulated dystrophin at the sarcolemma and also showed functional improvement in the 6-minute walk distance. In another study, using a morpholino-based AON to skip exon 51, dystrophin expression was increased in a dose-dependent manner at the sarcolemma\(^16,17\). Additionally, long-term evidence suggests that glucocorticoid treatment prolongs ambulation, reduces the need for spinal surgery, and increases both survival and quality of life\(^20-27\). The current recommendation for initiation of corticosteroids is to start treatment when boys with DMD have stopped gaining motor skills and not to wait until motor skills have begun to decline\(^28\). Considering the mean age of DMD diagnosis to be 5 years-of-age\(^29\), and loss of
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function can begin before this time, an earlier diagnosis could result in an earlier corticosteroid start time resulting in potential long-term benefits.

In 2004, a Workshop sponsored by the Centers for Disease Control (CDC) and Prevention was held to review experiences, benefits, and risks in conducting NBS for DMD. The workshop participants concluded that there was inadequate evidence showing medical benefit from early identification, but that early diagnosis of DMD could have other advantages for the family (http://www.cdc.gov/ncbddd/duchenne/documents/nbs_lay_report.pdf). Following the CDC workshop, funds were made available to explore the potential for establishing a model for DMD NBS in the United States and to address ethical issues identified by the work group. Through this funding, we implemented a voluntary DMD NBS program in Ohio initially through a pilot study in several birthing hospitals in Columbus and Cincinnati followed by expansion to birthing hospitals throughout the state. Here we describe a two-tiered method for conducting DMD NBS with initial screening for CK followed by DNA isolation and DMD gene analysis on the same dried blood spot. The addition of DNA analysis provides information useful in reducing the number of false positives based on CK alone and a path for initial follow-up of newborns with positive CK screening results. This study provides a model for conducting newborn screening for DMD.

Methods

Study Populations

This study included four phases. Phase I efforts were devoted to creating a population-based range of CK to serve as the first tier of analysis establishing a threshold that would trigger second-tier DMD gene mutation analysis. These studies were carried out at the Ohio Department of Health (ODH) using anonymous dried blood spots from male and female newborns. In Phase II of this study, parents of newborn males were
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invited to participate in a pilot NBS study conducted at one of four major birthing hospitals in Columbus and Cincinnati, Ohio between March 2007 and September 2008. Newborn male infants born at any one of the participating hospitals were eligible for the study. In Phase III, the DMD NBS program was expanded to include a total of 43 hospitals throughout the state of Ohio with recruitment starting in October 2008 and extending through September 2010. In Phase IV, the final phase of the study, de-identified blood spots from the newborn screening cards of males and females were again anonymously screened through ODH (June 2010-January 2011). The goal in the final phase was to increase the number of male samples and to include females. We included both genders, not with the expectation of identifying carriers of an X-linked disease like DMD, but with the specific intent of enhancing our chances of identifying mutations of autosomal genes validating the two-tier method of screening (CK on dried blood spot followed by DNA testing) to address the issue of elevated CKs not found to have DMD gene mutations.

Considering that false-positive CK elevation is a potential concern of NBS, we wanted to determine if mutations in other muscular dystrophy genes could be found in cases without DMD mutations.

Study Design

Population-based CK Analysis on Anonymous Dried Blood Spots

In Phase I of the study a population-based range of CK was built upon CK testing of 30,547 consecutive anonymous dried blood spot samples (Table 1, Fig 1). Previous concerns had been raised regarding variability related to gender, neonatal weight, and age of time of sample collection. The current study is important in establishing future guidelines for CK since we found no significant difference in the mean values between males (251.52 ± 113.85 U/L) and females (246.39 ± 113.86 U/L) with a minimal effect related to birth weight. Another point of interest is that although our target group for NBS focuses
Newborn screening for Duchenne muscular dystrophy on dried blood spots collected within the first 48 hours, concerns have also been raised that CK activity diminishes over time. The data we collected shows little effect out to 5 days (>120 hours). From this database we were able to design our protocol for the two-tier testing protocol. Initially we chose to launch DNA testing for the \textit{DMD} gene at a CK $\geq 600$ U/L, three standard deviations from the mean [(0.75 % of screened population, \textit{Table 1, Fig 1})].

