Statewide Newborn Screening for Severe T-Cell Lymphopenia

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INFANTS WITH SEVERE T-CELL LYMPHOPENIA, including severe combined immunodeficiency (SCID), often appear normal at birth and have no family history of immunodeficiency.1 Consequently, many infants with severe T-cell deficiencies are not identified until life-threatening infections occur. This is an important issue because the long-term prognosis of infants with SCID and other serious immunodeficiencies is markedly improved if the diagnosis is made early, before the onset of serious infections.1

For example, hematopoetic stem cell transplantation is more successful when performed in the first 3 months of life.1 Administration of attenuated vaccines that are recommended in early infancy and which can cause serious infection in infants with T-cell lymphopenia can also be avoided.2

Prior studies indicated that the enumeration of T-cell receptor excision circles (TRECs) by real-time quantitative polymerase chain reaction (RT-qPCR) on DNA extracted from routine newborn blood screening (NBS) cards could identify infants with SCID regardless of genetic cause.3,4 T-cell receptor excision circles are small, episomal pieces of DNA that are formed during the differentiation of T cells in the thymus as a result of the rearrangement of T-cell receptor genes. One particular TREC, the δRec-ψα TREC, is produced by approximately 70% of all T cells that express the α/β T-cell receptor.5 Quantitation of the δRec-ψα TREC by RT-qPCR using DNA isolated from peripheral blood serves as a surrogate marker for the number of naive T cells, which are T cells that have not responded to antigen.6 Therefore, the TREC assay using DNA isolated from NBS cards should identify any infant with profound T-cell lymphopenia and not just infants with SCID.

In a large-scale pilot study using nearly 6000 anonymized, stored NBS cards, we recently reported optimization of the TREC assay on NBS cards and adapted it as a high-throughput assay for potential use statewide as a screen for severe T-cell lymphopenia.7 In this study, we present data from 2008 to determine whether the TREC assay could identify infants with SCID.

Context A newborn blood screening (NBS) test that could identify infants with a profound deficiency of T cells may result in a reduction in mortality.

Objective To determine if quantitating T-cell receptor excision circles (TRECs) using real-time quantitative polymerase chain reaction on DNA extracted from dried blood spots on NBS cards can detect infants with T-cell lymphopenia in a statewide program.

Design, Setting, and Participants Between January 1 and December 31, 2008, the Wisconsin State Laboratory of Hygiene screened all infants born in Wisconsin for T-cell lymphopenia by quantitating the number of TRECs contained in a 3.2-mm punch (approximately 3 µL of whole blood) of the NBS card. Flow cytometry to enumerate the number of T cells was performed on full-term infants and preterm infants when they reached the equivalent of at least 37 weeks’ gestation with TREC values of less than 25/µL. Infants with T-cell lymphopenia were evaluated by a clinical immunologist.

Main Outcome Measures The number of infants with TREC values of less than 25/µL with T-cell lymphopenia confirmed by flow cytometry.

Results Exactly 71 000 infants were screened by the TREC assay. Seventeen infants aged at least 37 weeks’ gestation had at least 1 abnormal TREC assay (TREC values <25/µL), 11 of whom had samples analyzed by flow cytometry to enumerate T cells. Eight infants demonstrated T-cell lymphopenia. The causes of the T-cell lymphopenia included DiGeorge syndrome (n=2), idiopathic T-cell lymphopenia (n=2), extravascular extravasation of lymphocytes (n=3), and a Rac2 mutation (n=1). The infant with the Rac2 mutation underwent successful cord blood transplantation.

Conclusion In a statewide screening program, use of the TREC assay performed on NBS cards was able to identify infants with T-cell lymphopenia.

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performed on NBS cards can detect T-cell lymphopenia in newborn infants.

