Molecular Analysis and Chorionic Villus Sampling (CVS): Opportunities for Rapid Prenatal Diagnosis in the Military

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Prior to recent advances in the field of molecular biology, prenatal diagnosis of hemoglobinopathies was accomplished by direct analysis of the gene product(s) in samples obtained by fetoscopy or, more recently, percutaneous umbilical blood sampling. The use of the polymerase chain reaction enables quicker diagnosis than with indirect or cumbersome Southern blot techniques. This case, reporting a couple at risk for fetal sickle cell disease, is a model for rapid prenatal diagnosis and demonstrates the capabilities which exist in the military health care referral system. Within 24 hours of a couple's presentation for consultation and accomplishment of chorionic villus sampling (CVS), preliminary results of both cytogenetic and sickle cell status of an at-risk fetus were determined. Final analysis was accomplished by 48 hours. The combination of early referral, early sampling by CVS, and application of advances in DNA laboratory technologies offers tremendous improvements for care heretofore unavailable as a complete "package" in the military. The implications and limitations for prenatal testing for single gene disorders within the military medical health care system will be discussed.

Introduction

Fundamental to the provision of optimal prenatal diagnosis health care is the provision of both accurate and timely results of testing. While several technical advances have enhanced the availability of obtaining specimens earlier in pregnancy, namely by chorionic villus sampling (CVS) and early amniocentesis (for first and early second trimester diagnosis), these tissues still must be analyzed before results can be made available to the obstetrician and the patient.

Heretofore, testing which involved molecular biologic techniques, such as Southern blot hybridization,[1] often required days in preparation and analysis. Even with early sampling and improved turnaround for culture,[2] the results of restriction fragment length polymorphism hybridization and blot analysis testing, procedures often required for DNA analysis, still may take weeks to accomplish. The implementation of polymerase chain reaction (PCR), a revolutionary new technique for rapid enzymatic amplification of specific DNA sequences, has decreased the time required for molecular analysis from weeks to hours, and greatly improved the efficiency with which prenatal diagnosis may be accomplished.

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Herein we report our recent experience using PCR in the rapid prenatal diagnosis of sickle cell disease following CVS.

Case Report

The consultands are a 31-year-old G1P0000 American black woman and her American black husband. Both consultands had a positive family history of sickle cell trait, and an electrophoresis confirmed the hemoglobin AS genotype in both individuals; thus, the risk for any of this couple's offspring to be homozygous for hemoglobin SS was 25%.

Following extensive counseling regarding inheritance, clinical manifestations, and therapy for patients with sickle cell disease, the couple opted for first trimester prenatal diagnosis by CVS.

A dating ultrasound examination was performed, confirming fetal viability, posterior fundal placentation, and a singleton intrauterine pregnancy of 10 weeks' estimated gestational age.

Transabdominal CVS was performed by introducing (under concurrent ultrasound guidance) an 18-gauge intra-discal therapy needle into the substance of the placenta, being careful not to enter the amniotic cavity. Seven milligrams of chorionic villi were aspirated in a single pass, immediately inspected for quality, then transferred to the cytogenetics laboratory for processing. Fetal cardiac activity was confirmed following the procedure.

The CVS samples were processed by both the "direct" method, in which metaphases present in the villi are analyzed after brief incubation, and by the traditional culture method. For biochemical studies and DNA analysis, extracts of chorionic villi were initially prepared by essentially the same methods used to prepare fibroblasts or cultured amniotic fluid cells.

Crude genomic DNA was obtained by boiling 5 μg of dissected chorionic villi in DNA denaturing solution (0.1 M NaOH, 2.0 M NaCl, 0.5% sodium dodacyl sulfate) for 5 minutes, followed by centrifugation at 12,000 rpm for 10 minutes to pellet cellular debris. One microliter of supernatant containing the fetal DNA was mixed with 0.5 μl Taq polymerase using appropriate buffer (Perkin-Elmer-Cetus), 20 pmol of each dNTP, 50 pmol of each primer (5'-ATCACTTAGACCTCACCCTGGTGGAGCCA-3' and 5'-TCATTCGTCTGTTTCCCATTCTAAAC-3'), and deionized water to a final volume of 100 μl. The mixture was placed in a Thermal Cycler (Perkin-Elmer) for temperature cycling of 94°C for 1 minute and 2 minutes at 66°C for 3 cycles, followed by final extension at 72°C for 10 minutes.

