Br Heart J 1995;73:368–371

TECHNIQUE

Rapid methods for population-scale analysis for gene polymorphisms: the ACE gene as an example

S D O'Dell, S E Humphries, I N M Day

Abstract

***Objective*—To obtain rapid, high throughput genotyping of the angiotensin converting enzyme (ACE) gene intron 16 insertion/deletion polymorphism.**

***Methods*—DNA was obtained from whole blood samples by a simplé liquid phase methanol extraction procedure. The ACE gene was amplified by the polymerase chain reaction (PCR) using two oligonucleotide primers (ACE1 and ACE3) outside the insertion sequence and one primer (ACE2) inside the sequence. Microtitre array diagonal gel electrophoresis (MADGE) was used to determine genotypes.**

***Results*—84 and 65 bp PCR products indicating the presence of deletion (D) and insertion (I) alleles, respectively, were clearly resolved after electrophoresis on a 7·5% polyacrylamide gel. Up to 480 DNA samples on 5 gels could be genotyped in a single electrophoresis run, or up to 1000 samples in a working day.**

***Conclusions*—A simplified DNA extraction protocol coupled to the high throughput capability of the MADGE electrophoretic system for genotyping enables analysis of large populations for association studies of ACE genotype with cardiac disease events.**

(*Br Heart J* 1995;73:368–371)

Keywords: ACE gene polymorphisms; population analysis; rapid genotyping.

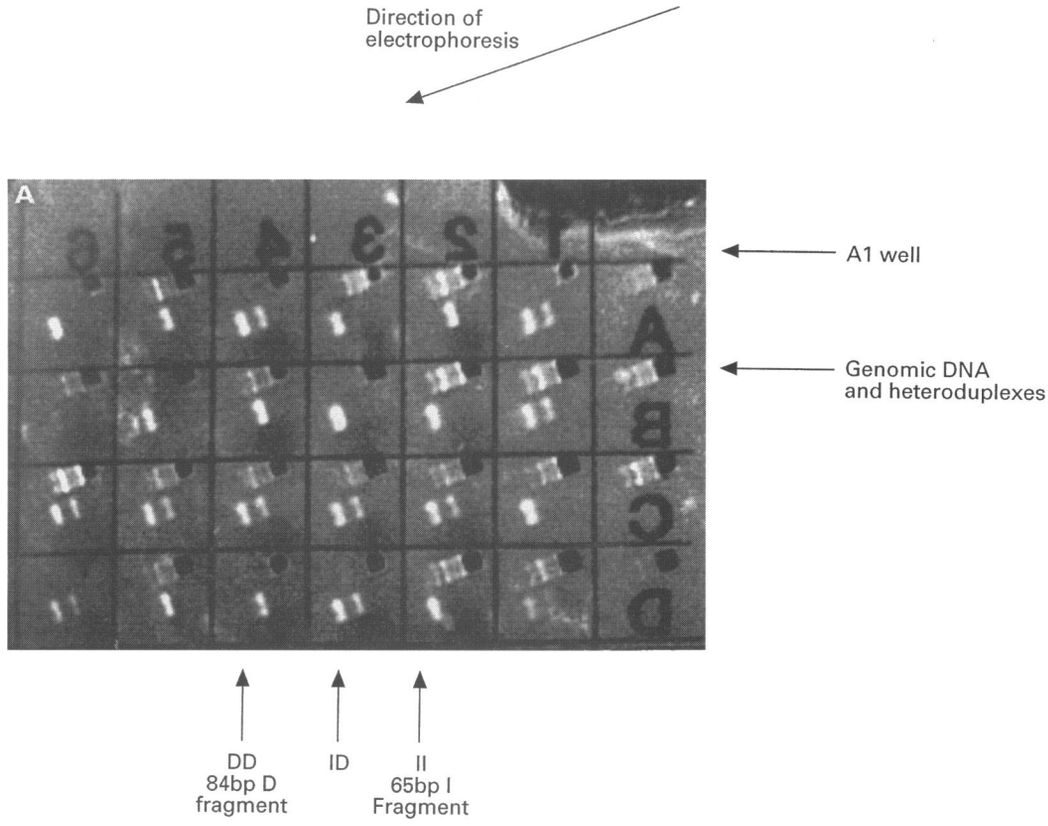
Division of Cardiovascular Genetics, Department of Medicine, University College London Medical School, The Rayne Institute, London
S D O'Dell
S E Humphries
I N M Day

