

A PCR based method to determine the Kalow allele of the cholinesterase gene: the E_1^k allele frequency and its significance in the normal population

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Abstract

The Kalow allele for cholinesterase is a quantitative variant of the usual gene and has been shown to result from a single base pair change in the DNA. A new method based on the polymerase chain reaction to distinguish Kalow alleles of the cholinesterase gene was developed. Using the amplification refractory mutagenesis system, two different reactions distinguished the presence of a guanine (normal E_1^u allele) from that of an adenine (Kalow E_1^k allele) at nucleotide 1615 within the coding sequences of the gene. The frequency of the Kalow allele in our sample of 51 persons was determined to be 20%. The mean total cholinesterase activity in heterozygotes was 90% of that in persons who typed as $E_1^uE_1^u$ homozygotes. Two $E_1^kE_1^k$ homozygotes were identified and their cholinesterase activities were the two lowest measured.

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The physiological function of serum cholinesterase (acyl choline acyl hydrolase, EC 3.1.1.8) is currently unknown. It has been shown, however, to be involved in the breakdown of the short acting muscle relaxant succinylcholine (suxamethonium, scoline). Failure to metabolise this drug can result in prolonged apnoea. Two loci, E_1 and E_2 , encode cholinesterase enzymes but only the E_1 locus is concerned with sensitivity to scoline. Multiple alleles at this E_1 locus alter the quality (E_1^s , E_1^l) or the quantity (E_1^s , E_1^k , E_1^j , E_1^h) of the enzyme produced.¹ Some of these cholinesterase variants can be distinguished by their activity in the presence of certain extraneous inhibitors, the most commonly used being dibucaine,² fluoride,² and Ro 02-0682 (dimethylcarbamate of (2-hydroxy-5 phenyl-benzyl) trimethylammonium bromide).⁴

The Kalow (E_1^k) allele for cholinesterase was first described⁵ as a quantitative variant of the usual gene producing a 33% reduction in the normal enzyme activity. Current inhibitor methods are unable to confirm the presence of the E_1^k allele unless the family is also segregating for E_1^s .⁶ Because of this difficulty in distinguishing the E_1^k allele, its frequency has been estimated by extrapolation and the effect of it on total cholinesterase activities has been deduced only from persons who could be genotyped because of their position in a family tree. Obligatory $E_1^uE_1^k$ persons have been reported⁷ to have 91% of the usual total en-

zyme activity. Evans and Wardell⁸ suggested that 1/76 persons could be homozygous for the E_1^k allele and this has been confirmed by Whittaker and Britten,⁷ who showed that slightly more than 1% of the British population could be homozygous for $E_1^kE_1^k$.

The gene for cholinesterase has been cloned and sequenced.^{9,10} The base change resulting in the E_1^k allele has been suggested¹¹ and confirmed.¹² The nucleotide at position 1615 in the coding sequences for the E_1^u allele is a guanine (G) and this codon translates to an alanine, giving the normal cholinesterase protein. An adenine (A) at this locus in the Kalow, E_1^k , allele changes the encoded amino acid to a threonine and results in the modified protein. We have developed a method based on the polymerase chain reaction (PCR) to detect the presence of the E_1^k allele and to determine its frequency in the general population. By choosing PCR primers differing by a single base, one compatible with the normal cholinesterase allele and another compatible with the Kalow allele, and adjusting the amplification temperature carefully, one allele only can be amplified in each reaction. The other allele is refractory to amplification, hence the description of the method as the amplification refractory mutagenesis system (PCR ARMS).¹³

A comparison of the genotypes determined by our new method and those assigned by the conventional inhibitor number method has been made.

Materials and methods

SUBJECTS

Healthy controls were drawn from laboratory staff; the total number was 51 of whom 36 were female and 15 male. The age range was from 20 to 58 years, mean 36.3 years, SD 10.3 years. From each volunteer a blood sample in EDTA was collected for DNA preparation and a serum sample for cholinesterase activity measurement and phenotyping. A drug history was also taken and pregnancy excluded.

DNA PREPARATION

DNA was prepared¹⁴ with increased resuspension times compared with the method described. The DNA was stored at -20°C until used.

PCR ARMS AND DNA SEQUENCING

Three 30 base oligonucleotides were purchased (BioMac, University of Glasgow) for the ARMS PCR: a common 3' primer se-

