Apolipoprotein B(Arg^{3500}→Gln) allele specific polymerase chain reaction: large-scale screening of pooled blood samples

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Abstract A two-step polymerase chain reaction (PCR) method for the rapid detection of the apolipoprotein B(Arg^{3500}→Gln) mutation in a mixture of pooled blood samples is described. In the first step PCR, a short gene fragment surrounding codon 3500 is amplified. Subsequently the reaction product is subjected to a second amplification in which a mutation-specific primer is used. A PCR product is generated only if the mutant sequence is present in the DNA pool. Individuals carrying the mutation can then be identified by PCR with mutagenic primers and *Msp*I restriction typing, essentially as described by Hansen et al. (*J. Lipid Res.* 1991. **32:** 1229-1233).—**Ruzicka, V., W. März, A. Russ, and W. Gross.** Apolipoprotein B(Arg^{3500}→Gln) allele specific polymerase chain reaction: large-scale screening of pooled blood samples. *J. Lipid Res.* 1992. **33:** 1563-1567.

Supplementary key words familial defective apolipoprotein B-100 • hypercholesterolemia • allele specific polymerase chain reaction • amplification refractory mutation system

Apolipoprotein (apo) B-100 is a major constituent of VLDL, IDL, and LDL. The interaction of apoB-100 with LDL receptors is responsible for the transfer of LDL-cholesterol from blood into the liver and most other cells in the body (1).

A mutation at codon 3500 of the apoB gene substituting glutamine for arginine has been shown to be associated with diminished LDL receptor binding. This disorder, which is also referred to as familial defective apoB-100 (FDB), is characterized by high plasma cholesterol and LDL levels (2, 3). It has been estimated to occur in 1-3% of hypercholesterolemic humans (4), thus being at least as frequent as classical familial hypercholesterolemia. Its impact on atherogenesis has not yet been fully elucidated. The main reason might be that it is difficult to recruit the number of affected individuals required for statistically meaningful studies.

Apart from LDL binding assays (5), genetic analysis methods have been described to detect FDB: hybridization with labeled allele-specific oligonucleotides (3), and gene amplification with mutagenic primers to generate artificial restriction enzyme recognition sites (6, 7). The former approach is labor intensive and not suitable for routine use. The latter method is rapid and reproducible, but it is limited to single DNA specimens, thus becoming costly, time-consuming, and laborious in large-scale screening endeavors. We have therefore developed a simple amplification refractory mutation system (ARMS) that is suitable for screening a blood pool consisting of up to 50 individuals.

MATERIALS AND METHODS

Preparation of pooled genomic DNA

Up to 50 blood samples (1 ml) containing EDTA were pooled. There was no correction for the leukocyte count of individual samples. DNA was isolated with a commercially available "blood DNA-kit" (Diagen, Düsseldorf, Germany) according to the manufacturer's instructions. Briefly, 0.5 ml of the pooled blood was loaded onto a Diagen spin column, spun, and washed four times with PBS containing 120 mM NaCl, 2.7 mM KCl, and 10 mM KH_2PO_4/K_2HPO_4, pH 7.4. Then, blood cells attached to the column matrix were lysed with 10% (v/v) Tween 20 for 30 min and spin-washed with 1 M KCl, 5 mM MOPS, pH 7.0, four times. DNA was eluted with 1.2 M KCl, 50 mM MOPS, pH 8.3. Finally, DNA was precipitated

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; apo, apolipoprotein; ARMS, amplification refractory mutation system; bp, base pair; DNA, deoxyribonucleic acid; FDB, familial defective apoB-100; MOPS, morpholinopropane sulfonic acid; PCR, polymerase chain reaction; TBE (1x), 89 mM tris(hydroxymethyl)aminomethane, 89 mM boric acid, 2 mM ethylenediamine-tetraacetate, pH 8.0; VLDL, LDL, and IDL, very low, low, and intermediate density lipoproteins, respectively.