METHODS

Study Population

All infants born in Wisconsin between January 1 and December 31, 2008, were included in the study. The secretary of the Wisconsin Department of Health and Family Services approved an administrative rule that added screening for SCID and severe T-cell lymphopenia using the TREC assay effective on January 1, 2008. According to Wisconsin state law, because screening for SCID and severe T-cell lymphopenia was part of routine NBS, the screening performed during 2008 was not considered research and no institutional review board approval for the expanded NBS was needed. Prior research to develop and evaluate the TREC assay was conducted under an exemption obtained from the University of Wisconsin–Madison Institutional Review Board pursuant to 45 CFR 46.101. The study protocol and consent procedure performed apart from the NBS for T-cell lymphopenia was approved by the institutional review board of Children's Hospital of Wisconsin. Wisconsin allows parents to opt out of NBS of their infant only for religious reasons. Wisconsin and Massachusetts are the only states to perform such screening. Massachusetts began screening infants in February 2009.

Algorithm for Use of TREC Assay to Detect T-Cell Lymphopenia in Newborn Infants

The TREC analysis was performed as previously described. Briefly, NBS cards were obtained, a 3.2-mm disk was punched from the dried blood spot, DNA was extracted, and RT-qPCR of TRECs and β-actin were performed. The TREC values were normalized to µL of blood based on the estimation that each punch contained 3 µL of blood. An algorithm of TREC analysis for NBS for SCID is shown in the eFigure (available at http://www.jama.com).

Using results from our prior study, a cutoff value was established at less than 25 TRECs/µL, representing the lowest 1.1% of all samples tested. This cutoff detected all blinded NBS cards spotted with blood from infants with SCID. All NBS cards with a TREC level of at least 25/µL were considered normal and no further analysis was performed. An NBS card with a TREC value of less than 25/µL led to a second round of analysis for TRECs and β-actin, which were performed with 2 new 3.2-mm punches from the same NBS card. The β-actin evaluation was performed in parallel with repunching to ensure the presence and integrity of the DNA extracted from the NBS card. If the TREC result remained less than 25/µL and the β-actin level was low, an inconclusive report was issued and a new NBS card was requested. If the TREC values remained less than 25/µL and the β-actin levels were normal, confirming DNA template integrity, an abnormal report was issued and the primary care physician was contacted. At this point, the primary care physician could either request a confirmatory flow cytometry screening test to validate the diagnosis of T-cell lymphopenia, which is the option recommended by the NBS program, or obtain a new NBS card for a repeat TREC assay. An abnormal TREC assay on the second NBS card resulted in a confirmatory flow cytometry screening test. All inconclusive TREC assays were repeated with new NBS cards until either a normal or abnormal result was obtained. All screened infants that had an abnormal TREC assay and low numbers of naive T cells by flow cytometry were referred to a clinical immunologist (J.M.R., W.J.G., or J.V.) for a complete evaluation.

During the initial weeks of the statewide screening program, NBS cards from a few premature infants were found to have low TREC numbers with normal β-actin levels, but T-cell enumeration by flow cytometry failed to demonstrate T-cell lymphopenia. These results were consistent with the previously reported high-false positive rate of NBS tests in the premature infant population. Based on this experience, all preterm infants (<37 weeks' gestation) with abnormal or inconclusive TREC assays have their TREC levels monitored until the infant reaches the equivalent of 37 weeks' gestation; then full-term criteria are applied (eFigure). This practice is the standard operating procedure for all premature infants tested in the Wisconsin NBS program.

Flow Cytometry Evaluation

A limited confirmatory flow cytometry screening test was developed that enumerated the total number of CD3 T cells, CD3 CD4 T cells, CD3 CD8 T cells, CD3 CD4 CD55RO memory CD4 T cells, and CD3 CD8 CD45RO memory CD8 T cells, CD3 CD56 natural killer cells, and CD19 B cells. One to 2 mL of whole blood collected in sodium heparin tubes was stained with antibodies to CD3-FITC (SK7), CD4-PE (SK3), CD8-PE (SK1), CD19-PE (SJ25C1), CD45-PE (2D1) (all from BD-Immunocytometry Systems, Franklin Lakes, New Jersey), CD56-APC (N901; Beckmann Coulter, Fullerton, California), and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey).

