Robust and inexpensive equipment design for polymerase chain reaction detection of sequence mutations

Cystic fibrosis in a mother and 2 children analysed

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Abstract **Every polymerase chain reaction (PCR) requires use of a temperature cycler for about 3 hours. Since there are many diagnostic tests using this technology, it is important that robust but inexpensive machinery is available. Such a stand-alone machine has been designed and used to analyse an interesting family in which a mother and her 2 children were diagnosed as having cystic fibrosis.**

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The polymerase chain reaction (PCR)[1] has the potential to revolutionise diagnostic pathology. Whenever a gene sequence has been determined, the sequence can again be read in the genome of any individual using DNA extracted from any source. Where a specific mutation site is known, a defined region, e.g. a few hundred base pairs, can be amplified so that the DNA can be studied directly by actual resequencing of the DNA, by observing loss of DNA due to deletions and therefore shorter PCR products, by using the susceptibility to restriction enzyme digestion to indicate DNA mutations, or by using single-stranded conformation polymorphism of DNA to indicate a mutation somewhere within the PCR product that can then be analysed further.

The power of the PCR technique relies on the fact that the DNA region of interest can be amplified millions of times with high fidelity. Applications range from diagnosing genetic mutations to determining the presence of viral or bacterial DNA or RNA.

Each application ties up a PCR machine — essentially a programmable thermal cycler — for a few hours at least, so a number of machines are required in any diagnostic laboratory. With this need in mind a robust but inexpensive thermal cycler was designed and tested.

Rapid diagnosis of mutations places in the hands of the genetic counsellor information that permits very precise recommendations for those families who have prudently sought advice. This is a distinct improvement on less direct methods, e.g. restriction fragment polymorphism mapping, that were until recently the mainstay for intrafamilial diagnoses such as that of cystic fibrosis.[2] The ease and accuracy with which a family can be analysed is described in a case report of a mother and her 2 children, all of whom have cystic fibrosis.

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Design of thermal cycler

In order to reduce costs we eschewed memory facilities and the need for external computer control. In the interests of rapidity of cycling cooling was assisted by circulating water through an aluminium block, which was intermittently heated by two attached elements. Feedback control from thermistors in the block was achieved with solid-state printed circuitry. Water flow

was regulated through a solenoid attached directly to the mains water supply.

Three cycles of differing temperature and duration are possible and each cycle can be held continuously if required. The temperature of the block is set for each cycle through potentiometers accurately calibrated from 0 to 100°C and the duration of each cycle can be set in 10-second steps up to 990 seconds. An automatic cut-out is activated if the block temperature rises above 95°C. The number of cycles is counted and the cycler stops at the synthesis temperature after a predetermined number of cycles.

The thermal cycler efficiency is increased by heating and cooling as little metal mass as possible, and the main barrier to even faster cycling is the thickness of the plastic of the Eppendorf tubes currently available (Fig. 1).

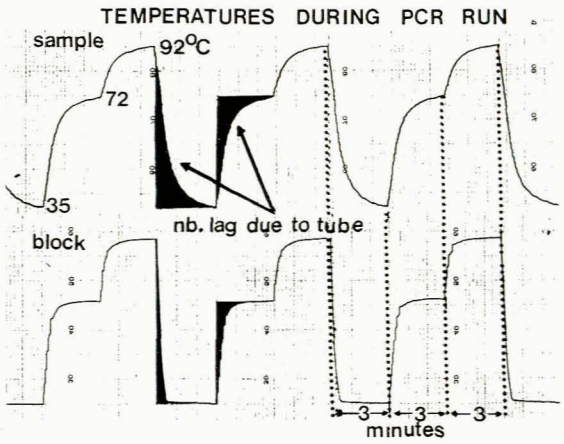

FIG. 1.
Temperature of sample and heating block measured with a thermistor probe.

Since the rate of heating and cooling is constant, only total cycle duration is measured. More rapid heating or block temperature overshoot in order to speed up the rate of sample heating leads to the outer shell of the sample being overheated, and this causes enzyme deterioration at an increased rate. We have therefore chosen a more gentle heating ramp rate to reach the required temperature. It should be noted that accurate *sample* temperature measurement requires very special equipment and that systems using feedback control through thermistors within a sample tube risk problems due to tube inhomogeneity, the mass of the thermistor itself, inadequate contact between tube and well, and inadequate or unrealistic comparison of the probe tube with the actual sample tubes with respect to their contents. We have catered for 48 × 50 µl samples in our system. A 1-volt analogue output is available that reflects block temperature. The effect of tube thickness on the change of temperature can clearly be seen when a sample tube and heating block temperatures are compared (Fig. 1).