After confirming the presence of the expected 725 base-pair amplification product, 10 μl of the final product mixture was digested with the restriction enzyme *Cvnl* at 37°C for 3 hours.

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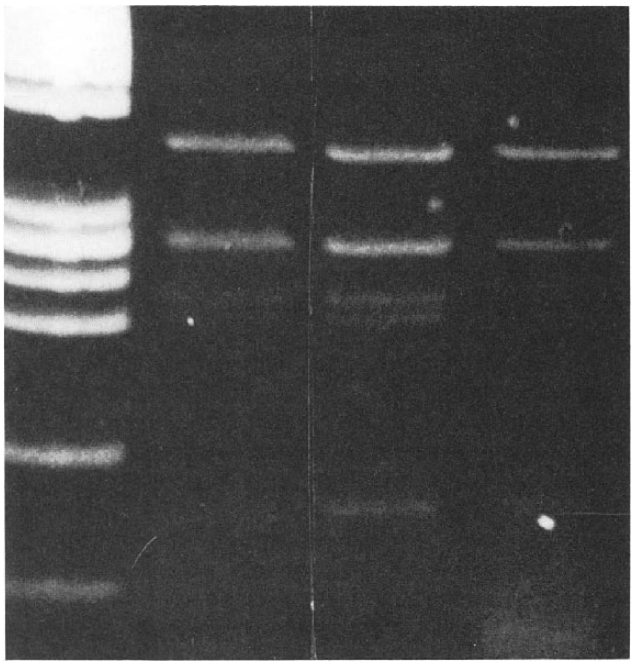

Fig. 1. Cvn1 restriction digest PCR Results.

The digest was then separated on a 4% agarose gel in TBE buffer at 75 V for 90 minutes. The gel was stained with ethidium bromide and the fluorescent bands visualized and photographed under ultraviolet light.

A similar process was followed for DNA obtained from parental blood, as well as other control patients, except that the DNA was extracted and purified using standard phenol/chloroform technique and 260 nm ultraviolet quantification.

The results of testing are shown in Figure 1. Both parents (mother and father) as well as the control AS heterozygote manifest the typical five-band pattern at the beta-globin locus (381 base pairs (bp), 256 bp, 201 bp, 180 bp, and 88 bp). The absence of the 180 bp/201 bp doublet in the fetal (fetus) samples shows that no wild-type alleles are present. Note that the fetal sample shows a faint extra band, consistent with incomplete digestion. Therefore, the predicted fetal genotype is hemoglobin SS, homozygous affected. (These results were later confirmed independently by investigators in the Department of Medical Genetics at the Johns Hopkins University.)

The complete process of counseling, invasive testing, and specimen analysis was accomplished in 24 hours.

Discussion

The prenatal diagnosis of sickle cell anemia is, of itself, not particularly noteworthy. This disease has been antenatally detected using genetic amniocentesis and CVS for several years, initially by linkage analysis and later using direct detection of the specific molecular mutation in the beta-globin gene.[3,4] As initially described, analysis using Southern blot technique was both accurate and reproducible; however, the technique often required one week to accomplish once adequate cells or tissue were available for analysis.[5,6] The clinical application of the polymerase chain reaction has remarkably improved the ability to rapidly accomplish molecular analysis, at no obvious expense of either accuracy or reliability.[7-9]

Prior to the introduction of PCR by Mullis et al.,[10] samples destined to be processed by molecular biologic techniques were generally of insufficient volume for immediate analysis when initially obtained by CVS, and certainly insufficient when obtained by genetic amniocentesis. Cell culture was necessary to insure an adequate volume of DNA for analysis. The most significant impact that PCR has had on prenatal diagnosis has been the ability to rapidly amplify DNA by several million-fold in a matter of hours. Thus, molecular analysis of specimens obtained early in pregnancy may be quickly and accurately accomplished as demonstrated in this report.