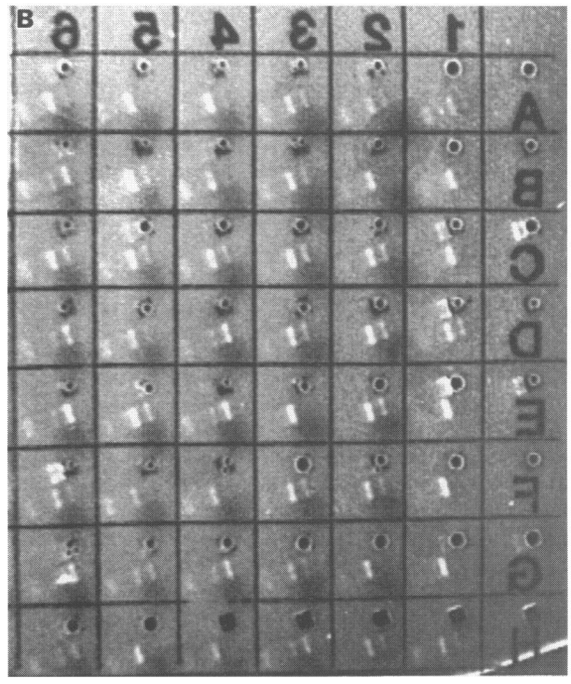
Correspondence to:
Dr S D O'Dell, Division of Cardiovascular Genetics, Department of Medicine, University College London Medical School, The Rayne Institute, 5 University Street, London WC1E 6JJ.

Accepted for publication 19 December 1994

Angiotensin converting enzyme (ACE) functions to convert angiotensin I to vasoactive angiotensin II and to inactivate the vasodilator bradykinin. The enzyme is predominantly located on capillary endothelial cells, but ACE activity is also detectable in serum. The biological role for ACE suggests that the ACE gene could be a candidate in predisposition to cardiovascular disease because the renin-angiotensin system is involved in the systemic control of fluid volume and blood pressure, induction of smooth muscle cell proliferation, and stimulation of myocardial cell hypertrophy. Plasma ACE concentrations are very stable within the individual, but there are large inter-individual differences, and studies have shown that approximately 50% of the variability of plasma ACE between individuals is the result of an insertion/deletion polymorphism in intron 16 of the ACE gene[1 2]; the insertion (I) and deletion (D) alleles being the result of the presence or absence respectively of a 287 bp *Alu* sequence.[3] Possession of the ACE gene polymorphic marker D has been shown to be a risk factor for coronary artery disease. Individuals homozygous for the deletion allele (about 35% of the population) have plasma ACE concentrations about 60% higher than individuals homozygous for the insertion allele. In a recent large myocardial infarction (MI) case control study from Europe the frequency of the deletion allele was found to be significantly higher in patients with MI than in controls (0·58 *v* 0·54).[4] When the MI cases were further divided into those with or without conventional cardiovascular risk factors (such as raised apoB or obesity), the frequency of the deletion allele was significantly higher in those without conventional risk factors than in the others (odds ratio of 3·2 *v* 1·1). Recently a synergistic effect between an angiotensin II type 1 receptor gene (AGT_1R) polymorphism and the ACE genotype has been reported.[5] Individuals homozygous for the rare AGT_1R allele (9% of the population) who are also ACE DD have about a 4-fold higher risk of MI, and the effect is greatest in those with low body mass index and apoB (relative risk 13·3). Thus the ACE and AGT_1R gene polymorphisms in combination seem to be a novel and powerful genetic risk factor for coronary artery disease (CAD) and may be having a large impact in patients considered to be at low risk according to conventional criteria.

Rigat *et al* developed a rapid assay based on the polymerase chain reaction (PCR) for identifying ACE genotypes, involving amplification of genomic DNA using paired primers flanking the insertion sequence, which allows discrimination of the three ACE genotypes—II, ID, and DD.[3] We have used the method described by Evans *et al*, which yields much shorter fragments corresponding to the I and D alleles and we have resolved these on a 7·5% polyacrylamide gel.[6] We describe a rapid approach to genotyping the ID polymorphism using a protocol in which DNA is amplified after simple methanol extraction from whole blood (O'Dell *et al*, unpublished).





PCR amplified fragments electrophoresed on a 7·5% polyacrylamide MADGE gel and stained with ethidium bromide. Each track contains 3·57 μl of the product of a 20 μl PCR reaction. The three ACE genotypes are shown. (A) Enlarged section of gel overlaid with grid. Coordinates shown in mirror image because of face-down orientation on platen of transilluminator. The longer fragment (84 bp) corresponds to the deletion allele (D), and the short fragment (65 bp) to the insertion allele (I), annotated in row D. (B) Half of a MADGE gel (48 samples). The faint faster migrating band represents unincorporated oligonucleotide.

After PCR, products are analysed by the microtitre array diagonal gel electrophoresis (MADGE) system which we have recently described[7 8] and which enables convenient genotyping by electrophoresis of thousands of products.

