Optimization of Genotyping of the Common Apolipoprotein E Gene Alleles by Restriction Analysis

Optimierung der Apolipoprotein E Genotypisierung mit der allelspezifischen Restriktionsanalyse

S. Gehrisch[1,2], M. Tiebel[1], W. Jaross[1]

Summary: Restriction isotyping is a convenient method to perform Apo E genotyping. However, some experimental conditions can affect the restriction isotyping procedure and can lead to results prone to misinterpretation. It was evaluated whether the concentration of template DNA or different methods of DNA isolation have an influence on restriction isotyping. Reproducible results were achieved with 0.05 - 1 µg genomic DNA in a 100 µl PCR mixture. Using less DNA resulted in the appearance of non-specific bands. This could lead to a misinterpretation of the band pattern. An influence of different DNA isolation procedures on restriction isotyping could be excluded. The appearance of the band pattern is influenced by the intensity of the bands which depends on the size of the fragment and on the formation of heteroduplexes and homoduplexes in the case of a heterozygote apo E genotype. A detailed interpretation of the band patterns obtained by restriction isotyping of the apo E gene is given with regard to the intensity of the bands following ethidium bromide staining of the gel and UV photography.

Keywords: Apolipoproteins E/genetics; Alleles; Gene-amplification; DNA Restriction Enzymes.

Zusammenfassung: Die Bestimmung der sechs häufigsten Apolipoprotein E Genotypen mit Hilfe der allelspezifischen Restriktionsanalyse findet eine zunehmende Anwendung. In der routinemäßigen Durchführung dieser Methode werden jedoch Störungen beobachtet, die zu einer fehlerhaften Interpretation der Bandenmuster führen. In der vorliegenden Arbeit wurde überprüft, ob die DNS-Konzentrationen oder die Methoden der DNS-Gewinnung einen Einfluß auf die Genotypisierung haben. In einem Konzentrationsbereich der DNS von 0,05 bis 1,0 µg bezogen auf einen 100 ml PCR-Ansatz würden gut reproduzierbare Resultate erzielt. Bei Unterschreitung dieser DNS-Mengen wurden unspezifische Banden beobachtet, die zu einer falschen Interpretation der Bandenmuster und damit zu einer fehlerhaften Genotypisierung führten. Ein direkter Einfluß verschiedener Gewinnungsmethoden auf die Restriktionstypisierung konnte ausgeschlossen werden. Die Intensität der verschiedenen Banden bei der Apolipoprotein E Genotypsierung wird bedingt durch die Größe der DNS-Fragmente nach der Spaltung mit der Restriktase und dem Verhältnis der verschiedenen Homo- und Heteroduplexe, wie sie bei den verschiedenen Genotypen auftreten. Es wird eine genaue Interpretation der DNS-Bandenmuster nach der Ethidiumbromidfärbung der Polyacrylamidgele und der anschließenden Dokumentation mit der UV-Photographie unter Berücksichtigung dieser Einflußfaktoren gegeben.

Schlüsselwörter: Apolipoproteine E/Genetik; Allele; Genamplifikation; DNA Restriktionsenzyme.

The apolipoprotein E gene is located on chromosome 19 and comprises 4 exons encoding a 299-amino acid protein [1,2]. Apolipoprotein E is participating in the uptake of triglyceride rich and cholesterol rich particles such as chylomicrons, VLDL and the remnants formed during their catabolism.

Three common isoforms of the protein (E2, E3, E4) were found to arise from three different alleles [3]. The most common ε 3 allele is characterized by the amino acid cystein at residue 112 and arginine at residue 158. In case of an ε 2 allele arginine at residue 158 is replaced by cystein and in case of an ε 4 allele both positions are occupied by arginine [4]. The presence of the ε 4 allele is associated with increased LDL cholesterol in a variety of population samples [5, 6]. 90% of patients with a hyperlipoproteinemia type III are homozygote for the ε 2 allele [7]. The ε 4 allele was identified as a susceptibility gene for an early manifestation of late-onset Alzheimer's disease [8].

The differentiation of the isoforms of the protein is usually performed by isoelectric focusing (IEF). Reading genotypes as IEF phenotypes may result in misclassification [9,10]. It could be caused by post-translational glycosylation of the apo E peptide [9,10]. Some rare mutations such as Cys 136 -> Arg or Lys 146-> Gln could also be the reason for misclassification exhibiting similar electric charges as the common

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isoforms during IEF [9, 11]. Apo E genotyping based on restriction isotyping can deliver unquestionable results under optimized conditions. However, an evaluation of the impact of certain conditions on restriction isotyping procedure is necessary for optimizing this method.