RESULTS

Results of NBS TREC Assay

The overall distribution of the number of TRECs per microliter of whole blood extracted from dried blood spots in 2008 is shown in Figure 1. The mean number of TRECs was 225/µL and the median was 186/µL. Exactly 71 000 infants were screened in Wisconsin in 2008; 64 397 infants (90.7%) were full-term, and 6603 infants (9.3%) were preterm (Figure 2). Inconclusive reports were issued on 23 full-term infants (0.032% of all infants tested) and 96 preterm infants (0.135% of all infants tested, 1.46% of premature infants). Thus, the rate of required repeat testing was 0.168%. Of the full-term infants with inconclusive results, 19 infants had normal TREC assays on repeat testing, 2 infants died in the perinatal period, 1 infant was not retested at the parent’s request, and 1 infant had an abnormal TREC test result. Of the 96 preterm infants with inconclusive results, repeat TREC assays were normal in 72 infants, 22 infants died, 1 infant had an abnormal TREC assay at

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![Image](https://jama.jamanetwork.com/figure1)
37 weeks' gestation, and 1 infant received care in a neighboring state and was diagnosed with DiGeorge syndrome.

Twelve full-term infants (0.017% of all infants tested) and 23 preterm infants (0.032% of all infants tested, 0.35% of all preterm infants tested) had abnormal TREC assays on the initial NBS. Of the 23 preterm infants who had an abnormal test result, repeat testing produced a normal test result in 12 infants, 3 infants had a normal lymphocyte enumeration by flow cytometry while still premature, 3 infants had an abnormal result at the equivalent age of 37 weeks' gestation, and 5 infants died.

Evaluation of Infants With Abnormal TREC Test Results

In total, 17 infants that reached 37 weeks' gestation had at least 1 abnormal TREC assay (12 full-term infants had abnormal results on the initial screen, 1 full-term infant and 1 premature infant had...
an initial inconclusive test result and had an abnormal result on repeat testing, 3 premature infants had an initial abnormal result that remained abnormal when achieving the equivalent of 37 weeks' gestation (Figure 2). In 4 infants, a repeat TREC assay using a new NBS card was performed and the test result was normal, 1 infant died of causes unrelated to immunodeficiency, and 1 infant was not tested at the parent's request. Therefore, 11 infants at the equivalent of 37 or more weeks' gestation with abnormal TREC assays were analyzed by flow cytometry to determine lymphocyte subset numbers. Three of the infants had normal T-cell numbers on flow cytometric analysis. Flow cytometric analysis of the remaining 8 infants demonstrated T-cell lymphopenia, which can be grouped into known disorders of T cells, disorders of lymphocyte extravasation, and idiopathic T-cell lymphopenia (TABLE).

**Known Disorders of T-Cell Lymphopenia**

Two infants with T-cell lymphopenia determined by flow cytometry were diagnosed with DiGeorge syndrome (22q11.2 microdeletion syndrome), a disorder known to be associated with T-cell lymphopenia (Table). One infant was found to have a high percentage of phenotypically unusual lymphocytes that did not express CD3, TCRαβ, CD4, CD8, CD19, CD34, CD56, CD117, or Flt3 (CD135), but did express CD7, CD16, CD38, and CD45. This infant’s peripheral blood was analyzed longitudinally and the atypical cells decreased over time, but the T-cell lymphopenia persisted, likely due to the lack of thymic tissue present in infants with DiGeorge syndrome.

**Disorders Associated With Extravasation of Lymphocytes From the Vascular Space**

Three infants with abnormal TREC analysis had comorbidities that resulted from lymphocyte extravasation from the vascular space (1 infant with a congenital chylothorax, 1 infant with gastroschisis, and 1 infant with meconium aspiration, pulmonary hypertension, and chyloperitoneum) (Table). In all cases, the T-cell lymphopenia resolved with appropriate medical therapy.

**Idiopathic Causes of T-Cell Lymphopenia**

Three infants were found to have idiopathic T-cell lymphopenia (Table). The first infant presented with low numbers of T cells and a high percentage of phenotypically unusual cells within the lymphoid gate, with the same surface markers as those found in the infant with DiGeorge syndrome. Due to the presence of a cleft palate, testing was performed for a 22q11.2 microdeletion and was negative. Apart from a cleft palate, this infant did not have the other characteristic features of DiGeorge syndrome. The population of unusual cells in the lymphocyte gate decreased over time, but unlike the infant with DiGeorge syndrome, there was a concomitant normalization of peripheral T-cell numbers.