\textit{Implementation of CK Testing on Newborns}

For Phases II and III, NBS was voluntary and required a signed consent by parent or guardian based on approved protocols by the Institutional Review Board (IRB) at Nationwide Children’s Hospital, Cincinnati Children’s Hospital Medical Center, ODH, University of Utah, the CDC, and every participating hospital. Prior to the start of recruitment for Phase II and III, we provided an in-service teaching session to the staff of the delivery suites so that they could appropriately consent parents of newborn males. Within 48 hours of birth, trained staff members approached parents of newborn boys about participation in the study. A brochure describing the NBS was provided describing benefits and risks. Consenters than proceeded to answer questions, and consent parents who wanted to participate. Ohio currently mandates testing (http://www.odh.ohio.gov/odhprograms/phl/newbrn/nbrn1.aspx) of all newborns for 35 disorders through dried blood spots obtained 24-48 hours post-delivery (extenuating circumstances can permit testing up to 5 days of age). The blood spot cards supplied by ODH include demographic information, the date and time of collection and five 100 $\mu$L blood samples obtained by heel stick imprinted in separate circles on a piece of filter paper attached to the top of the collection card. Two of the circles are used for the mandated NBS tests and two of the three remaining circles were used for DMD NBS as part of this research study. A sticker was applied to those cards with parental consent for \textit{DMD}
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Nationwide Children’s Hospital used a cross referencing system to confirm parental approval.

All results were reported through the mail to the primary care physician or directly to the family if requested at the time of consent. In the case of a positive DMD mutation, telephone contact was made with the family to schedule a face-to-face conference to include the primary care physician and a neuromuscular specialist from our team. For CK results on dried blood spots above the threshold for DNA testing but negative for DMD gene mutations, the primary care physician was notified by telephone and a repeat venous blood CK was requested at the expense of the research study. In cases where CK elevations were again found on repeat testing, our staff offered to make an appointment with nearest MDA clinic for further testing.

Materials

CK Testing

CK testing was performed on the dried blood spots obtained for all four phases performed at the ODH Laboratory using a previously published methodology\textsuperscript{1,2,7,30, 31}. Dried blood spots were punched using a Wallac DBS Puncher (PerkinElmer Inc.®) and placed in wells of a filter plate with the addition of diadenosine pentaphosphate (DAP, USB Corp). Following incubation at room temperature to inhibit red blood cell enzyme activity, the supernatant was removed permitting N-acetyl-L-cysteine to reactivate CK activity (Reagent Kit, Thermo Electron Corporation®). CK enzymatic activity catalyzed the transphosphorylation of ADP to ATP. A series of coupled reactions produced a reduced form of nicotinamide adenine dinucleotide (NADH) at a rate directly proportional to the CK activity, measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm by a fluorometer (Victor ²D with Stacker, PerkinElmer Inc.®). For each sample, five measures were taken over 5 seconds (kinetic method) and the difference between first
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and last reading of each sample was used to normalize differences in incubation time between samples. CK levels (U/L) were calculated for each sample by a linear formula generated independently for each plate using internal controls with predefined CK concentrations loaded on each plate.