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TABLE 1. Sequences of oligonucleotide primers

Primer	Sequence	Position[a]
BCF[b]	5'-CCAACACTTACTTGAATTCCAAGAGCACAC-3'	10550–10579
BCR	5'-GAATATATGCGTTGGAGTGTGGCTTCTCC-3'	10654–10682
BMSF	5'-TTCCAAGAGCACAaa-3'	10566–10580
BRSF	5'-CCAACACTTACTTGAATTCCAAGAGCACcC-3'	10550–10579

[a]The positions are numbered according to Law et al. (11) (cf. EMBL nucleotide sequence database, accession number M14162).

[b]The abbreviations BCF, BCR, BMSF, BRSF denote for apo*B* *c*ommon *f*orward, apo*B* *c*ommon *r*everse, apo*B* *m*utation *s*pecific *f*orward, and apo*B* *r*estriction *s*ite creating *f*orward primer, respectively. Nucleotides not matching the wild type sequence are lower case. The A in primer BMSF matching the G to A mutation responsible for apoB (Arg^{3500}→Gln) is underlined.

with 0.8 volumes of 2-propanol at room temperature, washed once with 70% (v/v) ethanol, dried under vacuum, and dissolved in water. Two hundred fifty ng DNA was used for a 50-μl PCR.

Polymerase chain reaction

All three PCR mixtures (50 μl) contained 12.5 mM Tris-HCl, pH 8.3, 62.5 mM KCl, 2.25 mM $MgCl_2$, 10% (v/v) dimethyl sulfoxide, 0.01% (w/v) gelatine, 0.11% (v/v) Nonidet P-40, 0.11% (v/v) Tween-20, 100 ng of each primer, and 1.5 units *Taq* polymerase (Perkin Elmer Cetus, Emeryville, CA). The primer sequences are compiled in **Table 1.** The protocol for detecting mutant DNA consists of two amplification reactions (**Fig. 1**). In the first step PCR both wild type and mutant templates are amplified nonselectively. The second step PCR is allele-specific. It provides a product only if mutant template is present in the starting DNA pool.

First step PCR. Template DNA (250 ng) in 2.5 μl H_2O was added to the above reaction mixture containing the primer pair BCF-BCR and processed as follows: pretreatment at 97°C for 1 min, denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Thirty cycles were performed. Subsequently, 5-μl aliquots were checked for successful amplification by electrophoresis in 3% (w/v) agarose gels containing 1 mg/l ethidium bromide. The gel was run in 1 × TBE at 100 V for 30 min.