Methods

Isolation of DNA
DNA was recovered from citrate anticoagulated human blood applying three different isolation procedures described previously: the Triton X -100 lysis method [12], the conventional phenol chloroform extraction of the DNA [13] and the salting out method [14]. In addition, DNA was recovered from cervical smear by a modified Triton X- 100 lysis method.

Oligonucleotides
Primers for polymerase chain reaction were synthezised by standard cyanoethylphosphoramidite chemistry using a Gene Assembler Plus (Pharmacia Biotech, Freiburg). Primers were purified by sephadex G-25 column.
Primer sets were as follows:
5' primer BE31, 5'- CGGGCACGGCTGTCCAAGGA - 3'
3' primer E23, 5'- CTCGCGGGCCCCGGCCTGGTA-3'
The 5' primer was biotinylated.
32 pg of each primer were used in a 100 µl PCR mixture and 8 pg in a 25 µl reaction mixture.

Amplification of genomic DNA
Genomic DNA was amplified in a 25 µl reaction mixture. Reaction conditions were as recommended by Perkin Elmer Cetus. The amplification was performed on a Perkin Elmer Cetus 9600 Thermal Cycler and entailed 5 min of denaturation at 94 °C followed by 10 three-step cycles of 30 sec at 94 °C, 30 sec at 62 °C touching down to 52 °C and 1 min at 72 °C and by 30 cycles of 30 sec at 94 °C, 30 sec at 52 °C and 1 min at 72 °C.

Restriction isotyping
15 µl of the PCR mixture were used subsequently for an overnight digestion of the amplificate with 10 units of the restriction enzyme CfoI (Boehringer Mannheim, Mannheim) and the recommended buffer SuRE/Cut L. Fragments were size separated on an 8% polyacrylamide gel for 1.5 hours using the TBE electrophoresis buffer (89 mmol/l Tris base, 89 mmol/l boric acid, 2 mmol/l EDTA) and visualized by ethidium bromide staining of the gel in a 0.01% solution and UV photography [1].

DNA sequencing
PCR products for sequencing were obtained by amplification of genomic DNA in a 100 µl reaction mixture. The sequence of the downstream primer was 5', - TAGCGGCTGGGCCGGCCAGGGA - 3' (corresponding to amino acid residues 203 to 197). Single-strand solid-phase sequencing was performed on a 373 A sequencer (ABI) according to the manufacturers protocol. The primer E23 served as sequencing primer.

Results

The influence of different concentrations of template DNA and methods of DNA isolation on the restriction isotyping procedure was investigated. Fig.1 shows the gel-separated products of the CfoI digested PCR amplificate of an apo E 2/4 and an apoE 3/4 genotype. In this cases the interpretation of band patterns is usually most difficult. Clear and reproducible results were achieved when 0.05-1 µg DNA were used in a 100 µl PCR mixture. The application of 0.001- 0.01 µg template DNA led to a shift in the intensity of the bands and to the appearance of non-specific bands making the genotyping impossible.

Different methods of DNA isolation such as the salting out procedure, the phenol-chloroform extraction and the Triton X - 100 lysis method did not affect the results obtained by restriction isotyping under standardized conditions (Fig. 2). The advantage of the salting out and the phenol - chloroform DNA extraction procedures is the isolation of clean and stabile DNA which can be kept for years even at 4 °C (results not shown).

A further critical step is the interpretation of the band pattern obtained after the Cfo I digestion of the 237 basepair fragment previously amplified by touch down PCR. Depending on the genotype there is the possibility of different combinations of nine fragments of the length of 91, 80, 72, 48, 32-Biotin, 32, 19, 18 and 16 basepairs. Fig. 3 shows the CfoI cleavage maps of the common alleles. A typical band pattern of the most common genotypes are demonstrated in Fig. 4. The intensity of the bands could be estimated following staining of the gel with ethidium bromide and UV-photography. In case of homozygosity (E2/2, E3/3, E4/4) the digestion of the PCR product will result in an equal amount of molecules of each generated fragment. However, shorter fragments will bind less ethidium bromide and will have a lower intensity. The 91 basepair band should have the highest intensity. The size of the fragment is one critical factor in the determination of the theoretical intensity of the obtained bands. Another factor is the generation of homoduplexes and heteroduplexes by PCR in case of a heterozygote genotype. Four different amplificates are generated by PCR. There will be formed homoduplexes and

Non-standard abbreviations: Apo E, Apolipoprotein E; ARMS, amplification refractory mutation system; ASO, allele-specific oligonucleotide; IEF, isoelectric focusing; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism, TBE; Tris borate EDTA.

