*MUTATION IN BRIEF*

# The Spectrum of Aldolase B (*ALDOB*) Mutations and the Prevalence of Hereditary Fructose Intolerance in Central Europe

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**We investigated the molecular basis of hereditary fructose intolerance (HFI) in 80 patients from 72 families by means of a PCR-based mutation screening strategy, consisting of heteroduplex analysis, restriction enzyme digest, DNA single strand electrophoresis, and direct sequencing. For a subset of patients mutation screening with DHPLC was established which turned out to be as fast and as sensitive as the more conventional methods. Fifteen different mutations of the aldolase B (ALDOB) gene were identified in HFI patients. As in smaller previous studies, p.A150P (65%), p.A175D (11%) and p.N335K (8%) were the most common mutated alleles, followed by c.360_363delCAAA, p.R60X, p.Y204X, and c.865delC. Eight novel mutations were identified in eight families with HFI: a small indel mutation (c.1044_1049delTTCTGGinsACACT), two small deletions (c.345_372del28; c.841_842delAC), two splice site mutations (c.113-1G>A, c.799+2T>A), one nonsense mutation (c.612T>G (p.Y204X)), and two missense mutations (c.532T>C (p.C178R), c.851T>C (p.L284P)). By mutation screening for the three most common *ALDOB* mutations by DHPLC in 2,000 randomly selected newborns we detected 21 heterozygotes. Based on these data and after correction for less common and private *ALDOB* mutations, HFI prevalence in central Europe is estimated to be 1:26,100 (95% confidence interval 1: 12,600-79,000).** © 2005 Wiley-Liss, Inc.

KEY WORDS: aldolase B; ALDOB; DHPLC; fructose; neonatal screening; Central Europe

## INTRODUCTION

Hereditary fructose intolerance (HFI, MIM# 229600) is an autosomal recessive disorder caused by a deficiency of aldolase B (E.C. 4.1.2.13), an enzyme of liver, intestine and renal cortex catalysing the metabolism of fructose of exogenous origin. Affected individuals may present with symptomatic hypoglycaemia, vomiting and life-threatening episodes shortly after the intake of fructose or related sugars. Prolonged ingestion may ultimately lead to death from severe liver and kidney failure. Patients may, however, develop a protective aversion to sweets and fruit, which is a reason that diagnosis is frequently missed, and which also explains that reliable prevalence numbers for different populations do not exist (Steinmann et al., 2001).

For years, the mainstay of diagnosis of HFI has been the metabolic response to an intravenous fructose load or an enzymatic assay of liver or intestinal biopsy samples (Steinmann and Gitzelmann, 1981), both of them being bothering and invasive. Therefore, the advent of molecular genetic techniques seemed to provide a promising tool


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in the diagnosis of HFI. The human gene for aldolase B (*ALDOB*) has been mapped to chromosome 9q21.3 - q22.2 (Lebo et al., 1985; Henry et al., 1985). The cDNA of approximately 1.6 kb has been cloned and the genetic structure of its genomic 14.5 kb of DNA with nine exons has been elucidated. Exons 2 through 9 encode a protein of 364 amino acids (Rottmann et al., 1984; Tolan and Penhoet, 1986).

At present, 35 *ALDOB* mutations have been reported (The Human Gene Mutation Database, Cardiff, http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/119669.html, Steinmann et al., 2001; Esposito et al., 2004). Among them, three amino acid substitutions p.A150P (Cross et al., 1988), p.A175D (Cross et al. 1990a), and p.N335K (Cross et al. 1990b) are frequently found while others seem to be rare or confined to single families. Allele frequencies, however, show regional differences and are different among ethnic groups. In a multicenter study, for example, the three common mutations accounted for more than 85% of HFI alleles in a pool of European patients (Cross et al., 1990), in contrast to 68% in North American HFI patients (Tolan and Brooks, 1992). In a recent study on the feasibility of neonatal screening in the United Kingdom, the heterozygote frequency for the most common mutation p.A150P was 1.32% (James et al., 1996). Corresponding data for other countries are not yet available.

A rational use of molecular techniques for the diagnosis of HFI and for molecular genetic screening programs of this relatively frequent and treatable inherited metabolic disorder requires knowledge of the allele frequencies of HFI mutations in the particular population under study. In order to obtain such data we established a HFI mutation screening program including HFI patients resident in the central part of Europe.

## MATERIALS AND METHODS

### Patients

The present evaluation is based on data of 80 individuals from 72 unrelated families with at least one patient with a diagnosis of HFI. Diagnosis was based on *(i)* a positive conventional test result, *i.e.*, an intravenous fructose load test, or an enzymatic assay in a liver or intestinal biopsy specimen, or *(ii)* suggestive clinical symptoms and the exclusion of other disorders together with the detection of mutations of the *ALDOB* gene on both the maternal and paternal chromosome 9. The ethnic origin of patients was German in 47 families, the others were immigrants mainly from Mediterranean countries. All individuals tested and/or their parents were informed about the nature of the study and gave their consent. The ethical committee of the local Board of Physicians (Ärztekammer Hamburg) gave its approval to extend mutation analysis to 2,000 anonymous DNA samples collected randomly from newborns that were investigated by the Hamburg neonatal screening program.

