Cystic Fibrosis: Molecular Approaches to Diagnosis*

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ABSTRACT

Whole blood collected on filter paper (Guthrie cards) has provided an excellent means for screening inborn errors of metabolism in neonates. Traditional biochemical methods adapted for use with this collection device have proven instrumental in the detection of many congenital defects such as phenylketonuria, galactosemia, hypothyroidism and hemoglobinopathies. The advent of molecular techniques, specifically polymerase chain reaction (PCR), has resulted in unparalleled advances in diagnostic sensitivity. Because of its ability to amplify small quantities of deoxyribonucleic acid (DNA), PCR has proven particularly successful for use with Guthrie card bloodspots in the identification of many genetic disorders including cystic fibrosis, sickle cell anemia and muscular dystrophy. Furthermore, it has been suggested that Guthrie cards represent a vast archive of genomic material yet to be explored. In this article we review our experience using Guthrie card bloodspots for PCR amplification of the cystic fibrosis gene, describe the advantages and limitations of this technology and speculate on future prospects for molecular diagnostics over the next 100 years.

Introduction

Cystic fibrosis (CF) is the most common genetic defect in Caucasians (1:2,500) with a heterozygote frequency of one in 25.¹² In 1989, the gene encoding the CF transmembrane conductance regulator protein (CFTR) was identified and localized to the long arm of chromosome 7 (7q31).³⁴ The CF gene spans approximately 230,000 bases and contains 27 exons. The CF gene encodes a moderately large (1,480 amino acids, about 168,000 daltons) chloride channel protein regulated by cyclic adenosine monophosphate (cAMP).³⁴,⁵ The most common defect (approximately 66 percent individuals with CF) is the AF508 mutation, a three base pair deletion (exon 10) within one of the two nucleotide binding domains of the CFTR.⁶⁻⁷ Since the identification of the CF gene, polymerase chain reaction (PCR) has facilitated the molecular characterization of over 600 mutations.⁷⁻⁸⁻⁹ Of these, only a few occur with greater than 1 percent frequency.⁸⁻⁹ Because of the large heterogeneity of CF genetic abnormalities, approximately 70 mutations must be screened for in order to obtain a diagnostic sensitivity approaching 90 percent.⁸⁻¹⁰⁻¹¹
Thus, the molecular techniques aimed at analysis of CF gene provide an excellent opportunity for the development of future diagnostic methods and strategies. In this paper we review pertinent highlights and focus on the experience of our laboratory.

**Sample Collection**

PCR amplification\(^1,2\) of genomic DNA for CF has been successfully performed on a wide variety of biological samples including peripheral whole blood, saliva, buccal smears, amniotic fluid, paraffin-embedded tissue blocks, and chorionic villi.\(^13,14,15,16,17,18,19\) However, due to its widespread use as a neonatal screening tool, human capillary whole blood absorbed on filter paper (Guthrie cards) is perhaps the most common sampling device. Guthrie cards were first introduced as a neonatal collection device by Dr. Robert Guthrie in 1963.\(^20\) Guthrie cards have been instrumental in the early detection of inborn errors of metabolism including phenylketonuria, galactosemia, hypothyroidism, as well as various hemoglobinopathies. To date only one filter paper (Filter paper 903, Scheicher & Schuell, Inc., Keene, NH) has been specifically approved for use by the Food and Drug Administration of the United States of America. More recently, Guthrie card bloodspots have proven particularly helpful for the biochemical detection of elevated immunoreactive trypsinogen concentration (a CF marker).\(^21\) Although the presence of these contaminants may be minimized by including chelators and proteases, most frequently, selective separation of DNA by ethanol precipitation is required. Low levels of DNA (10–80 ng/µL whole blood) in Guthrie cards\(^22\) further complicate extraction schemes. In an effort to circumvent these limitations, several laboratories, including ours, developed direct methods for DNA amplification.\(^15,16,17,24,26,28\)

**Extraction of Guthrie Card DNA**

Investigators have used traditional chaotropic-based nucleic acid extraction protocols for Guthrie card DNA.\(^23,24,25,26,27,28,29\) These methods typically involve the use of organic solvents (phenol/chloroform, guanidium thiocyanate) and can be combined with detergents (sodium dodecyl sulfate), chelators (EDTA, Chelex-100) and proteases (proteinase K).\(^23,24,25,36,37,38,39\) These methods, while reliable, are generally labor-intensive, requiring multi-step separation schemes, and thus are not easily automated. Furthermore, the use of strong denaturants to extract DNA results in the simultaneous extraction of whole blood PCR inhibitors (heavy metals, heme, protein).\(^23,28,29\) Although the presence of DNA (10–80 ng/µL whole blood) in Guthrie cards\(^22\) further complicates extraction schemes. In an effort to circumvent these limitations, several laboratories, including ours, developed direct methods for DNA amplification.\(^15,16,17,24,26,28\)