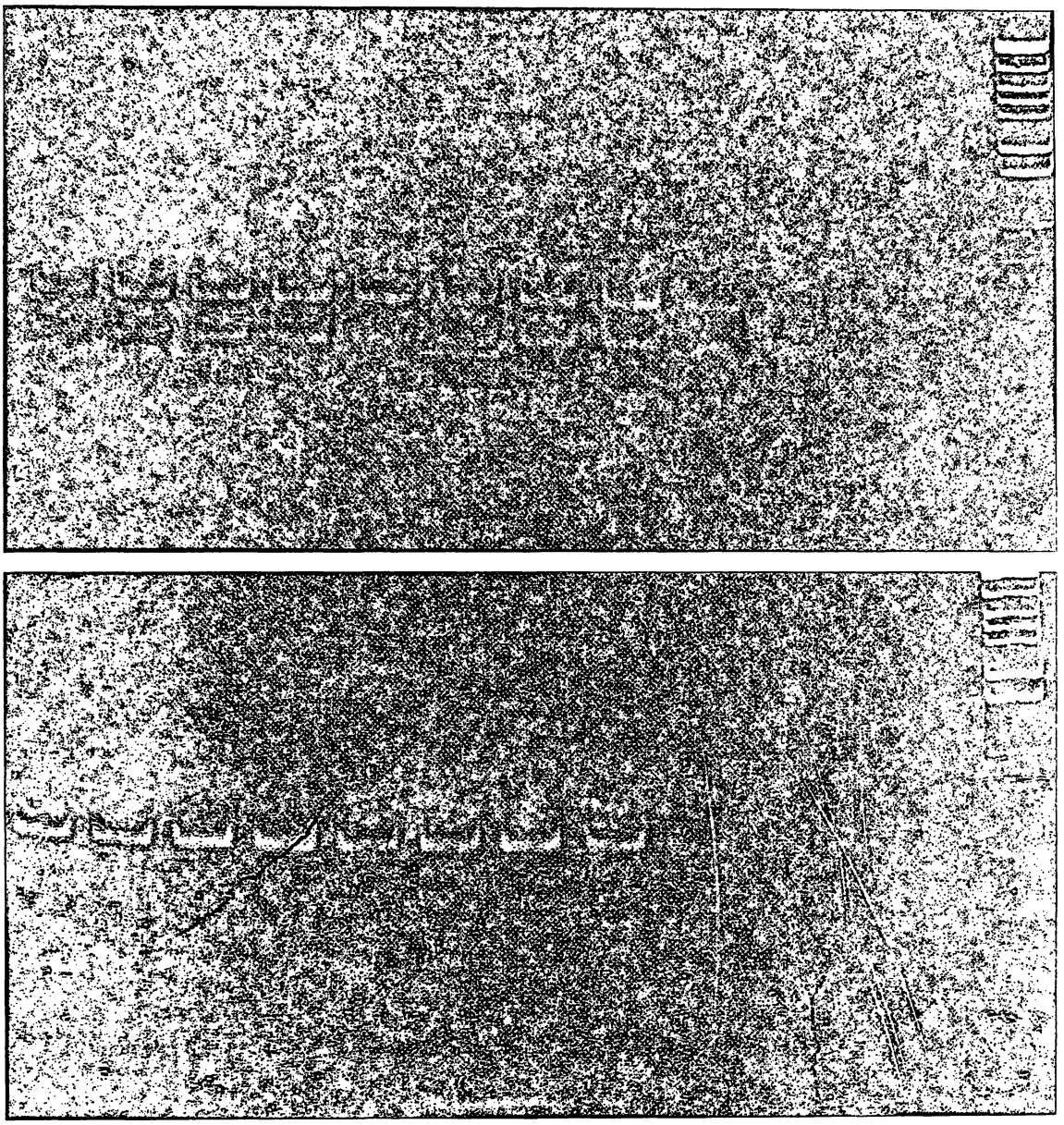

Figure 1 The influence of different concentrations of DNA used in the PCR on the results of restriction isotyping. Shown are [illegible] 0.10, 0.05, 0.01, 0.001 µg of DNA in a 100 microlitre PCR mixture were used (L- pBR322 Msp I ladder)