The second was a male infant who had very low numbers of CD4 and CD8 T cells but normal numbers of natural killer cells and B cells. There were increased numbers of CD45RO memory T cells, which are T cells that previously responded to antigen. Further immunological evaluation demonstrated

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<th>Table. Etiologies of T-Cell Lymphopenia (n = 8 Infants)</th>
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<td>Known disorders</td>
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<td>DiGeorge syndrome (22q11)</td>
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Abbreviation: TREC, T-cell receptor excision circle.

a Normal ranges for lymphocyte subpopulations shown in parentheses.

b Negative for 22q11 microdeletion.
normal serum immunoglobulins, normal tetanus and diphtheria responses, normal lymphocyte proliferation to mitogens, negative human immunodeficiency virus PCR, normal adenosine deaminase and purine nucleoside phosphorylase analysis, and normal radiation sensitivity studies. Interestingly, this infant’s older sister, who was born before the institution of the Wisconsin NBS program for T-cell lymphopenia, also exhibited CD4 T-cell lymphopenia with increased numbers of CD4 memory T cells and normal numbers of natural killer cells and B cells. At 18 months, her CD8 T-cell count was elevated and an abnormally high percentage of the CD8 T cells were activated. Similar to his sister, the boy also has developed an increased number of activated CD8 T cells over time. The sister has evidence of immunodysregulation with diffuse mild inflammatory bowel disease and immunodeficiency with treatment-resistant esophagitis and vaginitis due to Candida albicans, recurrent skin abscess formation, recurrent otitis media, pyelonephritis, and borderline failure to thrive. The increased numbers of activated CD8 T cells and memory CD4 T cells in the sister is consistent with the clinical picture of immunodysregulation and recent or persistent infections. The sister is currently in preparation for hematopoietic stem cell transplantation. The boy is being closely monitored for evidence of immunodeficiency or immunodysregulation.

The final infant with idiopathic T-cell lymphopenia was a male born at term to nonconsanguineous parents. Lymphocyte subset analysis showed low numbers of B cells and decreased numbers of CD4 and CD8 T cells, but no evidence of increased numbers of activated T cells or memory T cells. The infant was followed up closely by the clinical immunologist because of persistent T-cell lymphopenia. Within the first 8 weeks of life, this infant was admitted for omphalitis and a paratracheal abscess. Evaluation for neutropenia, chronic granulomatous disease, leukocyte adhesion deficiency 1, and leukocyte adhesion deficiency 2 were all negative. Subsequent workup for other etiologies causing a neutrophil defect demonstrated a G → A substitution in exon 3 at codon 57 in the Rac2 gene, resulting in the substitution of Asp57 to Asn(D57N), which is the same mutation of Rac2 in the only other case previously reported.8,9 This mutation was not present in the infant’s parents.

**COMMENT**

In this study, we present the results of the first statewide NBS program to our knowledge to detect T-cell lymphopenia by enumerating TRECs by RT-qPCR using DNA extracted from dried blood spots of NBS cards. Only 17 of 64,397 infants older than 37 weeks’ gestation screened by the TREC assay (approximately 0.026%) had TREC values below the cutoff limit (<25 TREC/µL blood). Eleven infants subsequently underwent a confirmatory flow cytometry screening test, which confirmed the presence of T-cell lymphopenia in 8 infants.

Some of the causes of T-cell lymphopenia, such as extravasation of T cells outside the vascular compartment, DiGeorge syndrome, or idiopathic T-cell lymphopenia, were consistent with the immunological findings known to be associated with these disorders. Importantly, 1 of 2 infants with DiGeorge syndrome did not have a heart defect at birth or other stigmata associated with DiGeorge syndrome. The diagnosis was made by a clinical immunologist, who evaluated the infant after flow cytometry confirmed the diagnosis of T-cell lymphopenia.