Methods

In our DNA extraction method, blood anticoagulated with 5 mM potassium EDTA and stored in 500 μl aliquots frozen at −20°C was thawed and 50 μl added to 50 μl methanol in the deep wells of a Beckman titre plate (No. 267001, Beckman, High Wycombe), and evaporated to dryness using a hot air dryer (Elton Sales, Hemel Hempstead) at a temperature not exceeding 55°C. 100 μl sterile distilled water was then added. The plate was then covered with its flexible plastic lid and the samples heated in a 100°C water bath for 25 minutes. The aqueous DNA

extract was withdrawn with a multichannel pipette and 4 μl aliquots transferred to a 96 well Omniplate (Hybaid, Teddington). The extracts were then dried at room temperature before use in the PCR reaction.

The sequences of the PCR primers used were as described by Evans *et al*[6] and the primer ratios correspond to the 50 pmol ACE1 and 3 and 15 pmol ACE2 used by them in a 50 μl reaction, giving amplification products of 84 bp for allele ACE D and 65 bp for allele ACE I. Our amplification conditions were as follows: 1 cycle 95°C 5 min.; 40 cycles 95°C 1 min, 50°C 1 min, 72°C 5 min. 20 μl PCR reactions contained 50 mM KCl, 10 mM Tris HCl pH 8·3, 1·5 mM $MgCl_2$, 0·01 mg/ml gelatin, 200 μM each dNTP, 0·2 units Taq polymerase (Gibco BRL, Paisley), and 8 pmol of primers ACE1 and ACE3, outside the insertion (*Alu*) sequence, and 2·4 pmol of primer ACE2, inside the insertion sequence. Reactions were overlaid with 20 μl mineral oil. All 96 wells were always filled with reagents (mix or dummy reagents) to ensure a constant thermal mass on the block.

Shanmugam *et al*[9] reported some mistyping of ACE genotypes when they used the method of Rigat *et al*[2] and several DD genotypes among offspring of an II parent subsequently amplified as ID. On other occasions ID genotypes amplified as DD. They concluded that amplification of the *larger I* allele is sometimes suppressed in an ID heterozygote so it can be mistyped as DD. In our use of conditions described by Evans *et al*,[6] we were concerned that the *larger* PCR product from the *D* allele was suppressed in the presence of the I allele in our samples, so that the ID genotype appeared as II. This possibility was suggested by the fainter D band compared with the I band that appeared in ID genotypes. To confirm the accuracy of our genotyping, replica microtitre plates were set up using the primer pair ACE1 and ACE3, both at 8 pmol per 20 μl PCR reaction, in order to confirm the presence of the D allele.

Amplification products were visualised using 7·5% polyacrylamide microtitre array diagonal gel electrophoresis (MADGE) gels,[7 8] (figure). Briefly, these are open faced horizontal polyacrylamide gels attached to a glass support (genetiX, Wimborne). The wells preserve the exact configuration and dimensions of 96 well microtitre plates but are arranged diagonally relative to the electrodes so that 26·5 mm gel tracks are available, each track representing the main diagonal across a 2 × 4 rectangle of wells. To load, each gel was immersed horizontally in the electrophoresis tank. Using a multichannel pipette, 5 μl PCR product was mixed with 2 μl xylene cyanol/bromophenol blue loading buffer,[10] and 5 μl of this volume (containing 3·57 μl PCR product) was loaded into the MADGE gel wells. Electrophoresis was at 150 V (10 V/cm) for 45 minutes, which allowed the 84 and 65 bp bands to be resolved clearly. The unincorporated oligonucleotide smear migrated well ahead of the products. A set of MADGE gels were stacked directly on top of each other in one tank, running in the samples on one gel for a few minutes before sliding in the next gel horizontally. Up to 480 samples on 5 gels were electrophoresed in a typical small electrophoresis tank in this way, and once established, it was possible for one worker conveniently to undertake analysis from arrays representing nearly 1000 samples in one day.

Results

DNA was detected by placing the gel gel-side down on the transilluminator, because little ultraviolet light would pass through the glass to which the gel was attached. The fluorescent bands were viewed through the glass. The most cathodal well corresponds to well A1 of a microtitre plate. For photography, a transparency of a microtitre grid was overlaid on the glass plate, with letters and numbers orientated to identify the bands, and with the lines of the grid arranged to assist identification of the patterns. The figure shows that the 84 bp D allele amplification product and the 65 bp I allele product were clearly resolved. The check for D allele presence using paired primers ACE1 and ACE3 showed that approximately 1 to 3 samples on a plate of 96 had been mistyped using the 3 oligonucleotide PCR[6] as implemented in this laboratory.