heteroduplexes from both alleles. Each of those will count for 25% of the total amplified PCR product. Only the homoduplex (25%) containing the appropriate restriction recognition site will be cut by CfoI at residue 112 and / or 158. The other 75% of the amplificate will be digested only at the four non-polymorphic restriction recognition sites of the exon 4 of the apo E gene. The apo E 3/4 genotype should serve as an example. The amplificate is digested resulting in this case in fragments of 16, 18, 19, 32, 32-B, 48, 72 and 91basepairs. Only the homoduplex of the E4 allele by meaning only 25% of the total amplificate will be digested at residue 112. The digestion of this 25% of the amplificate at this particular residue is responsible for the generation of the fragments of the length of 19 and 72 basepairs. 75% of the PCR product which consists of the homoduplex of the E3 allele and of the heteroduplexes formed by both genetic variants will not be cut at residue 112. This is the reason why the intensity of the bands of the 72 and 19 basepair fragments is

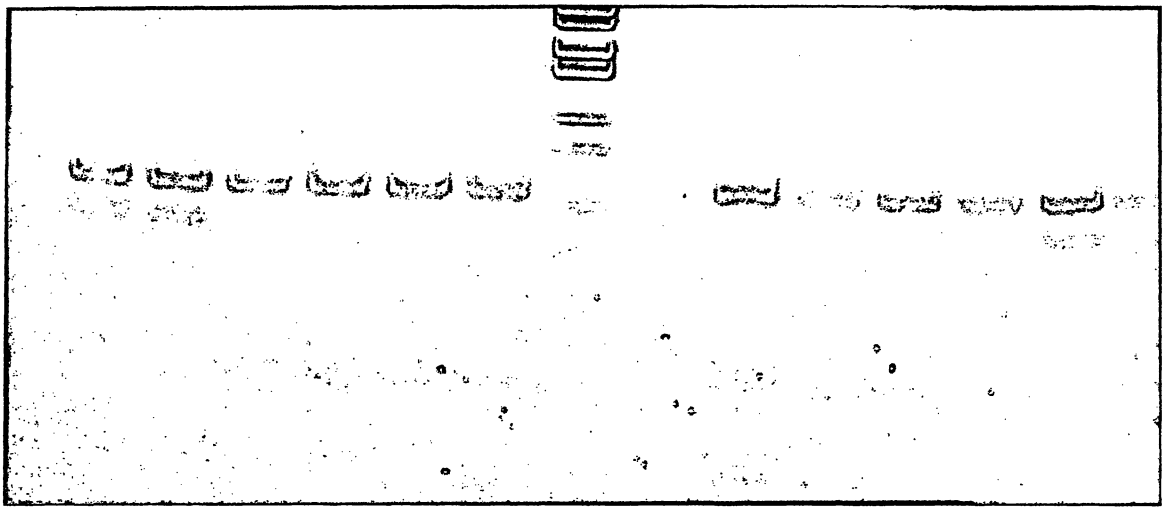

Figure 2 Restriction isotyping of the Apo E gene performed after isolation of template DNA by different methods. The lanes 1 to 4 leucocytic DNA was isolated using the Triton X- 100 lysis method. In lanes 5 to 8 the DNA was obtained from cervical smear. In lanes 9 to 10 leucocytic DNA was isolated by salting-out procedure. In the 11 to 14 leucocytic DNA was extracted by the phenol-chloroform method. Apo E genotypes:
lanes 1, 2, 10, 11, 12 — 3 / 4; lanes 5-9 — 3 / 3; lanes 3, 4, 13, 14 — 2 / 3
L - pBR322 Msp I ladder

