Hum Genet (1989) 81:363–366

Human Genetics

© Springer-Verlag 1989

Molecular genetic diagnosis of sickle cell disease using dried blood specimens on blotters used for newborn screening

David C. Jinks[1], Mikeanne Minter[2], Deborah A. Tarver[2], Mindy Vanderford[2], J. Fielding Hejtmancik[2,3], and Edward R. B. McCabe[2,4]

[1]Departments of Microbiology and Pediatrics, State University of New York at Buffalo, Buffalo, NY 14214, USA
[2]Institute for Molecular Genetics, [3]Department of Internal Medicine, and [4]Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA

Summary. The protein-based technologies used to screen newborns for sickle cell disease require confirmation with a liquid blood specimen. We have developed a strategy for rapid and specific genotypic diagnosis using DNA extracted from a dried blood spot on the filter paper blotter used to screen newborns. DNA could be microextracted from a specimen as small as a ⅛ inch diameter punched disc representing the dried equivalent of approximately 3 µl of whole blood. We utilized the DNA from a ¼ inch diameter specimen (12 µl equivalent) for polymerase chain reaction amplification of the β-globin region spanning the sickle cell mutation with detection by allele-specific oligonucleotide probes. Molecular confirmation of genotype from the original blotter would reduce the personnel costs associated with obtaining follow-up liquid blood specimens and would provide information to the family in a more timely and less equivocal manner.

Introduction

Neonatal hemoglobinopathy screening utilizes sensitive electrophoretic and isoelectric focusing techniques for determination of the abnormal β-globin (Schneider 1986; Garrick 1987). The primary purpose for hemoglobinopathy screening is early diagnosis and management of individuals with sickle cell disease (Wethers and Panel 1987). The incidence of pneumococcal septicemia in these patients is 10 per 100 person years, with a case fatality rate of 30% (Gaston and Rosse 1982). This high mortality rate from sepsis in infancy can be eliminated by penicillin prophylaxis (Gaston et al. 1986).

The developmental biology of the globin system results in neonates having a high proportion of fetal hemoglobin and a low proportion of the adult hemoglobin. Because the expression of the sickle (S) allele may be quite low in the neonatal period, the status of the individual as carrier (AS) versus homozygous affected (SS) may remain equivocal after the initial screen. The confusion generally can be resolved by confirmatory electrophoresis using a follow-up liquid blood specimen, though in a group of these patients the diagnosis may not be resolved until a subsequent specimen is obtained at 2 to 4 months of age. Direct DNA confirmatory diagnosis from the initial newborn filter paper blotter, as recommended by the National Institutes of Health Consensus Development Conference Statement on Sickle Cell Disease and Other Hemoglobinopathies (Wethers and Panel 1987), would permit rapid β-globin genotyping of these patients.

We have reported a method for DNA microextraction from the dried equivalent of 50 µl of whole blood on filter paper blotters and noted that this approach would be applicable to follow-up of positive newborn screens for disorders amenable to molecular genetic diagnosis, such as sickle cell disease (McCabe et al. 1987). We suggested that the microextracted DNA could be amplified by the polymerase chain reaction (PCR, Saiki et al. 1985) for analysis. Here we describe the microextraction of DNA from the dried equivalent of as little as approximately 3 µl of whole blood on filter paper blotters, and the PCR amplification and analysis of microextracted DNA using β-globin primers and allele-specific oligonucleotide (ASO) probes.

Offprint requests to: E. R. B. McCabe, Institute for Molecular Genetics, Room T526, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

Materials and methods

DNA extraction from blood dried on filter paper

Whole blood from adult volunteers was collected in heparinized tubes and spotted on Schleicher and Schuell no. 903 filter paper using a Pasteur pipet. The specimens were allowed to dry at room temperature for several hours until completely dried. We also analyzed dried blood specimens from newborn infants, diagnosed as being homozygous or heterozygous for the sickle cell trait. Blood spots from newborns were supplied by the Texas State Health Department Newborn Screening Laboratory, Austin. Steel punches (⅛ and ¼ inches) were used and were routinely washed in 0.25 *N* HCl for 5 min between specimens to depurinate DNA and prevent cross-contamination of specimens. Human total genomic DNA was labeled by nick translation (Boehringer Mannheim, Ribgy et al. 1977) and was hybridized to microextracted DNA to estimate the amount of DNA extracted by different procedures.