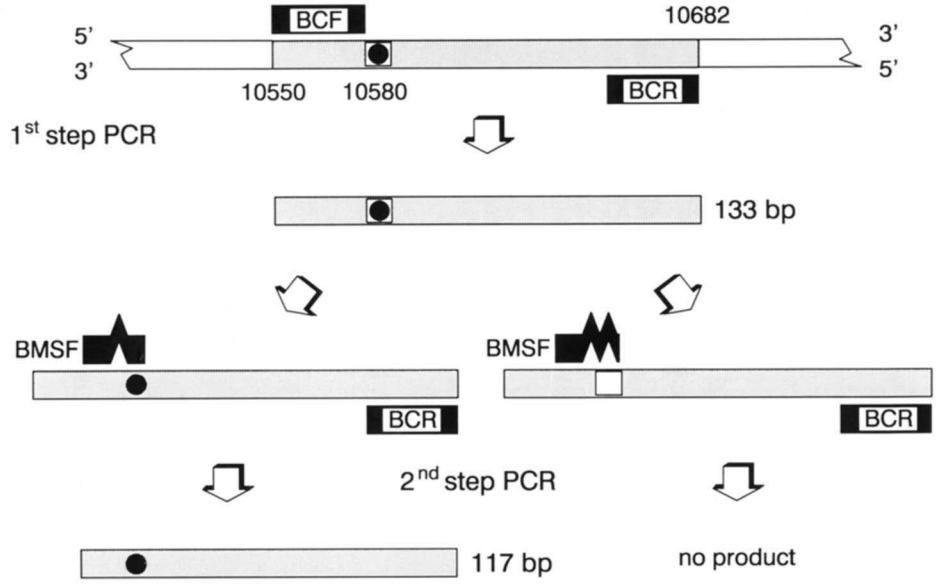

Fig. 1. Principle of the amplification refractory mutation system (ARMS) for the detection of the apoB (Arg^{3500}→Gln) mutation. The symbols denote: □, G in position 10580 of the apoB mRNA (wild type); ●, A in position 10580 of the apoB mRNA (FDB mutation); (⊙), mixture of templates either containing G or A in position 10580 of the apoB mRNA. The first step PCR is nonselective and generates a 133 bp product. It provides the template for the second step PCR. The second step PCR is allele specific. A 117 bp product is generated only if the 3'-terminal nucleotide of the mutation specific primer (BMSF) matches the template. Note that primer BMSF contains an obligatory mismatch penultimate to its 3' end.

Second step PCR. Five μl of the first step PCR product was diluted 1:100 with distilled water and 2.5 μl thereof was added to 50 μl of PCR reaction mixture, containing the oligonucleotide primers BMSF and BCR. Conditions were as follows: pretreatment at 97°C for 1 min, denaturation at 95°C for 30 sec, annealing at 40°C for 30 sec, extension at 72°C for 30 sec. Thirty cycles were performed. PCR products were analyzed as described above.

Identification of individual mutation carriers. DNA was prepared form the specimen that contributed to those pools displaying a mutation specific band in the second step PCR. Subsequently, these DNA samples were analyzed using a modification of the procedure described by Hansen et al. (6): 250 ng of genomic DNA in 2.5 μl H_2O was added to 50 μl PCR mixture containing primers BRSF and BCR, i.e., the *Msp*I cleavage site creating forward primer and the common reverse primer. Cycling conditions were those described for the first step PCR (see above).

Five μl of the PCR product was added to 5 μl "One phor all" restriction endonuclease buffer containing 10 U *Msp*I restriction endonuclease (Pharmacia, Uppsala, Sweden) and incubated at 37°C for 3 h. *Msp*I-treated PCR products were analyzed in 3% (v/v) agarose gels as described above and photographed with a Polaroid camera.

RESULTS

First step PCR

Fig. 2 (left side) shows amplification products of the first step PCR. Template DNA was obtained from the blood of a single mutation carrier, from pooled normal blood samples (each of which was individually reassessed by *Msp*I restriction typing), and from mixtures of both. The lengths of the amplification products correspond to the predicted value of 133 bp. Electrophoretically, products generated from both mutant and wild type templates cannot be distinguished, and both templates are amplified with similar efficacy.

Second step PCR

Results of the second step PCR are also shown in Fig. 2 (right side). One affected individual can be detected in a pool containing blood from as many as 216 subjects. Thus, the ARMS assay is very sensitive. It is also highly specific: no product is obtained in a pool solely containing mutation negative donors.

Identification of mutation carriers

In order to identify single affected individuals, we used a slight modification of the *Msp*I restriction typing method described by Hansen et al. (6). An example is

