Genetic analysis in cystic fibrosis using the amplification refractory mutation system (ARMS): the J3.11 *MspI* polymorphism

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Abstract

A new method of genetic analysis has been devised. The method, amplification refractory mutation system (ARMS), has been used to genotype the J3.11 *MspI* restriction fragment length polymorphism (RFLP) closely linked to cystic fibrosis (CF). The DNA sequence for both alleles of this dimorphism has been used to design ARMS primers. These allow genotyping of DNA isolated from blood, Guthrie cards, and buccal cells.

The ARMS method allows discrimination of alleles differing by a single base pair using a polymerase chain reaction (PCR) with selectively modified primers.¹ ARMS does not require DNA of the quantity and quality necessary for conventional filter hybridisation analysis of RFLPs and less than 100 ng of DNA is required.

Purified DNA from peripheral leucocytes and chorionic villus biopsy has been genotyped using ARMS.² In this report we describe the first use of this method on crude DNA extracts from mouthwash samples and Guthrie cards. These sources of DNA allow non-invasive and retrospective sampling, respectively. A further advantage of ARMS is the avoidance of restriction enzyme cleavage which is subject to problems with incomplete digestion.

The locus detected by the J3.11 probe is closely linked to CF and contains a dimorphism detected by

the restriction enzyme *MspI*.³ We describe the use of ARMS to determine the J3.11 *MspI* RFLP genotype of DNA from the sources described above.

Materials and methods

CHARACTERISATION OF THE J3.11 *MspI* DIMORPHISM
Genomic DNA from a number of healthy volunteers was subjected to PCR amplification using the upstream primer dAAGTTTGAGCATAGG-AAAAGTTCTGTGCC and the downstream primer dCATCCTTGACTCTTCTCTTCTCAAAGGCC. These primers flank the dimorphic *MspI* site. Direct sequence analysis was performed with primer dAGTTCTGTGCCAAAATTGCATCCAAG, which is nested with respect to the upstream amplification primer, using the method previously described.⁴ The sequence characterisation of the *MspI* dimorphism is shown in fig 1. Allele 1, which lacks the *MspI* site, and allele 2 are characterised by the sequences CCAG and CCGG respectively at the dimorphic *MspI* site. During sequencing, a second single base change A to G was noted 23 base pairs upstream of the *MspI* site. The G variant cosegregated with the *MspI* site in the samples sequenced. Extensive population studies were not carried out. Appropriate volunteers, who had genotypes 11, 12, and 22 for J3.11, provided blood, Guthrie spots, and buccal washes for ARMS analysis.

DESIGN OF ARMS PRIMERS

DNA polymerase isolated from *Thermophilus aquaticus* (*Taq* DNA polymerase) will synthesise DNA in a 5' to 3' direction from a primer annealed to a complementary template DNA. Previous studies have shown that alteration of the 3' terminal nucleotide of a primer, to create a mismatch with the template, is inhibitory to this process.¹ This inhibition depends on the nature of the nucleotide mismatch. A purine-pyrimidine mismatch is less inhibitory than either a purine-purine or pyrimidine-pyrimidine mismatch. Complete inhibition of primer extension has been achieved by introduction of further nucleotide

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Figure 1 Characterisation of the J3.11 polymorphism by PCR and direct sequencing. The left panel shows the genomic DNA sequence of the *MspI*+ allele (haplotype 2), the panel on the right shows that of the *MspI*- allele (haplotype 1).

mismatches near the 3' terminus of the primer.¹ For the J3.11 *MspI* dimorphism the base change (A→G, allele 1→allele 2) defined the 3' OH terminus of the discriminatory ARMS primers. Because these primers generate either an A–C mismatch or a G–T mismatch we chose to introduce further mismatched base 3 nucleotides from the allele specific 3' base. The primers were designed with an A–G mismatch at this position. Consequently, the ARMS primers were dTTATATTGTTGTGTCAGGACCAGCATTACA (allele 1) and dTTATATTGTTGTGTCAGGACCAGCATTACG (allele 2). The common primer was the downstream amplification primer described above. Allele specific amplification with each primer pair generated a 550 base pair (approx) product when the appropriate allele was present.

DNA EXTRACTION

DNA was prepared from buccal epithelial cells according to Lench *et al.*⁵ However, the cells were also resuspended and washed twice with saline solution. The cells were resuspended in 500 µl water, boiled for five minutes, and centrifuged at 12 000 *g* for five minutes. DNA was extracted from Guthrie cards by the dissection of individual dried

blood spots into small fragments. These were boiled in 0.05% 'Tween 20' (100 µl). Debris was pelleted by centrifugation at 12 000 *g* for five minutes.