After initial investigation of microextraction and fixation conditions the following procedure was routinely used. DNA was extracted from ¼ inch (6 mm) dried blood specimens on filter paper discs. The discs were quartered and placed in a 1.5 ml disposable microcentrifuge tube, or, equivalently, four ⅛ inch (3 mm) discs were used. Several drops of reagent grade methanol, enough to cover the discs, were added to each tube.

The tubes were incubated at room temperature for 5 min and the methanol was evaporated to dryness under vacuum. After the methanol had evaporated, 380 µl of 0.15 *M* NaCl containing 0.5% sodium dodecyl sulfate (SDS) was pipetted into the tubes, and they were incubated at 37°C for 1 h. When the incubation was complete, 20 µl of proteinase K (400 µg, 5.5 units) was added, and the tubes were incubated an additional 2 h at 37°C. Following the proteinase K digestion, the supernatant was removed from the filter paper discs. The tubes with the discs were rinsed with two 100-µl aliquots of 0.15 *M* NaCl containing 0.5% SDS and combined with the original supernatant. Phenol extraction and DNA precipitation were accomplished as reported previously (McCabe et al. 1987). DNA was quantified using a Hoefer TKO 100 fluorometer and Hoechst 33258 (bis-benzimidazole) fluorescence.

DNA was also obtained by boiling the specimens using an adaptation of the method for liquid blood specimens (Kogan et al. 1987). The ¼ inch, methanol-fixed, dried specimens were boiled for 15 min in 61 µl deionized water, which had been filtered through a 0.45-µm nylon membrane. The paper was pelleted by centrifugation for 15–20 min at 10,000 *g* and the supernatant was removed and used for PCR amplification.

Polymerase chain reaction (PCR)

DNA extracted from the dried blood on the filter paper was amplified using a modification of a procedure described by Kogan et al. (1987). *Thermus aquaticus (Taq)* polymerase (Perkin Elmer Cetus) was used to amplify the sequence of the β-globin gene desired. Amplification was accomplished in 100 µl of reaction mixture, which contained 50–100 ng extracted DNA. Reaction buffer and other components of the reaction mixture were the same as previously reported (Kogan et al. 1987). The 20-base synthetic oligonucleotide primers were identical in sequence to those of Saiki et al. (1985) and were synthesized on an Applied BioSystems 380B DNA Synthesizer. The times and temperatures of incubations used routinely were as follow: annealing for 2.0 min at 55°C, extension for 1.5 min at 72°C, and denaturation for 1.0 min at 95°C. At various times 37°C or 46°C were used for annealing and 2.0 or 3.0 min for extension. Forty rounds of amplification were typically used.

Analysis of amplified sequences

For slot blot analysis, 20–40 µl of amplified DNA was denatured for 7 min at 95°C, cooled on ice, and loaded into the wells of a Bethesda Research Laboratories slot blot manifold containing a nylon filter (0.45 µm, Zeta-Probe, BioRad). The filter had been soaked in 20 × SSPE (3.0 *M* NaCl, 200 m*M* NaH_2PO_4, and 20 m*M* EDTA, pH 7.4) for at least 2 h. Samples were blotted by vacuum through the filter, and the wells were rinsed with 400 µl of 20 × SSPE. Electrophoresis and Southern blotting were performed as previously described (McCabe et al. 1987).