In these protocols PCR is performed directly using a small piece of Guthrie card filter paper. The small size of the filter paper minimizes the concentration of inhibitors eluted into the PCR reaction mix. Using the direct method, we were able to amplify short stretches (98 base pairs) of the CF gene encoding the ΔF508 mutation.\(^15,26\) However, when we attempted to directly amplify larger DNA regions (491 bp), we found substantial variation in the quality and quantity of PCR amplification product.\(^28\) Further investigation revealed that whole blood contaminants including heme, heavy metals (iron and zinc), protein (albumin, carbonic anhydrase, hemoglobin) were responsible.\(^28,29\) The presence of these PCR inhibitors prompted our laboratory to develop a water-based extraction method which selectively eluted PCR inhibitors while retaining filter-bound DNA in amplifiable form.\(^28\) In this simple procedure, a 3 mm diameter blood spot punch is incubated with 1
mL ultrapure water twice. Hypotonicity promotes lysis of cellular elements, especially red blood cells. The avidity of DNA for the filter matrix results in retention of nucleic acid while water soluble contaminants are conveniently removed. The washed filter paper is transferred to a PCR reaction tube and direct amplification is performed (see below).

**Amplification of Guthrie Card DNA**

The size of the genomic region to be amplified can vary. Although it is possible to amplify many thousands of base pairs (bp), amplification of several hundred base pairs is frequently adequate for most diagnostic testing. For example, we have successfully PCR amplified both a small- (98 bp) and moderately-sized (491 bp) region surrounding the CF ΔF508 mutation. Amplification is performed in reaction mixtures by placing a washed 3 mm diameter Guthrie card filter paper (or portion thereof) into a standard PCR amplification recipe containing: 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 200 μmol/L each deoxyribonucleotide triphosphate (dATP, dTTP, dCTP and dGTP), 3 mmol/L MgCl₂, 1.25 U Taq polymerase, 0.01 percent (weight/volume) gelatin and 100 ng (each) oligonucleotide primer. To avoid nonspecific amplification of contaminating amplicons, we utilize carryover prevention by substituting dUTP for dTTP and including 0.5 U uracil N-glycosylase in each reaction mix. Depending on the purity of the starting material, thermal cycling is typically initiated at high temperature (95°C) for several minutes to inactivate endogenous nucleases. Thermal cycling generally consists of denaturation (94°C), annealing (55°C) and elongation (72°C), generally one minute each for a total of 35 to 40 cycles. The length and temperature of each step may be adjusted for optimization. A final longer elongation time (15 to 20 minutes) is used for maximal amplification of existing sequences. Since the rate of amplicon generation by Thermus aquaticus DNA polymerase is extremely rapid (about 10,000 bases/min) increasing the elongation period is generally not required even for large-sized amplicons. Although it is possible to overcome limiting template concentration by increasing cycle number, this practice may increase the likelihood of amplifying contaminating nucleic acid. Using our direct method, we demonstrated that PCR amplification could be successfully performed on filter paper as small as 0.4 mm². This piece of filter paper contains about 0.2 μL whole blood corresponding to 2–16 ng DNA. Using our direct method, we approximated that a total of 400 CF mutations could be individually amplified from a single 1.5 cm diameter Guthrie card bloodspot. If multiplex PCR amplification were employed, this number would increase dramatically. Alternatively, RNA may also be extracted from Guthrie cards and used for molecular diagnostics by reverse transcription. Quantitative determination of RNA level correlates to relative expression of genes. Despite the ubiquitous presence of ribonucleases, Guthrie card RNA appears sufficiently stable for reverse transcription amplification.