Polymerase chain reaction

The commonest cystic fibrosis mutation in exon 10 of the chloride conductor channel protein gene involves a 3-base pair deletion so that primers bracketing this region (corresponding to amino acid phenylalanine at position 509 in the amino acid sequence) will produce either a normal 50-base pair product from a gene or the smaller 47-base pair product with a 3bp deletion.[3-5] This size difference is readily seen in the amplified DNA when electrophoresed in agarose and visualised with ethidium bromide under fluorescent illumination (Fig. 2A). The primer sequence for each PCR and the reaction conditions used have been described.[3,4]

More than 70 mutations have been described in the cystic fibrosis gene. The additional mutation of relevance in this paper is found by amplifying part of exon 11 and detecting the mutation (G1784 → A) in that the PCR product from the mutated gene has acquired a new enzyme restriction site. The restriction enzyme Mbo 1 cuts the 425-base-pair product into two fragments 243 and 182 base pairs long if the gene is mutated (Fig. 2B). The chloride conductance channel protein has as a consequence a glycine at position 551 replaced by an aspartic acid (G551D).[3]

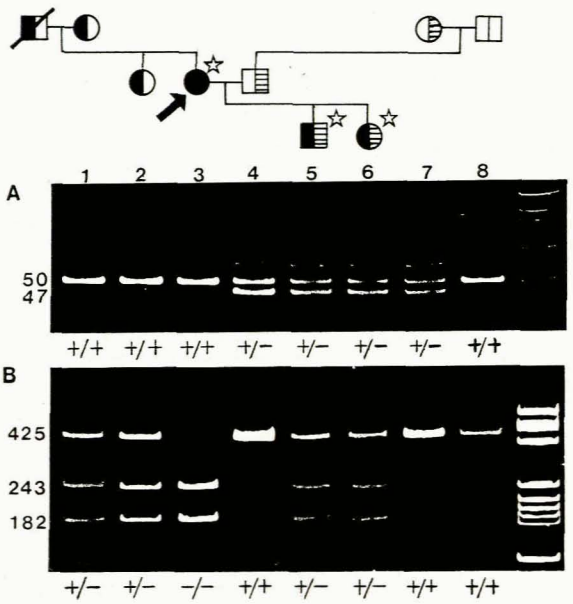

FIG. 2.
Cystic fibrosis family tree and analysis of PCR products and restriction enzyme fragments by agarose electrophoresis. A: F508 deletion: (+) = normal gene, 50bp; (–) = 3bp deleted gene, 47bp. B: G 551D mutation: (+) = normal gene, 425bp, not cut by Mbo I; (–) = mutated gene, cut by Mbo I into two fragments, 243 and 182bp.
In the family tree the propositus is indicated with an arrow and cases of clinical cystic fibrosis with a star. The deceased maternal grandfather would have been an obligate heterozygote.

Illustrative family history and genetic tree

The mother, who had a history of chronic chest disease since youth, developed chronic bronchitis and was only diagnosed as having cystic fibrosis when pregnant for the first time. She had also experienced several episodes of heat exhaustion. The breast-fed female infant failed to thrive and was first seen at the age of 2 months. Sweat tests on two occasions were positive and the infant was started on enzyme replacement therapy. She is now 11 years old and on the 50th percentile for weight. She has never required hospitalisation but is showing mild but progressive lung involvement due to *Haemophilus influenzae* infection. In spite of the first child's disease (although this was relatively mild), and genetic counselling, which advised that 50% of future offspring would be homozygous while the other 50% would be obligatory carriers, the mother delivered a second infant 4 years later. This child, a boy, also failed to thrive during the 1st month of life while breast-fed

until enzyme replacement therapy was started. The sweat test was positive. He is now 7 years old and just above the 10th percentile for weight. His disease has also been relatively uncomplicated, with relatively mild intercurrent chest infections due to *Staphylococcus aureus*. The mother now has clubbing, and is plethoric, mildly cyanosed and short of breath at rest.