The filters were baked under vacuum at 80°C for 1 h and then washed at 65°C for 40–60 min in 0.1 × SSC (20 × SSC is 3 *M* NaCl, 0.3 *M* Na_3citrate, pH 7.0) 0.5% SDS. Filters were prehybridized and hybridized as previously reported (Kogan et al. 1987). Nineteen-base allele-specific oligonucleotide (ASO) probes were the same as those reported by Studencki et al. (1985), and were synthesized as above. Probes were labeled by polynucleotide kinase using (γ-^{32}P)-ATP.

Optimal conditions for washing the filters were determined to be three times for 20 min each, in 0.2 × SSC at 37°C, followed by 20 min in 0.2 × SSC at 68°C. Filters were exposed to film at −70°C overnight.

Results

The initial investigations were designed to determine the sensitivity of, and optimal conditions for, DNA microextraction from dried blood specimens on filter paper blotters. Figure 1 shows that DNA could be microextracted in detectable quantities from specimens as small as the ⅛ inch diameter punched disc routinely used in newborn screening laboratories and representing the dried equivalent of approximately 3 µl of whole blood (National Committee for Clinical Laboratory Standards 1985). The yield of microextracted DNA was clearly decreased by autoclaving the specimens, and methanol fixation appeared to improve the yield, compared with no treatment of the specimens prior to microextraction.

Blood discs, ¼ inch in diameter, from normal adults were microextracted under the standard conditions and yielded 145–500 ng DNA. Since a ¼ inch disc contains the dried equivalent of approximately 12 µl whole blood (National Committee for Clinical Laboratory Standards 1985), this represents yields of 12–42 ng/µl whole blood. The yield of DNA from ¼ inch discs from neonatal specimens was 520–940 ng or 43–78 ng/µl whole blood.

Blotters were prepared using blood from adult volunteers, genotype AA, and microextracted DNA underwent PCR amplification. Figure 2 shows results comparing a 25% aliquot of the amplified microextracted AA DNA with amplified cloned β^A sequence and genomic AA DNA. Signal is evident with the β^A ASO but not with the β^S ASO.

When authentic screening blotters from neonates were microextracted and DNA was amplified by PCR, the genotypes could be clearly distinguished by differential hybridization with the β^A and β^S ASOs. It was important to wash the punches in 0.25 *N* HCl for 5 min to depurinate residual DNA on the punches from the previous sample. Otherwise, signal was occasionally seen in the SS DNA with the β^A ASO and in the AA DNA with the β^S ASO.

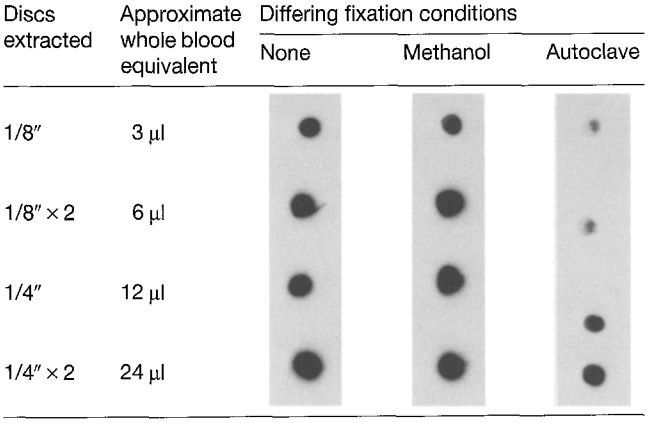

Fig. 1. DNA microextraction from dried blood spots on filter paper blotters using punched discs of differing diameters and with varying fixation conditions. This was probed with human total genomic DNA labeled by nick translation (Rigby et al. 1977)