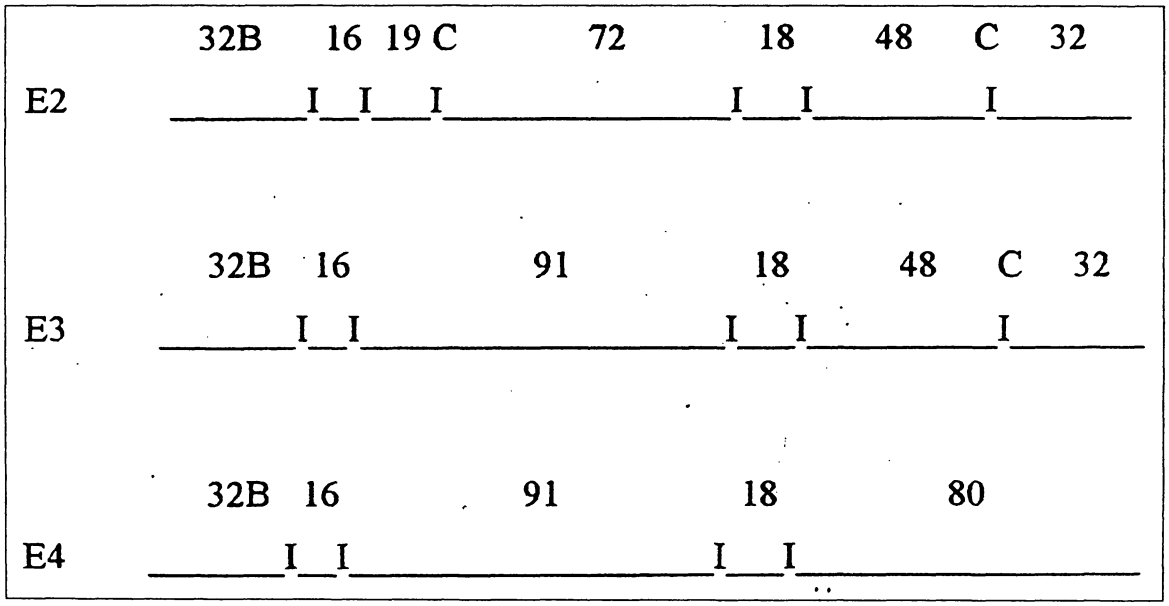

Figure 3 Cfo I cleavage map for each isoform of the apo E gene is shown c.f.[1] (modif.). The distance (bp) between all Cfo I cleavage sites is shown including the polymorphic Cfo I sites (C) that distinguish isoforms.

weaker than could be expected considering only the size of the fragment.

A formula to describe the theoretical intensity (TI) of the bands could be as follows:TI = K x L / 91
K = part of 1 (= part of the whole amplificate) which is digested by CfoI leading to the generation of the particular fragment
L = size of the fragment (bp)
91= size of the longest possible fragment (bp)

The theoretical intensity of the most important bands for genotyping are presented in Table 1. The band pattern shown in Fig. 4 can be characterized with regard to the calculated theoretical intensities of the bands.