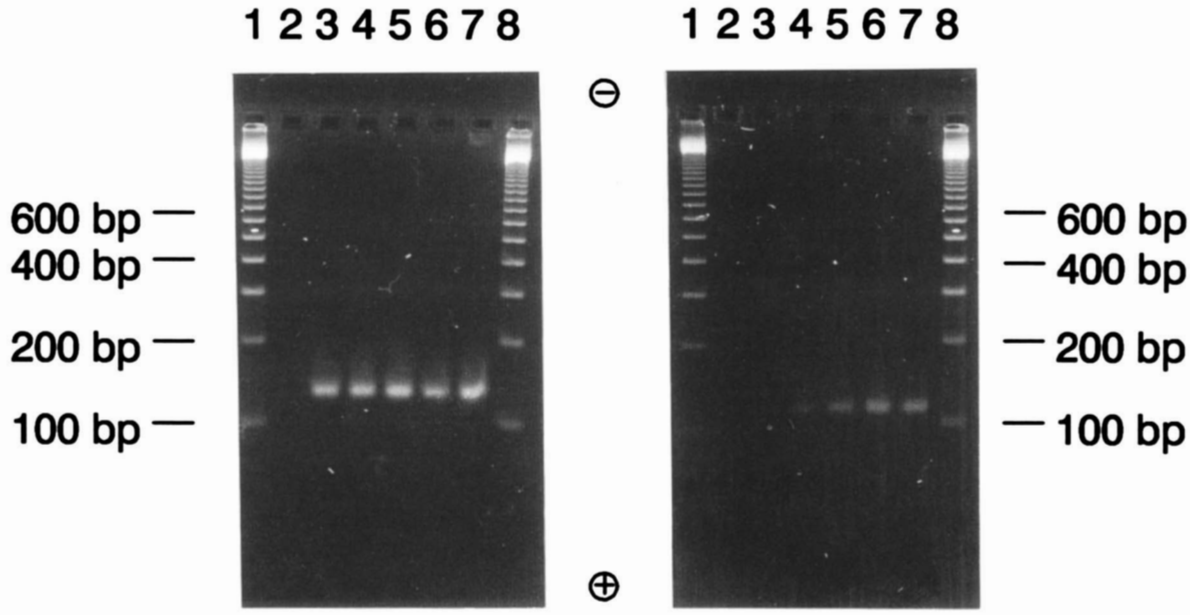

Fig. 2. Amplification refractory mutation system (ARMS) for the detection of the apoB (Arg^{3500}→Gln) mutation. Left panel: First step nonselective PCR. Lanes 1 and 8, 100 bp ladder; lane 2, no template added; lane 3, mutation negative blood pool from 50 donors, no mutation carrier added; lane 4, mixture of blood from a mutation carrier and a pool of 50 mutation negative donors, ratio 1:216 by volume; lane 5, ratio 1:36 by volume; lane 6, ratio 1:6 by volume; lane 7, mutation carrier only. Right panel: Second step mutation-specific PCR. The first step PCR products shown on the left panel were diluted 1:100, and 2.5 μl thereof was used as the template for the second step PCR. Lanes 1 and 8, 100 bp ladder; lanes 2 to 7 contain the second step PCR products (lane numbering corresponds to the left panel). The apoB (Arg^{3500}→Gln) mutation can be detected in a pool of as many as 216 blood samples (lane 4). Note that no PCR product can be seen in lane 3 (no mutation carrier present).

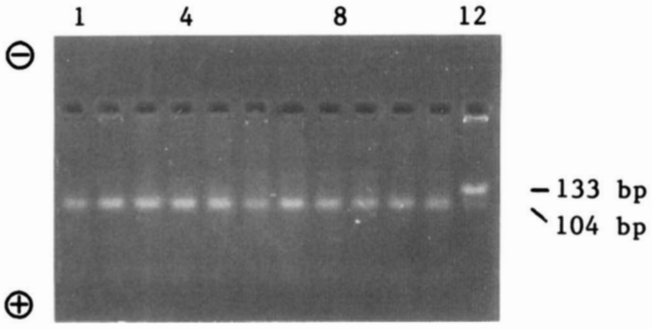

Fig. 3. Identification of a single carrier of the apoB (Arg^{3500}→Gln) mutation in a DNA pool. DNA was prepared from individual samples present in a pool that was positive in the ARMS assay, and then studied using a modification of the assay described by Hansen et al. (6). In this assay, a cleavage site for *Msp*I is introduced by PCR into the normal, but not into the mutant allele. Consequently, the normal allele gives a band of 104 bp, and the mutant one gives a band of 133 bp. A section of a gel containing a mutation carrier (lane 12) is shown. PCR products derived from mutation negative subjects are in lanes 1 to 11.

shown in **Fig. 3.** Genomic DNA was prepared from those individuals who constituted a pool of 50 donors positive for the apoB (Arg^{3500}→Gln) mutation in the ARMS assay. Using *Msp*I restriction typing, the mutation carrier responsive for the positive reaction could be identified unequivocally.