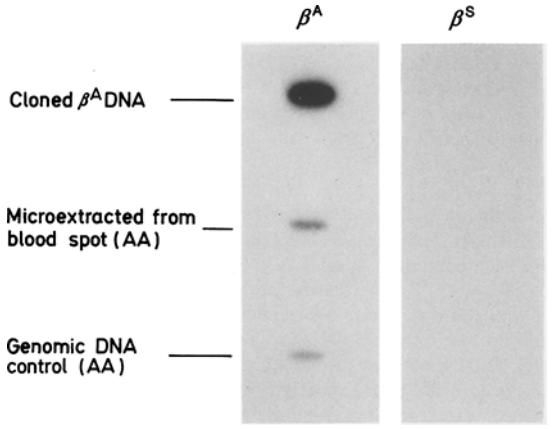

Fig. 2. Polymerase chain reaction amplification and slot blot analysis using β^A or β^S allele-specific oligonucleotide probes. *AA* Homozygous normal

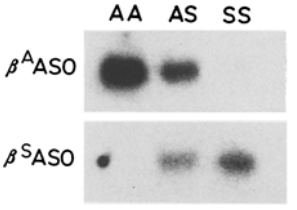

Fig. 3. Southern blot analyses of products after polymerase chain reaction amplification of DNA obtained from boiled homozygous normal *(AA)*, carrier *(AS)* and homozygous affected *(SS)* neonatal screening specimens. The only bands identified by autoradiography were the 109-bp bands shown. The *upper panel* shows hybridization of the β^A allele-specific oligonucleotide *(ASO)* with the 109-bp bands from the *AA* and *AS* (but not the *SS*) specimens, and the *lower panel* shows the hybridization of the β^S *ASO* with the 109-bp bands from the *AS* and *SS* (but not the *AA*) specimens

DNA could also be obtained in sufficient quantity for PCR amplification by boiling specimens from newborn screening blotters and collecting the supernatants. Southern blot hybridizations revealed appropriately sized amplification products, which permitted genotypic analysis (Fig. 3). The β^A ASO hybridized with the amplified 109-bp bands from the AA and AS specimens (upper panel) and the β^S ASO hybridized with the 109-bp bands from the AS and SS specimens (lower panel).

Discussion

These investigations demonstrate the applicability of molecular genetic techniques for β-globin genotype analysis directly from the dried blood specimens on filter paper blotters used for the initial newborn screening. DNA microextraction could be achieved with material from a ⅛ inch punched disc, representing the dried equivalent of approximately 3 µl of whole blood. Using a ¼ inch punched disc (approximately 12 µl dried equivalent), 145–500 ng DNA was obtained from adults and 520–940 ng from neonatal specimens. These differences undoubtedly represent the relatively higher white blood count and the presence of circulating nucleated erythrocyte precursors found in neonates. The yield of DNA was influenced significantly by the treatment of the specimen prior to microextraction. Routine treatment of specimens in newborn screening laboratories varies. Our results showed that the yield was diminished after autoclaving the specimens and that methanol fixation may be superior to no treatment at all. The microextraction method used in these investigations involved incubation of the dried specimens serially in SDS and proteinase K, similar to that described by Gill et al. (1985).

The results described here also showed that the DNA obtained by microextraction could be used for PCR amplification and analysis with an ASO. PCR amplification was routinely performed using a ¼ inch punched disc, but the slot blot analysis generally required only one-fourth or less of the amplified DNA. DNA could also be rapidly obtained for amplification by boiling the dried blood specimens, using an adaptation of a method originally described for liquid blood specimens (Kogan et al. 1987).

These investigations demonstrated the extreme sensitivity of this amplification strategy and the need to minimize contamination during sample collection and handling. Current laboratory procedures for newborn screening do not involve routine washing of specimen punches between samples. However, we found that it was necessary to wash these tools in a depurinating solution to prevent sample cross-contamination by this route. We also found that samples prepared in the laboratory gave a clean background with the probe of the opposite allele, whereas actual neonatal screening specimens occasionally showed cross-hybridization. This may represent contamination at the time of specimen collection, immediately after collection before the spots have fully dried, or during subsequent handling.