DNA Testing

DNA Extraction and whole genome amplification (WGA)

DNA testing off the initial dried blood spot was performed for samples with elevated CK. The dried blood spot was sent to the clinical DNA sequencing laboratory at the University of Utah. Genomic DNA was purified using the MasterPure Genomic DNA Extraction Kit (Epicentre cat. no. MC89010, Madison, WI.). A punch (2 mm²) from each blood spot card was submerged in 300 µl of cell lysis solution containing 50 µg of Proteinase K, and incubated at 50°C for 16 hrs. 160 µl of MPC Protein Precipitation Reagent was added, the samples were vortexed and placed on ice for 30 min. The debris was pelleted by centrifugation for 10 min. at 10,000 x g in a microcentrifuge. The supernatant was transferred to a fresh tube containing 600 µl of isopropanol, mixed and placed at -20°C for 30 min. DNA was pelleted by centrifugation at 4°C for 10 min at 10,000 x g, rinsed with 70% ice-cold EtOH, air-dried and resuspended in 20 µl of TE buffer, pH 7.6. Whole genome amplification (WGA) of this purified DNA from each blood spot was performed with the Qiagen-REPLI-g kit (no.150045). 5 µl of genomic DNA was denatured for 3 min. at room temperature, neutralized according to the manufacturer’s specifications, and a 50 µl final volume reaction containing REPLI-g DNA Polymerase was incubated at 30°C for 16 hours, followed by heating at 65°C for 3 min. to inactivate the enzyme.

Mutational analysis
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Deletion and sequencing analysis of the DMD gene was performed on WGA template DNA using the two-step SCAIP method, as described in detail elsewhere\textsuperscript{32}. This method uses PCR amplification and capillary-based fluorescent DNA sequencing to screen for deletions and point mutations in all 79 coding exons and approximately 50 nucleotides of flanking intronic sequences of the major mRNA transcript isoform in muscle plus the 5’ UTR, 3’ UTR and 6 alternate promoters. PCR was carried out in 10 µl reactions using Platinum Taq DNA polymerase (Invitrogen). Each PCR reaction contained 0.14 µl of WGA template and 93 total PCR reactions were analyzed per sample. Enzymatic clean-up was performed with the ExoSAP-IT reagent (Affymetrix) and the treated samples were sequenced using ABI BigDye Terminator v.3.1 chemistry. Samples were run on an ABI 3730xl sequencer, and analyzed using the base-calling sequence software described previously\textsuperscript{32}. Nucleotide positions were determined according to the standard reference DMD sequence used for mutation analysis (GenBank accession number NM_004006.2). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to established guidelines (www.hgvs.org/mutnomen).

All samples were analyzed for single/multi-exon deletions/duplications in the dystrophin gene using multiplex ligation-dependent probe amplification (Salsa MLPA kit P034/P035 DMD/Becker MLPA; MRC Holland, Inc.) as described\textsuperscript{33}. 1 µl of WGA template in 5 µl of TE buffer was fragmented at 98°C for 5 min, cooled and split between two separate tubes. 1.5 µl of SALSA P034 and P035 primers were added to the tubes, respectively, incubated at 95°C for 1 min. followed by an annealing at 60°C for 20 hrs. DNA ligase buffer and enzyme were added to each reaction in a total volume of 20 µl, incubated at 54°C for 15 min. followed by 5 minute incubation at 98°C. 5 µl was subsequently used in a 25 µl PCR reaction that consisted of 35 cycles: 95°C for 30 min,
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60°C for 30 min, 72°C for 1 min. followed by a final incubation at 72°C for 20 min. 1.5 µl of the sample was run on an ABI3730xl instrument and analyzed for fragment size, peak height and peak area using the GeneMapper software (Applied Biosystems). DMD exon copy number was determined by dosage quotient analysis generated for each MLPA peak by using 10 individual flanking peaks as reference peaks. Control ratios were calculated from MLPA assays using WGA genomic DNA from three non-DMD controls and the mean of these ratios formed the denominator in the dosage quotient formula. The diagnostic accuracy of the MLPA assay on WGA template purified from dried blood spots was validated by blinded analysis of blood spots obtained from consented DMD patients and parents with known mutations, including 7 exonic deletions and 6 exonic duplications, and showed 100% accuracy on these samples.