DISCUSSION

We here present an allele-specific PCR (8, 9) for the detection of the apoB (Arg^{3500}→Gln). Hansen and coworkers (6) have already attempted to establish an allele-specific PCR method for the mutation, but they failed to distinguish the wild type sequence from the mutant one.

Our procedure not only distinguishes between the wild type and the mutant apoB allele, but also opens up the opportunity to screen a large number of individuals. It is a two-step method. Its first step is nonselective. This ensures that the mutant DNA is sufficiently preamplified and thus avoids hazards of false negative results.

The second step is the mutation-selective PCR. In this step, a control template (for instance a plasmid with entirely unrelated sequences) can be included in order to prevent the possibility of false negative results due to a failure of the PCR for other reasons than the absence of mutant template.

The major problem of ARMS assays is their specificity. If the allele-specific primer (in this case the BMSF oligonucleotide) allowed any amplification of wild type alleles, falsely positive results would be generated. This has also been experienced during our initial attempts to set up the ARMS method. In those experiments, we used mutation-specific primers that were 31 bases in length, and even three 3' terminal mismatches did not prevent a successful amplification of the wild type sequence, although the reaction efficacy was low (data not shown). We suppose that this was caused by the high thermal stability of the complex formed between the 31-mer primer and the template DNA. Another explanation for the failure of this approach could be the presence of small amounts of 3' recessed primers lacking the discriminating 3' terminal nucleotides that could have primed the reaction unselectively.

Therefore, in the proposed method we used a short mutation specific primer (15-mer) containing only one mismatched base at position 2 from the 3' end and one 3' terminal A matching only the mutant sequence.

In line with considerations by Wu et al. (8) and Newton et al. (10), we believe that the thermodynamic properties of primers suitable for ARMS methods must meet the requirements for both high primer-template dissociation rates and sufficient DNA polymerase elongation rates. Too long primers have low template/primer dissociation rates that result in sufficient DNA polymerase elongation rates even for wild type mismatched sequences, and this ultimately leads to nonspecific amplification. In contrast, too short primers do not allow any amplification at all due to both high primer/template dissociation rates and low DNA polymerase elongation rates.

An important novel aspect of our present method is the possibility to pool blood samples from numerous donors. Consequently, we are now able to exclude the mutation simultaneously in as many as 50 subjects without performing individual testing. In routine work, however, we prefer to screen smaller pools, consisting of 6 to 12 samples, depending on whether hyper- or normocholesterolemic subjects are to be studied.

The method of DNA isolation we applied in this work is commercially available. It is rapid, convenient, and fortunately avoids the use of hazardous chemicals such as phenol and chloroform or labile proteolytic enzymes such as proteinase K.

Considering other possible ARMS designs, we rejected the use of primers that are specific either for the mutant or the wild type sequence and that differ from one another by their length. In such a procedure, the mutant sequence would give rise to a PCR product differing in length from the wild type sequence. This method may hardly be compatible with pooling of specimens because the underrepresented mutant template would result in a faint band adjacent to a much more distinct band representing the wild type template.

Another approach would have been to perform both the first step (nonselective) PCR and the second step mutation-specific PCR in a single tube using a limiting concentration of the wild type-specific primer. In this case, the concentration of the first step (nonspecific) product would be too low to allow for detection, whereas the concentration

of the mutation-specific primer would be sufficiently high to produce a distinct band. Such an assay would demand tedious titration and adjustment. Therefore, in our opinion, it would be error-prone and difficult to establish in a clinical setting.

In conclusion, we have presented a simple ARMS for the detection of the apoB (Arg^{3500}→Gln) mutation. In its current version the method should be insensitive to slight variations in experimental conditions, for instance brought about by the use of other thermocycler models. The technique permits screening of a large number of individuals in 1 or 2 days. It will enable studies on the prevalence and penetrance of the mutation in general populations, and possibly help to elucidate the significance of familial defective apoB-100 in premature atherosclerosis.

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