The synthesized primers and ASO were identical to those previously described (Saiki et al. 1985), and utilized the standard approach to differential ASO hybridization (Conner et al. 1983; Studencki et al. 1985). Amplification was carried out using a thermostable polymerase isolated from *Thermus aquaticus (Taq)* which allows for semi-automation of this process using a programmable heat block (Saiki et al. 1988b; Oste 1988). Advances, including nonradioactive detection methods (Saiki et al. 1988a), will facilitate routine diagnostic application of this technology. Additional diagnostic approaches will offer options to differential ASO hybridization (Caskey 1987; Embury et al. 1987; Wong et al. 1987; Saiki et al. 1988b; Landegren et al. 1988).

The value of newborn screening and follow-up in the prevention of mortality from sickle cell disease has been shown in a study of 88 patients screened by hemoglobin electrophoresis of cord blood during the period 1976–1980 (Nussbaum et al. 1984). Gaston et al. (1986) observed an 84% reduction in the incidence of pneumococcal septicemia by penicillin prophylaxis in a study of 215 children. The placebo group experienced thirteen episodes of infection and three fatalities, whereas the penicillin group experienced two episodes of infection and no fatalities. They concluded that the fulminant nature of the sepsis in these patients requires antibiotic prophylaxis and this should be initiated no later than 4 months of age. We would speculate that clarification of the initial screening results with direct genotypic diagnosis from the original newborn filter paper blotter should provide a more timely diagnosis and convey to the families an appropriate sense of urgency and immediacy regarding the diagnosis of sickle cell disease. In addition, direct diagnosis using the original specimen will reduce a major expense to newborn screening programs, i.e., the personnel time required to contact the families to obtain the confirmatory liquid blood specimen.

The molecular genetic approach to the follow-up of newborn screening will be applicable to other disorders as well.

This approach has been reported for phenylketonuria (PKU, Lyonnet et al. 1988) and cystic fibrosis (Williams et al. 1988) using PCR amplification of DNA extracted from dried blood spots. At this time 60% of the mutant PKU alleles can be diagnosed using PCR and 90% may be accessible in the future (DiLella et al. 1988). The value of this approach to PKU will increase as additional information is obtained correlating genotype and phenotype (Guttler et al. 1987). Follow-up for positive cystic fibrosis screens may also be amenable to this approach, especially if the prediction is accurate that there is a single mutant allele (Estivill et al. 1987). Development and refinement of DNA microextraction, amplification, and detection may have eventual applications in such areas as primary newborn screening (McCabe et al. 1987) and evaluation of fetal cells in the maternal circulation (Covone et al. 1984).

Acknowledgements. Supported in part by grants from Maternal and Child Health (MCJ-480566 and MCH-000435), U.S. Public Health Service.

References

Caskey CT (1987) Disease diagnosis by recombinant DNA methods. Science 236:1223–1229

Conner BJ, Reyes AA, Morin C, Itakura K, Teplitz RL, Wallace RB (1983) Detection of sickle cell β^S-globin allele by hybridization with synthetic oligonucleotides. Proc Natl Acad Sci USA 80:278–282

Covone AE, Mutton D, Johnson PM, Adinolfi M (1984) Trophoblast cells in the peripheral blood from pregnant women. Lancet II: 841–843

DiLella AG, Huang W-M, Woo SLC (1988) Screening for phenylketonuria mutations by DNA amplification with the polymerase chain reaction. Lancet I:497–499

Embury SH, Scharf SJ, Saiki RK, Gholson MA, Golbus M, Arnheim N, Erlich HA (1987) Rapid prenatal diagnosis of sickle cell anemia by a new method of DNA analysis. N Engl J Med 316: 656–661

Estivill X, Farrall M, Scambler PJ, Bell GM, Hawley KMF, Lench NJ, Bates GP, Kruyer HC, Frederick PA, Stanier P, Watson EK, Williamson R, Wainwright BJ (1987) A candidate for the cystic fibrosis locus isolated by selection for methylation-free islands. Nature 326:840–845