Discussion

The PCR is widely used in diagnostic research and whole blood is one of the most readily available samples in clinical investigation. Commonly the DNA in PCR assays is extracted and purified according to one of several complex multistep processes[11 12] which are not compatible with high throughput diagnostic or epidemiological research programmes. We used an efficient method to amplify DNA after simple methanol extraction from whole blood. Methanol is routinely used in the extraction of DNA from dried Guthrie blood spots,[13] but our method uses liquid phase manipulation which is more convenient in high throughput diagnostic genotyping. Furthermore the extract can be dried in microtitre arrays and stored for several weeks before use in PCR assays.

Electrophoresis of DNA has usually been performed in either an agarose or acrylamide gel[10 14] with acrylamide offering the highest resolution for smaller fragments such as those from PCR (typical size range 50–500 bp). Acrylamide does not polymerise in the presence of air and hitherto the configurations for gel preparation have led to electrophoresis in the vertical dimension. The MADGE device allows preparation and manipulation of horizontal acrylamide gels, with the open-faced format allowing loading of arrays of wells which preserves the configuration and dimensions of the 96-well microtitre plates routinely used in PCR procedures. The MADGE system readily enables pattern recognition analysis of mobility differences of $\geqslant$10% for

fragments in the 50–500 bp range. As well as ACE gene polymorphism genotyping, the system could also be applied to analysis of other genetic factors involved in cardiovascular disease. MADGE methods are also applicable to determining AGT_1R genotype, for example by allele specific oligonucleotides or by allele-specific amplification techniques.

Conclusion

A rapid DNA extraction procedure together with the high throughput capability of the MADGE system for genotyping by electrophoresis has enabled the accurate genotyping by a single worker of 1000 DNA samples in a day. The speed with which these results can be routinely obtained makes it feasible to undertake large scale population studies to test for association between candidate genetic variation and cardiac disease.

This work is supported by UK Medical Research Council ROPA Award G9414230 to INMD. INMD is the recipient of a British Heart Foundation Intermediate Fellowship. SEH is supported by an Award and Program Grant RG16 from the British Heart Foundation. The MADGE method is subject to patent application.

1 Rigat B, Hubert C, Alhenc-Geles F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I converting enzyme accounting for half the variance of serum enzyme levels. J Clin Invest 1992; 86:1343–6.

2 Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien C, Soubrier F. Evidence from combined segregation and linkage analysis that a variant of the angiotensin I converting enzyme (ACE) controls plasma ACE. *Am J Hum Genet* 1991;**51**:197–205.

3 Rigat B, Hubert C, Corvol P, Soubrier F. PCR detection of the insertion/deletion polymorphism of the human angiotensin I converting enzyme gene (DCPI) (dipeptidyl carboxypeptidase 1). *Nucleic Acids Res* 1992;**20**: 1433.

4 Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D, *et al.* Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* 1992;**359**:641–4.

5 Tiret L, Bonnardeaux A, Poirier O, Rickard S, Marques-Vidal P, Evans A, *et al.* Synergistic effects of angiotensin-converting enzyme and angiotensin-II type 1 receptor gene polymorphisms on risk of myocardial infarction. *Lancet* 1994;**344**:910–3.

6 Evans AE, Poirier O, Kee F, Lecerf L, McCrum E, Falconer T, *et al.* Polymorphisms of the angiotensin-converting enzyme gene in subjects who die from coronary heart disease. *Quart J Med* 1994;**87**:211–4.

7 Day INM, Humphries SE. Electrophoresis for genotyping: microtitre array diagonal gel electrophoresis (MADGE) on horizontal polyacrylamide (H-PAGE) gels, Hydrolink or agarose. *Anal Biochem* 1994;**222**: 389–95.

8 Day INM, Humphries SE. Electrophoresis for genotyping: devices for high throughput using horizontal acrylamide gels (H-Page) and microtitre array diagonal gel electrophoresis (MADGE). *Nature (Product Review)* June 1994;36–7.

9 Shanmugam V, Sell KW, Saha BK. Mistyping ACE heterozygotes. *PCR Methods and Applications* 1993;**3**:120–1.

10 Maniatis T, Fritsch EF, Sambrook J. *Molecular cloning: a laboratory manual.* New York: Cold Spring Harbour, 1982;149–85.

11 Gross-Bellard M, Oudet P, Chambon P. Isolation of high molecular weight DNA from mammalian cells. *Eur J Biochem* 1973;**36**:32–8.

12 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;**16**:1215.

13 Nelson PV, Carey WF, Morris CP. Gene amplification directly from Guthrie blood spots. *Lancet* 1990;**336**: 1451–2.

14 Rickwood D, Hames BD, eds. *Gel electrophoresis of nucleic acids. A practical approach.* Oxford: IRL Press. 1982.