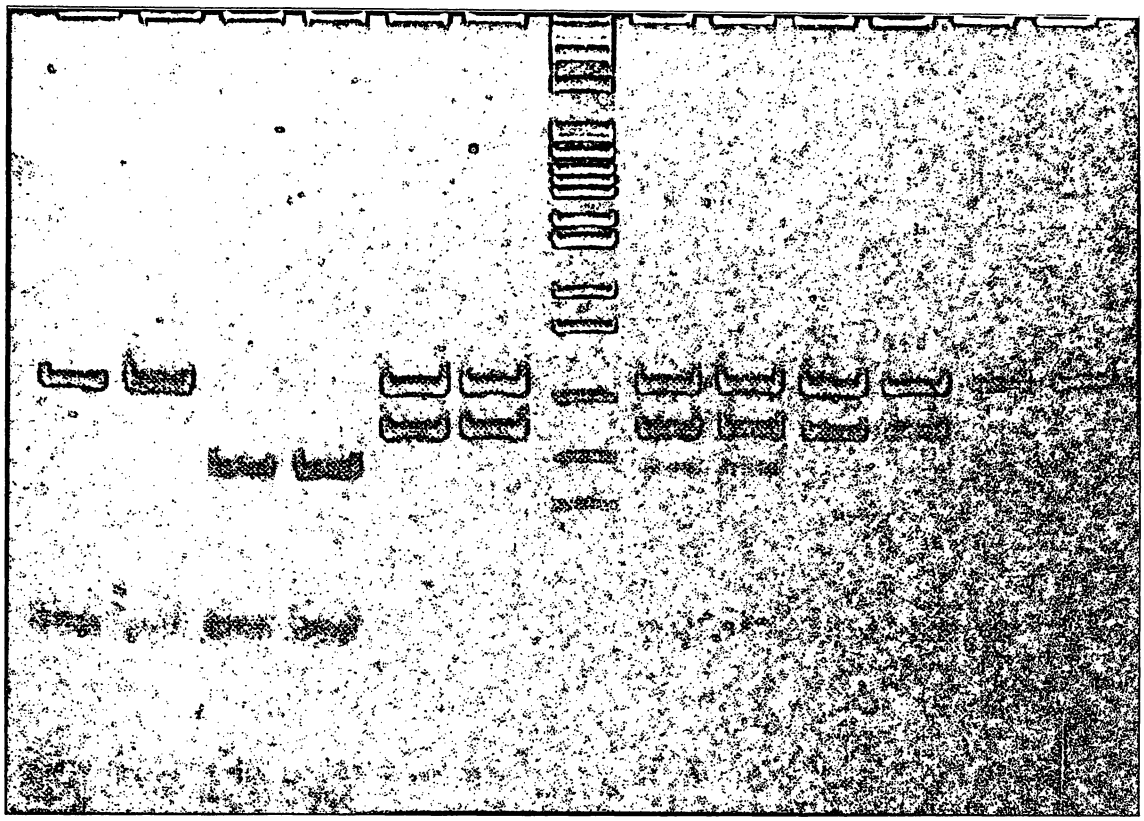

Figure 4 The genotyping of the six common genotypes was performed by restriction isotyping method [1]. The PCR product was digested with Cfo I. The fragments were size separeted on an 8% polyacrylamide gel. The gel was stained with ethidium bromide and UV-photographed. The fragments of the sizes of 16, 18 and 19 bp are not essential for restriction isotyping. They are not shown in the photograph

It should be mentioned that the sequence analysis is still the gold standard for every analytical investigation of genomic DNA. We have generated a standard for each apo E genotype by sequencing. The sequenced probes served as positive controls in the restriction isotyping procedures for diagnostic purposes (Fig. 3).

Discussion

Apo E genotyping is a necessary step to investigate patients with disorders of lipoprotein metabolism or an early manifestation of atherosclerosis and Alzheimer's disease. The presence of an E2 or E4 allele might play a role in the early manifestation of the mentioned diseases [5-8]. The individual risk should be evaluated, because of possible consequences regarding therapeutic efforts. It should be of interest for advising those patients and their relatives. The genotyping procedure has to be reliable and reproducible results have to be achieved.

Restriction isotyping seems to be the preferable procedure in the case of apo E genotyping. In our labora-

Table 1 Theoretical intensity (TI) of the bands most important for Apo E genotyping. K is the part of the total amplificate which was digested by Cfo1 leading to the generation of the particular fragment.

size of the fragment	homozygote 3/3		homozygote 4/4		homozygote 2/2		heterozygote 2/4		heterozygote 2/3		heterozygote 3/4	
(bp)	K	TI	K	TI	K	TI	K	TI	K	TI	K	TI
91	1	1.00			1	1.00	0.75	0.75	1	1.00	0.75	0.75
80					1	0.88	0.75	0.66	0.75	0.66		
72			1	0.79			0.25	0.20			0.25	0.20
48	1	0.53	1	0.53			0.25	0.13	0.25	0.13	1	0.53
Biotin-32	1	0.35	1	0.35	1	0.35	1	0.35	1	0.35	1	0.35
32	1	0.35	1	0.35			0.25	0.09	0.25	0.09	1	0.35

tory we have performed the apo E genotyping of more than 1000 patients applying restriction isotyping. In contrast to *Appel* et al. [15] the conventional method always delivered stabile results considering the particular features described above. There was no doubt about an influence of the DNA concentration on restriction isotyping but until now quantitative investigations have not been performed. As it is shown in the results we recommend the use of at least 0.05 µg of genomic DNA in a 100 µl PCR mixture. The method of DNA isolation from cells could also have an impact on restriction isotyping. This may be caused by extraction of bad quality DNA containing for example inhibitors of the PCR like haemoglobin or SDS. However, we did not find an influence of preparative procedures on the outcome of restriction isotyping. An incomplete digestion of the PCR product by CfoI is considered to be responsible for misinterpretations [15]. In accordance with *Hansen* et al. this seems to be unlikely as the DNA amplificate contains four non- polymorphic recognition sequences for CfoI in addition to the two polymorphic sites [16]. Thus there are four invariable internal controls for CfoI cleavage [16]. Applying the amount of DNA, primers and enzyme as previously desribed we could not verify problems caused by the conditions of the restriction enzyme digestion of the PCR product.