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quence 5'-TATTAATTAGAGACCCACA-CAACTTCTTT-3' which corresponds to the coding sequence for the last seven amino acids of the protein, the stop signal, and six downstream (3') nucleotides. The E_1^u and E_1^k allele specific primers terminate at the G (for E_1^u allele) or A (for E_1^k allele) and the second last base of the primer sequence is also destabilised in that it should be an A but a cytosine (C) was synthesised instead. This second destabilised base is to help to avoid false positives.¹⁵ The primer for E_1^u therefore was 5'-TTCCATATTTTACAG-GAAATATTGATGACG-3' and that for E_1^k was the same except the last base was an A. Presence of the specific ARMS product was indicated by a band of 146 base pairs. In each tube a positive PCR control was incorporated. A region in the gene for apolipoprotein B was targeted because all subjects have this gene. The primers were 5'-GGAGCAGTTGACCACA-AGCTTAGCTTGGAA-3' which started at nucleotide number 10551,¹⁶ and 5'-CAGGGT-GGCTTGCTTGATGTTCTCCGTT - 3' which ended at nucleotide number 10895. This control resulted in a band of 345 bp to indicate that the polymerase chain reaction had worked in each tube. The thermal cycling buffer was as recommended by the manufacturers of the polymerase, Promega, Southampton. The conditions were: 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0 at 25°C), 1% Triton X-100, 3.75 mmol/l MgCl₂, 0.2 mmol/l each dNTP. PCR ARMS primers were used at 1 µmol/l each and control primers at 0.25 µmol/l each in a final volume of 30 µl with 1.5 units of *Taq* polymerase enzyme per tube. Thermal cycling was carried out on a water cooled Techne PHC-2 Intelligent Heating Block (Scotlab, Glasgow).

Conditions to distinguish E_1^u and E_1^k alleles were as follows: 36 cycles of 95°C for one minute followed by 65°C for 45 seconds, and 72°C for one minute 30 seconds. For the first cycle, 95°C was maintained for five minutes rather than one minute in the subsequent cycles. Finally, 72°C was maintained for 10 minutes to complete any partially copied templates. Ramp rate 4 (the fastest) was used throughout except for the step from 65°C to 72°C where ramp rate 2 was used. PCR was carried out in two tubes, both with the common primer and the control primers and each with one or other of the E_1^k or E_1^u specific primer. The products were analysed by separation on an 8% polyacrylamide gel in Tris borate buffer.¹⁷

To sequence across the region a 28mer primer from exon 3 was synthesised, 5'-GGCTTTTGTATTCGAAATTATTTT-CAG-3', and used in a PCR reaction with the common 3' primer above, using the same PCR conditions except 55°C was used as the annealing temperature. The resulting PCR products were cut out of an agarose gel and sequenced from the 28mer primer using standard dideoxy chain termination methods.

CHOLINESTERASE METHOD

Total cholinesterase was measured at 25°C using benzoyl choline as substrate.¹⁸ Inhibition of

cholinesterase activity by the addition of dibucaine,² fluoride,³ or Ro 02-0682⁴ to the assay was calculated. The inhibitor number is the percentage of inhibition of cholinesterase activity in the presence of the respective inhibitor. Using reference data these inhibitor numbers can be used to assign phenotype to each sample. All measurements were made on a Phillips PU 8730 uv/vis scanning spectrophotometer.

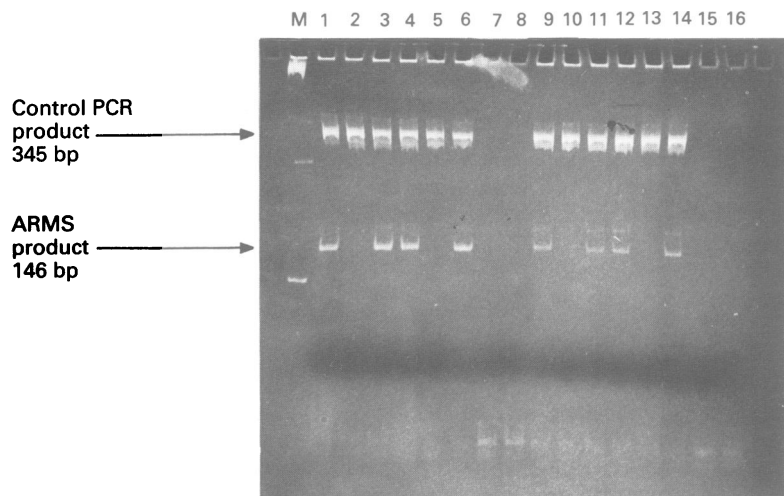
Results

To ensure a reproducible band of 146 base pairs after PCR, a great variety of temperature conditions had to be tried. Both the annealing and extension temperatures were varied. Temperatures which were too low tended to give false positives (all subjects typing as $E_1^uE_1^k$) while temperatures which were too high gave no bands at all. The magnesium concentration of the PCR mixture affected the results at any given set of temperatures. Negative controls (no DNA) were carried out simultaneously to check every batch of PCR done. The results in tracks 1 to 8 of the figure are for the conditions finally chosen and described in Materials and methods. The G-A Kalow change was verified by direct sequencing of the PCR product generated using the flanking primers from a person of each genotype (gel not shown). The total allele frequency of E_1^k was 19.6% (table). Using the Hardy-Weinberg equilibrium formula, we calculated the expected number of the three genotypes from the total allele frequency and they were 33, 16, and 2, for genotypes $E_1^uE_1^u$, $E_1^uE_1^k$, and $E_1^kE_1^k$ respectively, which were exactly the numbers obtained for each genotype (table).