There was no history of consanguinity or other cases of cystic fibrosis in the parents' families. The mutations found by the PCR technique clearly demonstrate the unfortunate concurrence of genetic predispositions in the family and its consequences (Fig. 2). The mother, the propositus, was homozygous for a less common mutation (G551D) and her mother and sister were heterozygous carriers. The untested, deceased maternal grandfather would have been an obligatory heterozygote. Both affected children are compound heterozygotes, the other mutated gene being the commonest found in cystic fibrosis, viz. deletion of F508, and inherited through the paternal grandmother (frequency in Caucasian populations 1:25 - 1:30).[5]

Discussion

The PCR and related techniques are major developments, and it can be predicted with confidence that use of the technique will have a rapidly growing impact on the practice of medicine. The need for robust but inexpensive machinery is self-evident. The temperature cycler designed has been tested for 18 months now without any problems and is in widespread use.

Diagnosis of cystic fibrosis in cases with the F508 deletion and other mutations is now feasible in the routine laboratory, not only for the purposes of genetic counselling but also to detect new cases and screen selected populations.

The family history presented illustrates a useful application of PCR and is in itself clinically very interesting. A number of sensitive issues can also be raised, such as: (*i*) should cystic fibrosis screening be applied more generally?; (*ii*) should selected clinical populations be screened routinely, e.g. for chronic lung disease, heat exhaustion, neonatal intestinal disturbances with abnormalities apart from meconium ileus, adolescent diabetes and cases of malabsorption?; and (*iii*) should genetic screening become a normal form of medical documentation?

In the case history presented the mother was homozygous for a less common mutation and it is remarkable that the cystic fibrosis was mild enough to permit her to become pregnant twice. Infertility is common in affected females, while in males sterility usually occurs secondarily to aplasia of the epididymis and vas deferens (7 out of 17 cases were reported to be heterozygous for the F508 deletion).[6] Unfortunately our patient's spouse was heterozygous for the commonest mutation causing cystic fibrosis, viz. the F508 deletion, so that in the marriage 50% of his progeny could be expected to be compound heterozygotes and to manifest cystic fibrosis. In this case both children were unfortunately affected, but the clinical pattern of their cystic fibrosis was similar to the milder form shown by the mother.

Would routine genetic screening have helped this family? At the present the answer has to be negative, since there are too many rare mutations to screen for them routinely and this approach is therefore beyond the capabilities and costs of our current PCR systems. Restriction fragment length polymorphism phenotyping can be used to assess the likelihood of transmission of mutated genes[2] within families.

The rapid diagnosis of cystic fibrosis by genetic means has proved to be very useful in investigating new cases and is used as the initial test in neonates and severely ill children in whom sweat for conventional measurement of electrolyte concentrations is difficult, if not impossible, to obtain. Sweat testing should of course be done routinely and with great care in all other cases, including those in which PCR tests are negative, which will still account for about 20% of cases in whites. In several Malay families in the Cape we have found only other rarer mutations (not published), and so far no cases in blacks have been detected.[7]

Recent developments in the understanding of the working of the gene product affected in cystic fibrosis — viz. the chloride conductance channel protein — have opened up new potential modes of therapy for the respiratory tract using amiloride and possibly trinucleotides to cause the chloride channel to function properly.[8] Prospects also look very promising for gene therapy in the form of adenovirus vector-mediated transfection of the bronchial epithelium in the not-too-distant future. The biochemical lesion in cystic fibrosis and its consequence (defective glycosylation of proteins in the poorly acidified trans-Golgi apparatus) have provided the means to study transfection strategies *in vitro*.[9]

The PCR machine was designed in the laboratories of the Department of Chemical Pathology of the University of Cape Town at Red Cross War Memorial Children's Hospital in collaboration with Mr J. Thornthwaite and is currently manufactured under licence by ESU Electronic Supplies, PO Box 4274, Stikland, Cape Town. The current cost is R6 000. The project was supported by the UCT Organisation for Applied Research, the South African Medical Research Council and the UCT Ethics and Research Fund. The assistance of Professor M. Bowie and colleagues at the Cystic Fibrosis Clinic at the Red Cross Hospital is gratefully acknowledged. Dr J. Herbert of the Department of Human Genetics at the University of Stellenbosch and Dr M. Ramsay of the Department of Genetics at the University of Witwatersrand provided points of view on genetic counselling that we appreciate and value. The primers for exon 11 were a gift from Dr M. Ramsay.

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