The determination of the theoretical intensity of the bands could be very helpful in the interpretation of the band pattern in an effort to make the interpretation of the obtained results more objective.

Alternative methods to perform Apo E genotyping are sequencing, the ASO hybridization, SSCP and ARMS [2, 17, 18]. Even if sequencing is the gold standard of genotyping it is a time consuming and expensive method in case when genotyping should become a routineous procedure in the laboratory. A hybridization analysis with allele-specific oligonucleotides also requires optimal conditions [2] . Four different hybridization reactions and a high quality dot blot have to be performed. The exon 4 of of the Apo E gene is an excellent target for SSCP based on its high GC content [18]. The problem is that rare mutations show similar patterns in SSCP leading to an incorrect determination of the genotype.

In contrast, restriction isotyping is an easy method to perform that does not require much expensive technical equipment in the laboratory. The improvement of the performance of taq polymerases over recent years and the amplification of the genomic DNA by touch down PCR result in an increased specificity of PCR. The ethidium bromide staining of the gel can be replaced by silverstaining. However, by taking precautions with regard to the toxicity of the substance the ethidium bromide staining is still the method of choice in our daily routine.

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Mitteilung

Erweiterung der Indikation zur Testung auf E. coli 0157 in Großbritannien auf sämtliche durchfälligen Stühle

Das Advisory Committee on the Microbiological Safety of Food hat bereits im Jahre 1995 empfohlen, sämtliche durchfälligen Stühle, insbesondere auch solche ohne Blutbeimengungen, auf E. coli 0157 zu untersuchen. Die britischen Kollegen begründen diese Empfehlung damit, daß die Hälfte der mit E. coli 0157 infizierten Patienten kein Blut im Stuhl haben und daß einige dieser Patienten ein hämorrhagisch-urämisches Syndrom (HUS) entwickeln, ohne daß es zu irgendeinem Zeitpunkt zu einer hämorrhagischen Colitis gekommen wäre.

Der Inzidenzgipfel läge zwar im dritten Quartal, sowohl in Großbritannien als auch in USA seien aber große Epidemien auch im Winter ausgebrochen. Deshalb könne zu keiner Zeit auf die generelle Untersuchung aller durchfälligen Stühle auf E. coli 0157 verzichtet werden.

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Dr. *R. Seuffer,* Reutlingen

DIN-Mitteilung

Aus dem DIN-Institut

Der Arbeitsausschuß C 6 „Hämatologie" (Obmann: *T. Seeger*) des Normenausschusses Medizin im DIN gibt das Erscheinen der folgenden Norm bekannt:
DIN 58932-1 Hämatologie - Bestimmung der Blutkörperchenkonzentration im Blut - Teil 1: Blutentnahme, Probenvorbereitung, Einflußgrößen, Störfaktoren.
Diese Norm gilt für die Blutentnahme zur Bestimmung der Blutkörperchenkonzentration im Humanblut, das durch Zusatz von Antikoagulantien oder durch hohe Verdünnung mit einer isotonen wässrigen Lösung ungerinnbar gemacht wurde.

Stand der Akkreditierung Medizinischer Laboratorien

Eine Mitteilung der GLP Kommission der AML

Der Begriff Qualität hat im Produktions und Dienstleistungsbereich global erheblich an Interesse gewonnen und dabei eine Ausdehnung auf neue Dimensionen erfahren (Tabelle 1), die zu einem umfassenden Konzept, als total quality management (TQM) bezeichnet, geführt haben. Wesentlicher Teil dieses Konzepts ist die Einführung eines umfassenden Qualitätsmanagement, das alle Managementaspekte eines medizinischen Laboratoriums umfaßt. Anleitungen finden sich in mehreren Normen und Richtlinien, wie z.B. den Normenreihen DIN EN ISO 9000, DIN EN 45 000 und dem ISO Guide 25.

Die Umsetzung dieser Normen bedeutet einen wesentlichen Schritt in Richtung TQM. Obwohl dieser Schritt zur Zeit freiwillig ist, sollte er jedoch aus mehreren Gründen erwogen werden (Tabelle 2). Ist ein TQM implementiert, kann dies nach DIN EN ISO 9001f zertifiziert, bzw. bestätigt werden (Konformitätsbestätigung). Wer sich jedoch die kompetente Umsetzung bestätigen lassen möchte, sollte sich nach DIN EN 45001 akkreditieren lassen (Kompetenzbestätigung).