The three inhibitor numbers determined for each of the 51 subjects were compatible with the $E_1^uE_1^u$ genotype. Persons were grouped according to genotype using the PCR ARMS method, and the means of their cholinesterase activities determined (table). This was carried out including all subjects and also separately, excluding those on oral contraceptives (n=8), or hormone replacement therapy (n=1), or tamoxifen (n=1), drugs known to affect cholinesterase activity. Persons with one E_1^k allele had enzyme activities on average 90% of that of the $E_1^uE_1^u$ homozygotes, but using a two sample *t* test these differences were not significant. The two subjects who were $E_1^kE_1^k$ had much reduced activities.

Discussion

For the PCR ARMS method, the conditions used had to be very precise especially to avoid false positives. However, the final conditions we determined were robust and completely reproducible. For convenience, we chose conditions so that E_1^k alleles and E_1^u alleles could be distinguished using the same thermal cycling conditions. Results for annealing temperatures of both 65°C and 66°C can be seen in the figure, but owing to the stronger bands, we considered the former temperature more suitable for routine analysis. The method as described here used DNA prepared by a quick



Ethidium stained polyacrylamide gel of PCR products. Annealing temperature 65°C for tracks 1–8, 66°C for tracks 9–16. Odd numbered tracks result from PCRs with the U primer, even numbers had the K primer. Tracks 1, 2, 9, and 10 are from the same $E_1^u E_1^u$ subject. Tracks 3, 4, 11, and 12 are from the same $E_1^k E_1^k$ subject. Tracks 5, 6, 13, and 14 are from the same $E_1^k E_1^k$ subject. Tracks 7, 8, 15, and 16 are the corresponding negative controls (no DNA) for the reactions. The marker track (M) contains 1 μ g 123 bp ladder (Life Technologies, Paisley).

Total cholinesterase activity (U/l) related to genotype

| Genotype | All subjects | | | Excluding drugs | | |
|---------------|--------------|------|-----|-----------------|------|-----|
| | No | Mean | SD | No | Mean | SD |
| $E_1^u E_1^u$ | 33 | 1007 | 241 | 26 | 1030 | 235 |
| $E_1^u E_1^k$ | 16 | 905 | 222 | 13 | 935 | 233 |
| $E_1^k E_1^k$ | 2 | 548 | * | 2 | 548 | |

* Values 536, 561.

The results are presented for all subjects in the left section of the table. On the right, the 10 subjects taking drugs known to affect cholinesterase activity measurements have been omitted. The inhibitor numbers for all these subjects excluded E_1^u or E_1^k alleles.

method, but we also had DNA prepared by the Triton Lysis Method¹⁹ available for five of the same subjects. Identical genotypes by the PCR ARMS method were determined no matter what the method of preparation of the DNA (data not shown).

The frequency of the E_1^k allele in our normal population was 19.6%. Calculating the expected frequency of $E_1^k E_1^k$ homozygotes from the observed frequency of E_1^k alleles, 3.8% of our random population was expected to be $E_1^k E_1^k$. This agrees with our finding of two $E_1^k E_1^k$ homozygotes (4%) in our population and is higher than the predicted 1.3%.⁸ The highest published estimate of $E_1^k E_1^k$ frequency was by Bartels *et al.*,¹¹ who looked at 40 unrelated persons and predicted 1.5% homozygote frequency. It is likely that the lower earlier estimates were based on indirect methods of assessing E_1^k allele frequency. The two $E_1^k E_1^k$ homozygotes that we detected using the PCR ARMS method would have escaped identification by any other published screening technique.

In our population $E_1^u E_1^k$ subjects have, on average, 90% of the total cholinesterase activity of $E_1^u E_1^u$ subjects although the difference is not statistically significant. This compares well with the measured activities of confirmed $E_1^u E_1^k$ heterozygotes (80% for six obligatory $E_1^u E_1^k$ subjects⁸ and 91% for 38 obligatory $E_1^u E_1^k$ subjects⁷). The two $E_1^k E_1^k$ homozygotes had very low total activities, outside the 5%

confidence limit compared with the means for the $E_1^u E_1^u$ homozygotes. Neither of the $E_1^k E_1^k$ persons was currently taking any medication.

This ARMS method looks at only one sequence change within the gene. There may be other sequence changes of functional significance on the same allele which will not be detected by this method; for example, all the relatively rare alleles, E_1^s , E_1^j , E_1^h or even E_1^a or E_1^f will type as E_1^u because of being identical to the normal allele at this sequence position. However, we can be certain that there were no examples of the E_1^a or E_1^f alleles in this sample because they would have been detected by a distinctive pattern of inhibitor numbers. Some of our normal subjects (without an E_1^k allele) had low total enzyme activity which may have been because of the presence of an undetectable rare allele leading to low total activity.

This method to identify the presence of E_1^k alleles is the only method in current use (other than direct sequencing) which will find $E_1^k E_1^k$ homozygotes.

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