Mutation analysis was performed on nine anonymous samples (2 females, 7 males) that had CK levels >2000 and did not have a mutation identified in the DMD gene. The seven most common genes causing limb-girdle muscular dystrophy (LGMD) were selected and prioritized for analysis according to the following hierarchy (DYSF, CAPN3, SGCA, SGCB, SGCG, SGCD and FKRP). DYSF and CAPN3 were sequenced with 15 µl of WGA template using SCAIP methodology. These tests surveyed for point mutations in the DYSF gene (reference mRNA transcript, NCBI Accession: NM_003494.3, 55 exons encoding the 237 kDa dysferlin protein) and in the CAPN3 gene (reference mRNA transcript, NCBI Accession: NM_000070.2, 24 exons encoding the calpain-3 isoform a 94 kDa protein). In samples not found to have DYSF or CAPN3 mutations, sequence analysis on coding exons was performed on the following genes SGCA (NM_000023.2), SGCB (NM_000232.4), SGCD (NM_000337.5), SGCG (NM_000231.2) and FKRP (NM_024301.4).

Results
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Newborn Screening CK Studies: Phase II and Phase III Analyses

A Phase II pilot study screening 6,928 newborns was done at the major birthing hospitals in Columbus and Cincinnati, Ohio. In this phase of the study we tracked the number who declined consent and found it to be 6.0% (n = 478) of those authorized to give approval. We found that 110 subjects exceeded the CK ≥600 U/L testing threshold requiring DNA analyses. Only the two subjects above CK ≥2000 U/L (2461 and 2675) were found to have proven DMD gene mutations. The false positive rate for this phase was 1.6% (108/6,926).

The pilot study provided the impetus to move the CK threshold for the statewide, Phase III program to ≥750 U/L. The CK data collected from enrollment of an additional 10,937 newborn males led to the identification of 58 with elevated CK. One newborn was found to harbor a DMD mutation, and his CK was again above 2000 U/L (2003 U/L). The false positive rate for Phase III was 0.52% (57/10,936). Increasing the CK threshold from 600 U/L to 750 U/L reduced the number of newborn males requiring DNA testing by 68%. The number declining enrollment in this phase of the study was not accurately tracked. Forty-three individual birthing sites were responsible for consenting in the statewide program, exceeding our tracking capabilities.

Of additional interest regarding a frequently expressed concern of CK testing in the newborn period is the potential contribution of enzyme elevation from trauma as the neonate progresses through the birth canal. We have examined this by checking CK levels on follow up venous bloods obtained through the primary care physician for participants in Phase II and Phase III studies. We were able to obtain samples on only 43 of 165 subjects who were negative for DMD gene mutations in whom CK was elevated on dried blood spots (distributed as follows: 35 between 600-999 CK U/L; six between 1000-1499 CK U/L; two between 1500-1999). In most cases the follow up venous CK was lower.
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compared to the blood spot derived CK (Fig 2). Of particular note, the highest of the non-DMD group was 1700 U/L yet the repeat venous blood showed a CK 46 U/L. In only two cases the venous CK remained slightly elevated above 500 U/L on follow up (888 reduced to 672, 809 reduced to 656). This confirms that CK elevation on dried blood spots can be attributed to birth trauma and accounts for most values above normal, findings similar to previous reports. In one of the infants with a documented DMD gene mutation whose dried blood spot CK was 2,462 U/L had a repeat venous blood at six weeks showing a dramatic elevation to 8888 U/L.

Phase IV Newborn Screening CK Study

In the fourth and final phase of this study, in order to increase the sample size to further validate the two-tier approach for DMD identification in the newborn period, we screened a large cohort of de-identified newborn samples anonymously. This increased our sample size by 19,884 newborn males (total 37,649). Based on results of Phase II and Phase III studies, we limited DNA screening on dried blood spots to those males with CK ≥750. There were 308 CKs found to be above 750 U/L, and ten above 2000. In this final phase of the study, we also included anonymous CK analysis on dried blood spots of 18,763 newborn females. For the females, CK was >750 U/L in 242, with CK > 2000 on two anonymous dried blood spots.