Die GLP-Kommission hat sich nach Rücksprache mit den Vorständen der in der AML zusammengeschlossenen wissenschaftlichen Fachgesellschaften entschlossen, alle Aktivitäten bei der ZLG (Zentralstelle der Länder für Gesundheitsschutz bei Medizinprodukten, 53115 Bonn, Sebastianstr. 189), einer von den Ländern getragenen Behörde, zu fokussieren. Dadurch konnte die Einrichtung einer kostenintensiven Akkreditierungsstelle durch die Fachgesellschaften vermieden werden.

In enger Kooperation mit der AML wurde von der ZLG das Sektorkomitee V Medizinische Laboratorien eingerichtet, das die Funktion eines wissenschaftlichen Beirates wahrnimmt. In den letzten 18 Monaten wurden intensive Vorarbeiten geleistet, um ein Handbuch zu erstellen, das alle wichtigen Informationen zur Vorbereitung und zur Durchführung von Akkreditierungen medizinischer Laboratorien enthält und zum Preis von 198 DM über die ZLG bestellt werden kann. Voraussichtlicher Erscheinungstermin ist Anfang 1997. Das Handbuch wurde in Form eines Ringbuches erstellt, um spätere Aktualisierungen leichter vornehmen zu können. Außerdem wurden inzwischen mehrere von den Fachgesellschaften vorgeschlagene Gutachter geschult, so daß die Akkreditierung durch die ZLG Anfang 1997 aufgenommen werden kann.

Wer an einer Akkreditierung interessiert ist, kann sich bei der ZLG anmelden und die notwendigen Informationen einholen. Wichtiger als die Akkreditierung ist jedoch die Vorbereitung, von der jedes Laboratorium unabhängig von dem Erhalt einer Akkreditierungsurkunde profitieren wird. Wenn ein Laboratorium keinen Nutzen für die Verbesserung seiner Dienstleistungen erkennen kann, ist das Bemühen um TQM und Akkreditierung nutzlos und sollte wegen des damit verbundenen Einsatzes von Ressourcen unterbleiben.

R. Haeckel
Vorsitzender der GLP-Kommission der AML und des Sektorkomitees Medizinische Laboratorien der ZLG

Tabelle 1 Qualitätsebenen in medizinischen Laboratorien

1. Strukturqualität: Verfügbarkeit und Organisation humaner, physischer und finanzieller Ressourcen (Personal, Raum, Ausrüstung, Verbrauchsmaterialien, usw.) für ein Produkt oder eine Dienstleitung.
2. Prozeßqualität: alle Aktivitäten, um ein Produkt oder eine Dienstleitung zu liefern (z. B. Befundbericht, Interpretation, Beratung).
3. Outcome-Qualität (Wirksamkeit, Effektivität): einschließlich Strategien für Diagnose, Verlauf und Prognose von Krankheiten im Sinne von Good Use of Laboratory Medicine (totales Qualitätskonzept, das den Kunden miteinschließt).
4. Managementqualität: Optimierung aller Management-Aufgaben; bei Zielkonflikten muß der für die Patienten effektivste Kompromiß gefunden werden.

Tabelle 2 Gründe für eine Akkreditierung medizinisch-diagnostischer Laboratorien

1. Zum Nachweis des Nachkommens der gesetzlichen und standesrechtlichen Verpflichtung zur Qualitätssicherung.
2. Für die Erhebung klinischer, biologischer und sonstiger Daten im Rahmen der Konformitätsbewertung von Medizinprodukten werden sich Benannte Stellen, Industrie und Behörden zunehmend auf akkreditierte Laboratorien stützen.
3. Einzelne Fachdisziplinen wie die Transfusionsmedizin und Immunhämatologie unterliegen aufgrund ihrer Tätigkeit bereits gesetzlichen Regelungen (Arzneimittelgesetz, in Zukunft dem Transfusionsgesetz) und der Überwachung von Bundes- und Landesbehörden.
4. Die BÄK bereitet eine Akkreditierung für Referenzlaboratorien vor.
5. Konkurrenzdruck zwischen den Laboratorien.
6. Kommerzielle Partner der medizinischen Laboratorien werben bereits mit Zertifikaten.