**Detection of PCR Amplified Sequences**

Polymerase chain reaction amplified DNA sequences (amplicons) can be easily detected by electrophoresis in agarose or polyacrylamide matrices. Because of their double-stranded structure, amplicons avidly intercalate ethidium bromide and can be visualized with ultraviolet lighting. Although resolution of DNA amplicons is generally poor using these media (up to 30,000 bp), separation may be substantially enhanced by combining the two media or simply adjusting the relative proportion of acrylamide to its cross-linker bis-acrylamide. In fact, we demonstrated that it was possible to electrophoretically resolve small differences in amplicon size including the ΔF508 mutation (95 bp) from normal (98 bp). Larger-sized amplicons (491 bp) are not readily resolved by this technique. Under these circumstances it may be possible to detect the ΔF508 mutation by monitoring heteroduplex formation. Amplicon resolution may be substantially improved using pulse field electrophoresis (up to 20 million base
pairs). Sensitivity may be enhanced following transfer of amplicons to filter matrices (ie, nitrocellulose or nylon), heat denaturation (to single-stranded structures) and hybridization to allele-specific oligonucleotide (ASO) probes (dot and Northern blotting). Although historically favored for autoradiographic detection, radionuclide $^{32}$P- or $^{35}$S-labelled probes have been supplanted by nonradioactive electrochemiluminescent technology. Microtiter plate-based methodology has been adapted for amplicon detection using a variety of techniques. In one method a specific antibody for double-stranded DNA sequences is used. Double-stranded hybrids may be detected using an enzyme-labelled (alkaline phosphatase, horseradish peroxidase) secondary antibody and monitoring conversion of substrate to product spectrophotometrically. Because the same anti-double-stranded DNA antibody can be used for detection of any competent hybrid, the benefits of this technology are obvious. Alternatively, a biotin-labelled dNTP may be incorporated into the amplicon during amplification. Avidin-associated enzymes may then be used to probe for the formation of competent hybrids to ASOs fixed to a solid support (microtiter well, nylon/nitrocellulose membrane). Increased sensitivity may be obtained by oligonucleotide ligation assay (ALO), which can detect a single base pair mutations. Other suitable labels have included digoxigenin, fluorescein, as well as rare earth metals such as Europium (Eu), Samartium (Sm) and Rubidium (Rb).

GUTHRIE CARDS AS A GENOMIC REPOSITORY

It has been proposed that Guthrie card bloodspots provide a unique repository of genetic information. Because of their use as a neonatal screening tool, the collection of whole blood on filter paper is simple and widely established. Samples can be transported easily by mail and have minimal storage requirements (generally at room temperature). Furthermore, in contrast to adults, neonatal whole blood contains more DNA due to the presence of nucleated red blood cell precursors. Filter paper DNA appears relatively stable (>17 years in one study). In a limited study in our laboratory, we found that storage at room temperature (up to 5 y) did not affect the stability of nucleic acid on filter paper, although the elution properties of PCR inhibitors were altered. Whole blood components, which act as natural PCR inhibitors, become increasingly fixed to the filter paper matrix during storage. As storage times increase, this effect may complicate direct amplification methods, including ours. Although fixation did not appear to result from sample dehydration (based on weight), the use of Guthrie cards stored for very long periods of time may require extraction with organic solvents and detergents (ie, phenol/chloroform, guanidium thiocyanate, sodium dodecyl sulfate). In an attempt to address storage and stability issues, filter paper impregnated with guanidine thiocyanate (Schleicher & Schuell, Keene, NH) was recently introduced as a whole blood collection device designed specifically for molecular diagnostics. Because of the potential to serve as a DNA data base, many neonatal screening laboratories have indicated that they plan to establish more permanent DNA collections. Thus, it is anticipated that molecular methods aimed at utilizing this repository of genetic information will grow.

FUTURE CONSIDERATIONS

What will the next 100 years bring us? It is anticipated that virtually all of the estimated 100,000 human genes will be identified by the year 2005. As the human genome project progresses, fast, accurate and ultrasensitive molecular diagnostics will be required. To prevent cross-contamination, these systems will be self-contained and thus most likely require miniaturization (microfluidic technology). Screening for thousands of mutations will take place simultaneously on silicon "chips" impregnated with mutation-specific oligonucleotide sequences. Alternatively, high throughput sequencers will be developed to...
sequence entire genes, some spanning up to 2 million base pairs. The ability to sequence large regions of genomic material will provide more accurate correlation between genotype and disease presentation. Thus, unequivocal identification of large nucleic acid sequences such as the CFTR gene will no longer represent an overwhelming technical obstacle, but rather a routine laboratory test. But what will be the role of the physician in the future? Despite these achievements, genetic identification does not offer a panacea.55,56,57 The role of the physician will not diminish—it must expand and evolve to meet the scientific, ethical and social challenges which await in the new millenium.

Acknowledgements

The authors would like to extend their deepest appreciation to Ms. Francesca L. Nadeau, Ester L. Davis and Kathy Kolakowski for their dedication, support and excellent technical assistance in the University of Connecticut Cystic Fibrosis Newborn Screening Laboratory.

References

27. Yang M, Hendrie HC, Hall KS, Oluwole OSA, Hodes


35. Lanzillo JL. Chemiluminescent nucleic acid detection with digoxigenin-labelled probes: a model system with probes for angiotensin converting enzyme which detect less than one attomole of target DNA. Anal Biochem 1991;194:45–53.