ARMS ANALYSES

DNA (10 to 30 ng) purified from peripheral leucocytes, 10 µl from supernatants of buccal cell DNA preparations, or 5 µl from the supernatants from the Guthrie card DNA isolations, was used directly. All ARMS analyses were performed with concurrent amplification of a 360 bp or 220 bp region of the human α_1 -antitrypsin (AAT) gene⁴ as internal control. The reactions were carried out in pairs using the common primer with the *MspI* RFLP allele 1 specific primer in one reaction and the *MspI* RFLP allele 2 specific primer in the other. All reactions were performed in 100 µl containing 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.2 mmol/l MgCl₂, 0.01% gelatin, 2 units *Taq* polymerase, the four deoxynucleoside triphosphates (100 µmol/l each), and the appropriate ARMS and internal control primers (1 µmol/l each). All reactions were overlaid with 50 µl paraffin oil. The amplification protocol was as follows: denaturation at 92°C for two minutes, enzyme addition (at

Figure 2 Ethidium bromide stained agarose gel analysis of ARMS reaction products. (A) ARMS analysis of purified genomic DNA. The internal control is a 360 bp fragment of the AAT gene. (B) ARMS analysis of Guthrie card extracts. The internal control is a 220 bp fragment of the AAT gene. (C) ARMS analysis of buccal cell extracts. The internal control is as in (A). m denotes DNA fragment size markers and is an *HaeIII* digest of bacteriophage ϕ X174 DNA (1353, 1078, 872, 603, 310, 281, 271, 234, 118, and 72 base pairs). + and - describe zygosity for the J3.11 *MspI* dimorphism. In the left of each pair of lanes the allele 2 (*MspI*+) specific ARMS primer was used, in the right of each pair the allele 1 (*MspI*-) specific primer was used.

92°C), primer annealing and extension at 60°C for four minutes, and denaturation at 92°C for two minutes. Forty cycles were performed. The ARMS analyses are shown in figs 2 and 3.

Results

DEMONSTRATION OF PRIMER SPECIFICITY

The ARMS primers designed as above discriminated between the three test genotypes, defined by direct sequencing, in DNA samples from peripheral leucocytes, Guthrie cards, and buccal epithelial cells (fig 2).

Figure 3 (A) Pedigree of family A with the J3.11 *MspI* alleles, determined by filter hybridisation, of each subject shown directly below their ARMS analysis. The J3.11 *MspI* alleles as determined by ARMS are shown directly above their ARMS analysis. (B) As for family A. Each ARMS analysis is as described in the legend to fig 2. m denotes DNA size markers, also as described in the legend to fig 2. The internal control is a 360 bp product derived from the AAT gene. The CF status of the subjects in both pedigrees has been omitted for clarity because neither family was informative for CF using the J3.11 *MspI* RFLP.

FAMILY STUDIES

In order to verify that ARMS is an alternative to the conventional filter hybridisation analysis of the J3.11 *MspI* RFLP, two CF pedigrees (A and B) were studied (fig 3). These had been previously genotyped for the J3.11 *MspI* dimorphism as described.³ The results from ARMS and filter hybridisation studies were concordant (data not shown).

SECOND SEQUENCE VARIANT

As noted above, a second sequence variant was observed 23 base pairs upstream of the G to A base change at the dimorphic *MspI* site. Alternative ARMS primers incorporating this second variant gave identical results to those described above. Thus, a mismatch at this distance from the 3' terminus of the ARMS primers did not influence the specificity of the reactions.

Discussion

We have shown that ARMS analysis of DNA can distinguish RFLPs in a rapid and easy manner whether the DNA is isolated pure or as a crude preparation from buccal epithelial cells or from Guthrie cards. This method can be used for early prenatal diagnosis of CF since analyses can be performed on chorionic villus biopsy and at other CF

linked polymorphic loci.² Carrier detection can be performed using non-invasive sampling methods and at lower cost than invasive alternatives. ARMS analyses can be performed retrospectively using Guthrie cards; indeed, we have amplified DNA from Guthrie cards 21 years old.⁶ Therefore, retrospective analysis may be carried out when it is required to establish the phase of a linked RFLP in certain families where the key person has died or cannot be traced.

Even though the major CF mutation has been characterised,⁷ linkage analysis is still necessary in the majority of CF families.⁸ ARMS analysis will be of value in genotyping of the J3.11 MspI dimorphism until all the CF mutations are characterised.

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The ARMS method is the subject of European patent

application 0 332 435 and corresponding world wide patents on behalf of ICI plc.

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