### Mutation detection by RFL and SSC analysis

For diagnostic purposes, high molecular weight genomic DNA was prepared from leukocytes according to standard protocols (Sambrook et al., 1989). DNA from dried blood samples was extracted by the E.Z.N.A.® Tissue DNA kit II (Peqlab Biotechnologie, Erlangen, Germany). Sense and antisense primer sequences (Table 1) used for PCR amplification of exons 2 through 9 of *ALDOB* and their flanking intronic sequences were chosen from the published gene sequence (Mukai et al., 1987; RefSeq sequence NM_000035.2) or were obtained from literature (Cross et al., 1990; Santamaria et al., 1996). All PCR reactions were carried out in the TRIO-Thermoblock (Biometra, Göttingen, Germany) using reagents and Taq polymerase from Gibco BRL (Eggenstein, Germany). In a first attempt, samples were tested for the presence of the three most common mutations by restriction fragment length (RFL) analysis with PCR conditions and enzymes given in Table 2. In case that these mutations were not found or found only on one chromosome, a mutation screening of exons 2 to 9 by means of heteroduplex analysis and/or single strand conformation (SSC) analysis at room temperature and 4°C was carried out.

**Table 1. Sense (sn) and Antisense (asn) Primers Used for the Amplification of *ALDOB* Exons 2 Through 9 with Flanking Intron Sequences**

| Exon | Primer | $T_a$ | Reference |
|---|---|---|---|
| **2** | sn 5'-caa ctc tcg cac act cat ttc c-3',<br>asn 5'-cta cgc tta ctg agt ctt ctg c-3' | 66 | *this study* |
| **3** | sn 5'-aga agg gtg aca gga aag c-3',<br>asn 5'-agt gtg ctt gga gtt tgc c-3' | 69 | *this study* |
| **4** | sn 5'-gtt caa gag ttc tgc ttg tg-3',<br>asn 5'-gcc ttc att tct agc tta ca-3' | 53 | Cross et al., 1990 |
| **5** | sn 5'-cct tcc ctt tat tag aag ccc-3',<br>asn 5'-cta gcc tac tct ttt tca gcc c-3' | 62 | *this study* |
| **5** | sn 5'-ttc cct tta tta gaa gcc cca tg-3',<br>asn 5'-aga gca CCT GCT GAC AGA TGC TA-3' | 64 | mmp, *this study* |
| **6** | sn 5'-atg tag tat ttc tcc ata atg g-3'<br>asn 5'-aga ttt ttc aac tag aat tgg g-3' | 57 | *this study* |
| **7** | sn 5'-gtc aag tgg ctc tat gac tag c-3',<br>asn 5'-tgt ggc tct cca aag aat gag g-3' | 64 | *this study* |
| **8** | sn 5'-tca ttg ctt gct ttc tca agc-3',<br>asn 5'-aag aaa aca atg ctt ctc cg-3' | 54 | Santamaria et al., 1996 |
| **8** | sn 5'-CTC AAC CTC AAT GCT ATC AG-3'<br>asn 5'-aag aaa aca atg ctt ctc cg-3' | 56 | mmp, *this study* |
| **9** | sn 5'-gtg aag gtt tga ctg gtt tcc-3'<br>asn 5'-aaa agt tgc tcc ctt tca gcc c-3' | 65 | *this study* |

*Lowercase* and *uppercase* letters represent intron and exon sequences, respectively. An *underlined* letter represents a mismatch. $T_a$, annealing temperature; mmp, mismatch primer

**DHPLC**

In our population-based investigation of newborns (n=2,000), products from a duplex PCR of exon 5 and exon 9 were primarily investigated by denaturating high performance liquid chromatography (DHPLC) (Oefner and Underhill, 1998) on a WAVE analyzer (Transgenomic Inc., Omaha, NE, USA). At a column oven temperature of 64 °C, 5 to 10 microliters of sample were injected into a preheated C18 reversed phase column based on non-porous poly (styrene-divinylbenzene) particles (DNA-Sep®, Transgenomic Inc., Omaha, NE, USA). Samples were eluted by a mixture of buffer A (0.1M triethylmmonium acetate (TEAA)) and buffer B (0.1M TEAA, 25% acetonitrile) with a linear gradient of 50% - 64% buffer B with a 2 percent point increase per minute. Since the elution profile for samples homozygous for p.A150P cannot be distinguished from the wild-type profile, all PCR products of the 2,000 newborns were primarily investigated after admixture of a known wild-type PCR product in order to allow for heteroduplex formation in the case of a homozygous aberration (Fig. 1).

**DNA Sequencing**

All PCR samples with an aberrant heteroduplex formation on polyacrylamide gel or with unusual patterns on SSC or DHPLC analysis were further analysed by direct cycle sequencing using the protocol for $^{33}$P labelled terminators provided with the Thermo-Sequenase cycle sequencing kit (Amersham/Buchler, Wenden, Germany). When necessary, particularly in the cases of heterozygosity for small deletions, the sequencing reaction was performed after cloning the respective PCR products into a phagemid vector according to the PCR Script Amp Cloning kit (Stratagene, Heidelberg, Germany).