DNA Analysis on Dried Blood Spots

Among a total of 37,649 newborn males screened for DMD (Phase II, III, IV), 6 males were found to have DMD gene mutations. All were single or multi-exon deletions, five out-of-frame and one in-frame mutation (Table 2); no point mutations or duplications were found. These exon deletion mutations followed a typical distribution seen from large cohorts, although the in-frame deletion (exons 5-41) has been reported only once.
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previously (http://www.leiden.nl), and was associated with DMD, likely due to a deletion that encompasses critical actin binding domains.

A striking finding in the mutation analysis was that all samples with DMD mutations had CK values ≥2000 U/L. Our attention was therefore drawn to subjects (7 males and 2 females) with CK ≥2000 in whom we did not find DMD mutations. We, therefore, extended the study to include analysis of mutations in the most common LGMD genes (DYSF, CAPN3, SGCA, SGCB, SGCC, SGCD, and FKRP). Mutations were found in one female (a DYSF point mutation) and two males (one with a point mutation in SGCB, and the other with a point mutation in FKRP) (Table 2). In none of these three instances was a second mutant allele detected.

Discussion

CK testing on dried blood spots to identify DMD cases in the newborn period was validated in 1979 and relies on enzyme activity to catalyze the transphosphorylation of ADP to ATP. As initially introduced, a luciferase-based bioluminescence assay established enzyme intensity; later modifications (as used here) utilized an NADH-based fluorometric read-out as a measure of CK activity. Table 3 tracks the sequential history of NBS for DMD from its early introduction in New Zealand through programs in Edinburgh, Germany, Canada, France, the USA (Western PA), Wales, Cyprus and Belgium. Antwerp is the only program that maintains NBS for DMD to this day. In this program, samples with elevated CK are re-tested through venous blood samples taken at about 6 weeks post-birth. Their nationalized health care system is positioned to accommodate multiple rounds of testing, concluding with DMD gene analysis if indicated. This approach can be challenging as evidenced by the recent closure in Wales of the longest running DMD NBS program in history.
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From its inception, our goals included creating a DMD NBS program that would fit the obstetrics practice in the USA where mother and child are discharged within 24-48 hours following uncomplicated deliveries and developing a method to readily distinguish false and true positives. Fulfillment of this task required a two-tier system of analysis permitting CK testing followed by DNA analysis on the same dried blood spot. The design introduced has similarities to the NBS program for cystic fibrosis based on two-tier molecular genetics testing that was first introduced in a pilot program in the state of Wisconsin. Prior to implementing a newborn screening program for DMD, two components had to be put in place. A validated method was required for extraction of genomic DNA from a small punch of the dried blood spot followed by whole genome amplification with analysis of single/multi-exon deletions/duplications in the dystrophin gene using SCAIP combined with MLPA. Preparatory trials provided confidence in the methodology based upon 100% accuracy in the blinded identification of 7 exonic deletions and 6 exonic duplications taken from DMD patients with known mutations (voluntary and IRB approved) placed on newborn screening cards at Nationwide Children’s Hospital and sent to the clinical DNA sequencing laboratory at the University of Utah. It was also necessary to establish a population-based range of CK on anonymous dried blood spots. This important undertaking was enabled by the full cooperation of the Laboratories of ODH facilitating a path forward for newborn screening. Through anonymous CK analysis on over 30,000 newborns (Fig 1) we established a starting point for DNA testing at a CK three standard deviations above the mean. Adding CK testing to the full battery of tests performed on dried blood spots at ODH was not overly burdensome and the cost for adding this one assay (to 35 others) was minimal (approximately $1.00 of raw materials). For those exceeding the CK threshold requiring DNA testing the cost at the University of Utah Laboratory was an additional $150.00 in raw materials.
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The results of our study support the two-tier system of analysis for newborn screening for DMD, perhaps in a way even more satisfying than anticipated. Over the course of this program we screened 37,649 males and found six males with DMD gene mutations, an incidence of 1:6291. The comparative incidence of newborn boys with documented DMD is lower than other studies throughout the world that ranged from 1:3802 to 1:6002 (taking all programs together 1:4087 Table 3), and would have to be viewed cautiously based on sample size and location in a single state in the USA. What is particularly notable about our study is that all of our patients with DMD (or dystrophinopathies) had CKs at birth ≥2000 U/L. This margin between documented cases of DMD and those with elevated CK not found to have a DMD mutation provides reasonable assurance for circumventing false positives enabling us to raise the threshold for DNA testing in Phase III of the study to CK ≥750 U/L. This reduced the number of newborns requiring DMD gene testing by about 68%, representing a significant cost saving for a NBS program. With additional confirmation of our findings these initial studies suggest that the threshold for DNA testing could be elevated even higher (e.g., CK ≥ 1000 U/L), improving the potential cost-benefit ratio for NBS.