One case of T-cell lymphopenia identified by the TREC assay was unexpected. The TREC assay identified an infant with a mutation of Rac2, the same mutation in Rac2 as has previously been reported.8,9 The protein translated by this mutant form of Rac2 (D57N) functions in a dominant negative manner and inhibits neutrophil migration and the production of superoxide by neutrophils in response to physiological stimuli.8,9 Consequently, the clinical manifestations of the immunodeficiency in both cases was predominantly characterized by a defect in neutrophil function with recurrent pyogenic infections.8,9 Recent studies demonstrate the importance of Rac1 and Rac2 in T-cell development. Mice deficient in both Rac1 and Rac2 exhibit a severe developmental block in T-cell maturation in the thymus.10 The mutant D57N Rac2 protein substantially inhibits the normal activity of the highly homologous Rac1 protein.11 Therefore, we hypothesize that the abnormal function of both Rac1 and Rac2 proteins led to T-cell lymphopenia in this infant. This infant received a cord blood transplantation and is currently engrafted and clinically well at age 12 months. The ability of the TREC assay to identify an infant with a Rac2 mutation and T-cell lymphopenia illustrates the power of this test to detect rare but life-threatening immunodeficiencies apart from SCID.

In comparison with full-term infants, the finding that more premature infants had either abnormal or inconclusive test results was not unexpected and in agreement with a prior study.3 There are several likely reasons for this result. Frequently, in very low birth weight premature infants, blood for the NBS card is obtained through an intravenous catheter. Although this is an acceptable practice, such blood may be diluted or contain a substance that may inhibit the RT-qPCR, resulting in an abnormal or inconclusive test result. Additionally, the cutoff value for an abnormal TREC assay was based on the distribution curve obtained on full-term infants,7 which is likely different in premature infants and may vary with gestational age. With additional data on TREC values in premature infants, it may be possible to derive a different cutoff value for premature infants compared with full-term infants. Importantly, the standard operating procedure for all premature infants in the Wisconsin NBS program adequately addresses the problem associated with abnormal or inconclusive TREC test results in premature infants.

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In 2006, a task force evaluated the potential inclusion of SCID in US state NBS programs. Criteria used for inclusion of specific diseases in state NBS programs include (1) the incidence of the disease should be at least 1:100,000 live births; (2) the disease must result in serious morbidity or mortality; (3) early diagnosis and treatment should significantly improve the prognosis; (4) the disorder must not be readily detected at birth by a routine physical examination; (5) a sensitive, specific, and inexpensive test must exist that uses existing NBS cards; and (6) a confirmatory gold standard test for diagnosis must be available. Based on these and other criteria, the task force concluded that SCID should not be included in US NBS programs at that time. One important factor in the task force’s decision was the lack of a sensitive, specific, and practical screening test to detect SCID.

This issue should be revisited in the broader context of NBS screening for profound T-cell lymphopenia and not just limited to SCID. The incidence of primary and secondary immunodeficiencies identified by the TREC assay (8:100,000) exceeds the required incidence of disease to institute screening. Even if one excludes the secondary immunodeficiencies that resolved with time, the TREC assay identified 5 cases of primary T-cell lymphopenia. Furthermore, the TREC assay is relatively inexpensive (approximately $5.50 per assay). Based on an analysis of potential cost-effectiveness of NBS for SCID, the relatively high incidence of T-cell lymphopenia and the low cost of the TREC assay suggest NBS may be cost-effective as well, although a formal cost-effectiveness analysis is needed.

One important limitation of our study is the inability to assess the total number of infants born in Wisconsin with clinically significant T-cell lymphopenia. Without such information, one cannot accurately calculate the true sensitivity and specificity of the TREC assay to detect severe T-cell lymphopenia. The TREC assay did not identify any infants with classic SCID during the first year of testing. However, there were no cases of SCID in Wisconsin during the screening program and the failure to identify an infant with SCID at this point is likely secondary to the relative rarity of the disorder (1:2,100,000) and not the lack of sensitivity of the TREC assay.

In conclusion, the Wisconsin screening program demonstrates the feasibility of the TREC assay performed on NBS cards to identify infants with primary and secondary forms of T-cell lymphopenia.

Author Contributions: Dr Routes had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Routes, Grossman, Verbsky, Hoffman, Baker.

Acquisition of data: Routes, Grossman, Verbsky, Laessig, Hoffman, Brokopp, Baker.


Drafting of the manuscript: Routes, Grossman, Verbsky.

Critical revision of the manuscript for important intellectual content: Routes, Grossman, Verbsky, Hoffman, Brokopp, Baker.

Statistical analysis: Verbsky.


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Additional Information: The eFigure is available at: http://www.jama.com.

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