Garrick MD (1987) Technical options for screening newborns for hemoglobinopathies. In: Therrel BL (ed) Advances in neonatal screening. Elsevier, Amsterdam, pp 417–423

Gaston M, Rosse WF (1982) The cooperative study of sickle cell disease: review of study design and objectives. Am J Pediatr Hematol Oncol 4:197–201

Gaston MH, Verter JI, Woods G, Pegelow C, Kelleher J, Presbury G, Zarkowsky H, Vichinsky E, Iyer R, Lobel JS, Diamond S, Holbrook CT, Gill FM, Ritchey K, Falletta JM, Prophylactic Penicillin Study Group (1986) Prophylaxis with oral penicillin in children with sickle cell anemia – a randomized trial. N Engl J Med 314:1593–1599

Gill P, Jeffreys AJ, Werrett DJ (1985) Forensic application of DNA "fingerprints". Nature 318:577–579

Guttler F, Lidsky AS, Ledley FD, DiLella AG, Sullivan SC, Woo SLC (1987) Correlation between polymorphic DNA haplotypes at the phenylalanine hydroxylase locus and the clinical phenotypes of phenylketonuria. J Pediatr 110:68–71

Kogan SC, Doherty M, Gitschier J (1987) An improved method for prenatal diagnosis of genetic diseases by analysis of amplified sequences: application to hemophilia A. N Engl J Med 317:985–990

Landegren U, Kaiser R, Sanders J, Hood L (1988) A ligase-mediated gene detection technique. Science 241:1077–1080

Lyonnet S, Caillaud C, Rey F, Berthelon M, Frezal J, Rey J, Munnich A (1988) Guthrie cards for detection of point mutations in phenylketonuria. Lancet II:507

McCabe ERB, Huang S-Z, Seltzer WK, Law ML (1987) DNA microextraction from dried blood spots on filter paper blotters: potential applications to newborn screening. Hum Genet 75:213–216

National Committee for Clinical Laboratory Standards (1985) Blood collection on filter paper for neonatal screening programs; tentative standards. (NCCLS publication LA4-T) NCCLS, Villanova, Pa

Nussbaum RL, Powell C, Graham HL, Caskey CT, Fernbach DJ (1984) Newborn screening for sickling hemoglobinopathies – Houston 1976 to 1980. Am J Dis Child 138:44–48

Oste C (1988) Polymerase chain reaction. BioTech 6:162–167

Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237–251

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Saiki RK, Chang CA, Levenson CH, Warren TC, Boehm CD, Kazazian HH Jr, Erlich HA (1988a) Diagnosis of sickle cell anemia and β-thalassemia with enzymatically amplified DNA and nonradioactive allele-specific oligonucleotide probes. N Engl J Med 319: 537–541

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988b) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Schneider RG (1986) Laboratory identification of hemoglobin variants in the newborn. In: Carter TP, Willey AM (eds) Genetic disease – screening and management. Liss, New York, pp 137–150

Studencki AB, Conner BJ, Impraim LC, Teplitz RL, Wallace RB (1985) Discrimination among the human β^A, β^S, β^C-globin genes using allele-specific oligonucleotide hybridization probes. Am J Hum Genet 37:42–51

Wethers DL, Panel (1987) Newborn screening for sickle cell disease and other hemoglobinopathies. National Institutes of Health Consensus Development Conference Statement. 6(9):1–22

Williams C, Weber L, Williamson R, Hjelm M (1988) Guthrie spots for DNA-based carrier testing in cystic fibrosis. Lancet II:693

Wong C, Dowling CE, Saiki RK, Higuchi RG, Erlich HA, Kazazian HH Jr (1987) Characterization of β-thalassemia mutations using direct genomic sequencing of amplified single copy DNA. Nature 330:384–386

Received August 29, 1988 / Revised October 10, 1988