As our program evolved we had more confidence in the identification of the great majority with DMD mutations but we were aware of limitations. Additional experience would be required to confirm that point mutations, present in approximately one-fourth of DMD patients and not detected in this study, were the result of mutation detection using WGA from DNA isolated from dried blood spots. However, we are confident that appropriate methodology has been applied in this NBS study based on our previous work demonstrating the detection of 506 point mutations (294 nonsense mutations) in the analysis of 1,111 dystrophinopathies representing 46% of subjects (over represented in this population because of study design)40. In addition, the group of dystrophinopathies
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manifesting predominantly as a cardiomyopathy accompanied by relative sparing of skeletal muscle (i.e., X-linked cardiomyopathy) will often be missed in any NBS protocol. It is well recognized that many patients in this group have reduced CK levels in venous blood, even some in the normal or near normal range. We were also aware of newborns on the other end of the spectrum with elevated CKs and no diagnosis of DMD. For this reason, we extended the study to address this potential limitation. In the final phase of this study we did DNA testing for the most common LGMDs if the CK was ≥2000 U/L in the absence of an identified DMD gene mutation. In this small sample we found one individual with a known single nucleotide insertion mutation in DYSF, one with a known missense mutation in FKRP, and another with a three nucleotide duplication in SGCB of unknown pathogenicity that has been reported in 5 sarcoglycanopathy patients (Leiden Database) (Table 2). These findings demonstrate proof of principle illustrating that LGMD gene mutations can be identified as part of the screening process. Only one pathogenic allele was detected in each case, a result that is not uncommon for these genes. Further characterization of these de-identified samples would be required to evaluate copy number changes indicative of a second, undetected large deletion or duplication.

Our completed study was not intended to address the question of whether or not NBS for DMD should be introduced but rather to provide a pathway for implementation given the recent reports of therapeutic benefit for DMD15-18. The Phase II DMD NBS program explored ethical issues involved by assessing parent and provider experiences through questionnaires; however, this topic has been reserved for a future manuscript.
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4-6 week time point, and 3) with a final step that requires an additional blood draw for DNA testing. The approach we have developed is a two-tier approach with all testing done using the original blood obtained from the heel stick within the first 24-48 hours. All testing is done from the same dried blood spot card. A threshold level of CK determines if DNA testing is to be done without additional blood obtained from the neonate. The DNA assay utilizes the most sophisticated technology available (and can be periodically modified if necessary). Whether treatment has advanced to the point of justifying newborn screening for DMD requires assessment through state and federal agencies with appropriate jurisdiction. If and when an early therapy that improves the health outcome for individuals with DMD becomes available, our study serves as a model for implementation of newborn screening for DMD. If the development of promising therapies for DMD continues to proceed at its current pace, newborn screening could be on the horizon for this disease, not only in the USA, but also in other countries of the world. If successful therapy for dystrophinopathies is available for newborns, guidelines will need to be established for referral to an appropriate muscle specialist. In addition, a pathway for referral could be built into the program for those with CK elevations in the absence of DMD mutations where there is the potential to identify other causative mutations as we have demonstrated in this report.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention

References


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Legends

Figure 1. A normal distribution of creatine kinase (CK) on dried blood spots was obtained during a Phase I population-based study of 30,547 de-identified male and female newborns. Based on this study we used CK 600 U/L as the threshold to trigger DNA testing for Phase II newborn screening. In Phase III testing we increased the threshold to CK 750 U/L.