As with Southern blot hybridization techniques, the optimal genetic diseases for PCR study involve those in which a specific, detectable method of direct analysis is possible. Diseases which are suboptimal for study include those in which the specific cause of the disorder is unrecognized and linkage analysis must be accomplished in addition to molecular analysis. A host of diseases prenatally detectable by molecular genetic techniques exists, and the list of disorders increases regularly. Many of these disorders are relatively uncommon; therefore, diagnostic testing is accomplished in several specialized laboratories. Some disorders, however, are sufficiently common that, given the unique health care referral system in the military, a single well-staffed and -funded genetics laboratory could accomplish the majority of prenatal diagnoses for patients at risk for the more common disorders detectable by molecular biologic techniques. The advantages of limited centralized testing for military health care recipients in a military laboratory are numerous.

First, a centralized laboratory functions as a valuable resource for information on clinical details such as specimen retrieval, critical members of the pedigree to test, and the likelihood of informativeness of any particular study in a given family. Since the results of linkage studies are given in terms of risk rather than as "predictions," variable factors such as paternity, meiotic recombination, and spontaneous mutation may be a source of confusion to consultands and referring physicians alike. With centralized analysis, test results (and the other issues listed, which are frequently not directly addressed by commercial companies) can be directly discussed with geneticists.

Second, typical commercial laboratory costs for family studies of common genetic conditions such as cystic fibrosis using PCR techniques range from $300–$1000, depending upon the complexity of the case and the informativeness of the pedigree. Moreover, personnel at individual hospitals, who often have little knowledge regarding consumer assessment of critical issues such as experience, technical expertise, and reproducibility of results, are forced to "shop" for a molecular biology laboratory.

Patients at risk for diseases which could be detected in the centralized system described herein are listed in Table I and include directly detectable disorders (e.g., sickle cell disease; the delta 508 cystic fibrosis mutation) as well as some indirectly detectable disorders (e.g., adult polycystic kidney disease; the hemophiliacs). Since the incidence of two diseases alone—sickle cell disease (heterozygosity in the American black population approximately 8%) and cystic fibrosis (het-

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TABLE I

PRENATAL DIAGNOSIS OF INHERITED DISEASES BY MEANS OF MOLECULAR BIOLOGIC TECHNIQUES IN A MILITARY LABORATORY

Direct detection
Sickle cell disease
Cystic fibrosis (delta 508 mutation)
Alpha-thalassemia (deletions)
Beta-thalassemia (most forms)
Duchenne muscular dystrophy (deletions)
Indirect detection (linkage analysis)
Adult polycystic kidney disease
Cystic fibrosis
Hemophilia (A and B)
Huntington's disease
"Fragile-X" syndrome (?)

erozygosity in the white population approximately 5%)—is very common, a large segment of military health care recipients are at risk for disorders potentially detectable by molecular genetic techniques. Moreover, in addition to being common genetic disorders, both of these inherited diseases are conditions in which early diagnosis and institution of medical therapy may afford improved outcome and which are theoretically candidates for treatments by so-called "gene therapy."[11-14]

This couple, and the management of their prenatal diagnostic needs, illustrate the potential which exists for providing rapid, accurate, and timely care in the socialized health care system that is military medicine. Clearly, we in the military are in the enviable position of being able to offer state-of-the-art testing, such as rapid early prenatal diagnosis and PCR analysis, to those who should be offered genetic testing. Given the complexity of the technology, the direct cost-savings to the Department of Defense by offering services within our health care system (for both chorionic villus sampling [$1200-$1500] and molecular analysis [$300-$1000 per family member tested]) is considerable.

In the vast majority of consultations, prenatal diagnosis gives reassuring results to couples who have considerable anxiety about their increased risks and the health of their fetus. While some parents would choose pregnancy termination following the diagnosis of a fetus with an abnormality, the patients in this report elected to continue the pregnancy. We were able to arrange prenatal counseling with both the neonatologist who will care for the infant in the nursery and the pediatric hematologist who will be providing long-term care for the child after birth. Thus, an early plan for management was instituted which offers a continuum of care and which facilitates entry of this couple and the proband into one of several national metabolic therapy programs prior to birth.

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