Figure 2. Forty-three subjects who were not found to have DMD mutations, but had elevated CK levels on dried blood spots agreed to be re-tested using venous blood at 6 weeks post-birth. CK was found to be lower in all cases (clinical follow up); two were slightly elevated (above 500 U/L) at 672 and 656 (Δ). These were far below the CK >2000 U/L found in all those identified with DMD.
Table 1. Population Based Creatine Kinase on anonymous newborns

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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2500 grams</td>
<td>27,506</td>
<td>250.61</td>
<td>115.99</td>
<td>366.60</td>
<td>480.16</td>
<td>593.72</td>
<td>707.28</td>
<td>820.84</td>
</tr>
<tr>
<td>&gt;2000 grams - 2500 grams</td>
<td>1,555</td>
<td>231.68</td>
<td>87.78</td>
<td>319.46</td>
<td>433.02</td>
<td>586.58</td>
<td>660.14</td>
<td>773.70</td>
</tr>
<tr>
<td>1500 - 2000 grams</td>
<td>538</td>
<td>210.41</td>
<td>71.01</td>
<td>281.41</td>
<td>394.97</td>
<td>508.53</td>
<td>622.09</td>
<td>735.65</td>
</tr>
<tr>
<td>&lt; 1500 grams</td>
<td>573</td>
<td>226.36</td>
<td>75.88</td>
<td>302.24</td>
<td>415.80</td>
<td>529.36</td>
<td>642.92</td>
<td>756.48</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>30,172</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age at Collection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 Hrs &amp; Less</td>
<td>27,065</td>
<td>253.37</td>
<td>116.99</td>
<td>370.36</td>
<td>483.92</td>
<td>597.48</td>
<td>711.04</td>
<td>824.60</td>
</tr>
<tr>
<td>&gt; 48 Hrs - 120 Hrs</td>
<td>2,572</td>
<td>207.56</td>
<td>68.51</td>
<td>276.07</td>
<td>389.63</td>
<td>503.19</td>
<td>616.75</td>
<td>730.31</td>
</tr>
<tr>
<td>&gt; 120 Hrs</td>
<td>596</td>
<td>201.64</td>
<td>63.54</td>
<td>265.18</td>
<td>378.74</td>
<td>492.30</td>
<td>605.86</td>
<td>719.42</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>30,233</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data from the CK testing on dried blood spots of 30,547 de-identified newborn samples (broken down by gender, age, weight, and age at sample collection).
Table 2. Mutations found in newborns with CKs >2000 U/L.

<table>
<thead>
<tr>
<th>Gender</th>
<th>CK value (U/L)</th>
<th>Gene</th>
<th>Mutation</th>
<th>cDNA</th>
<th>Frame</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>2462</td>
<td>DMD</td>
<td>deletion ex50</td>
<td>[c.7201-?_7309+?del]</td>
<td>Out</td>
</tr>
<tr>
<td>Male</td>
<td>2675</td>
<td>DMD</td>
<td>deletion ex5-41</td>
<td>[c.265-?_5922+?del]</td>
<td>In</td>
</tr>
<tr>
<td>Male</td>
<td>2466</td>
<td>DMD</td>
<td>deletion ex45</td>
<td>[c.6439-?_6614+?del]</td>
<td>Out</td>
</tr>
<tr>
<td>Male</td>
<td>2791</td>
<td>DMD</td>
<td>deletion ex45-48</td>
<td>[c.6439-?_7095+?del]</td>
<td>Out</td>
</tr>
<tr>
<td>Male</td>
<td>2688</td>
<td>DMD</td>
<td>deletion ex4-7</td>
<td>[c.187-?_649+?del]</td>
<td>Out</td>
</tr>
<tr>
<td>Female</td>
<td>2731</td>
<td>DYSF</td>
<td>frameshift ex39</td>
<td>[c.4200dupC]</td>
<td>Out</td>
</tr>
<tr>
<td>Male</td>
<td>2735</td>
<td>SGCB</td>
<td>3 nt. dup., ex1</td>
<td>c.21_23dup</td>
<td>In</td>
</tr>
<tr>
<td>Male</td>
<td>2984</td>
<td>FKRP</td>
<td>p.R143S missense</td>
<td>c.427C&gt;A</td>
<td>In</td>
</tr>
</tbody>
</table>
Table 3. History of Newborn Screening

<table>
<thead>
<tr>
<th>Year of report</th>
<th>Investigators/Country</th>
<th>Observations</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1975&lt;sup&gt;12&lt;/sup&gt;</td>
<td>USA</td>
<td>Introduced CK testing on DBS in normal newborns</td>
<td></td>
</tr>
<tr>
<td>1979&lt;sup&gt;1&lt;/sup&gt;</td>
<td>New Zealand</td>
<td>10,000 newborns screened; 2 DMD cases identified</td>
<td>1:5000</td>
</tr>
<tr>
<td>1982&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Edinburgh, UK</td>
<td>2336 newborns screened; No DMD cases identified</td>
<td>0</td>
</tr>
<tr>
<td>1986&lt;sup&gt;3&lt;/sup&gt;</td>
<td>West Germany</td>
<td>358,000 screened (10% &lt;4wks of age; 65% 4-6 wk of age; 23% 6wks-6 mo; 2% 6mo-1yr); 78 had DMD</td>
<td>1:4589</td>
</tr>
<tr>
<td>1988&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Manitoba, Canada</td>
<td>54,000 screened; 10 DMD cases identified</td>
<td>1:5400</td>
</tr>
<tr>
<td>1989&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Lyon, France</td>
<td>37,312 newborns screened; 7 DMD cases identified</td>
<td>1:5330</td>
</tr>
<tr>
<td>1991&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Western Pennsylvania, USA</td>
<td>49,000 screened; 10 DMD identified</td>
<td>1:4900</td>
</tr>
<tr>
<td>1993&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Wales, UK</td>
<td>34,219 screened; 9 DMD cases found</td>
<td>1:3802*</td>
</tr>
<tr>
<td>1998&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Cyprus</td>
<td>30,014 screened; 5 DMD cases found</td>
<td>1:6002</td>
</tr>
<tr>
<td>2003&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Antwerp, Belgium</td>
<td>281,214 newborns screened at 4-6 weeks; 51 DMD cases found</td>
<td>1:5500**</td>
</tr>
</tbody>
</table>

*Presentation in London, UK, March 18, 2011, reported 335,045 screened with an incidence of 1:5266;
**Only newborn screening program that continues to actively screen subjects for DMD.
Figure 1. A normal distribution of creatine kinase (CK) on dried blood spots was obtained during a Phase I population-based study of 30,547 de-identified male and female newborns. Based on this study we used CK 600 U/L as the threshold to trigger DNA testing for Phase II newborn screening. In Phase III testing we increased the threshold to CK 750 U/L.
Figure 2. Forty-three subjects who were not found to have DMD mutations, but had elevated CK levels on dried blood spots agreed to be re-tested using venous blood at 6 weeks post-birth. CK was found to be lower in all cases (clinical follow up); two were slightly elevated (above 500 U/L) at 672 and 656 (△). These were far below the CK >2000 U/L found in all those identified with DMD.

272x297mm (300 x 300 DPI)