In cases of negative results of the mutation screening on one or both chromosomes, and in cases of novel mutations, all coding exons were sequenced. Novel mutations were confirmed either by electrophoretic size determination of PCR products with deletions or, if available, by restriction enzyme digest in case of a simple nucleotide exchange (Table 2). For the latter method, PCR mismatch primers were constructed in some cases.

Amino acid residues are numbered and mutations are designated according to suggestions made by the Human Mutation Nomenclature Working Group (Antonarakis 1998). Nucleotides of the *ALDOB* cDNA sequence are num-

bered assigning +1 to the first nucleotide in the ATG initiation codon located in exon 2. The initiator methionine is designated p.M1 and the carboxyterminal tyrosine p.Y364.

**Table 2. Detection of 15 *ALDOB* Mutations in 72 HFI Index Cases**

| Exon / Intron | Nucleotide exchange | Translation effect* | Facilitated detection *RE;* others* | Remarks |
|---|---|---|---|---|
| IVS2 | c.113-1G>A | *-ss* | *None* SSCA | compound heterozygous German female |
| 3 | c.178C>T | Arg60X | *+Dde I* SSCA | |
| 4 | c.345_72 del28 | 115 (*fs*...) 152 X | *None;* EM, HD | German family with HFI in 2 generations, father p.A150P homozygous |
| 4 | c.360_363del CAAA | 119 (*fs*...) 152 X | HD | |
| 5 | c.448G>C | Ala150Pro | *+Bsa HI*; SSCA, **DHPLC** | |
| 5 | c.524C>A | Ala175Asp | *-Bfa I (mmp)* SSCA, **DHPLC** | |
| 5 | c.532T>C | Cys178Arg | *-Alw NI* **DHPLC** | compound heterozygous Swiss female |
| 6 | c.612T>A | Tyr204X | *None* SSCA | |
| 6 | c.612T>G | Tyr204X | *+Bcef I* SSCA | compound heterozygous German female |
| IVS7 | c.799+2T>A | *-ss* | *None* | homozygous Turkish male |
| 8 | c.841_842delAC | 280 (*fs*...) 334X | *None* SSCA | homozygous Turkish male |
| 8 | c.851T>C | Leu284Pro | *-MnlI* | compound heterozygous German female |
| 8 | c.865delC | 288 (*fs*...) 298 X | *+Alu I (mmp)* SSCA | |
| 9 | c.1005C>G | Asn335Lys | *+Dde I* SSCA, **DHPLC** | |
| 9 | c.1044-_1049delTTCTGG insACACT | 348 (*fs*...) 370 X | *None*; HD | compound heterozygous German female |

Novel mutations detected for the first time in this study are highlighted in gray. Furthermore, among 2,000 randomly selected neonates three showed an unclassifiable retention pattern and sequencing of exon 5 revealed heterozygosity for c.400 C>A [**Arg 134 Ser**], c.488 C>T [**Ala 163 Val**], and c.538 C>A [**Gln 180 Lys**], respectively.
***** *(-ss...)* denotes the loss of a splice site, *(fs...)* indicates a frameshift starting after the respective codon. Facilitated detection refers to the creation (+) or the loss (-) of a recognition sequence for the respective restriction enzyme (*RE*) in some cases after creation of an amplicon with the use of a mismatch primer (mmp) or a change in electrophoretic mobility (EM). HD denotes mutation detection by *h*etero*d*uplex formation; SSCA denotes detection and discrimination by single strand conformation analysis. DHPLC means that this mutation was detectable by denaturating high performance liquid chromatography. Numbering refers to the RefSeq sequence NM_000035.2

## RESULTS

### Mutation analysis

A total of 15 different mutations was detected in the 72 index patients (Table 3). Among them, seven have been described before, another 8 were novel defects (Table 2). The nature of 6 of these novel mutations (2 small deletions, 1 small *indel*, 2 splice site, 1 nonsense mutation) implies a causative role. The two missense mutations have only been detected in association with HFI and have never been observed among randomly chosen individuals: p.C178R and p.L284P were not observed among 95 random samples when investigated by restriction enzyme digest; moreover, p.C178R was not found in 2,000 neonates whose exon 5 was analyzed by DHPLC,

By screening only for the 3 most common mutations (p.A150P, p.A175D, p.N335K), the diagnosis of HFI was confirmed in 52 of 72 index patients (72%) (Table 3). Additionally, nine patients with p.A150P and 2 patients with p.A175D were compound heterozygotes for less common mutations (15%). Compound heterozygosity or homozygosity for rare alleles was observed in 4 patients (5.6%). In summary, we were able to confirm the diagnosis of HFI in 67 of 72 index patients (93%) by our molecular genetic approach. In 5 patients with an established diagnosis of HFI only one heterozygous mutation (4x p.A150P, 1x p.A175D) was detected. A specific ethnic origin was found for the mutation p.N335K which was detected in 4 of 5 patients from Bosnia who all were homozygotes. p.N335K was only detected in 2 patients whose ethnic origin was in Central Europe.

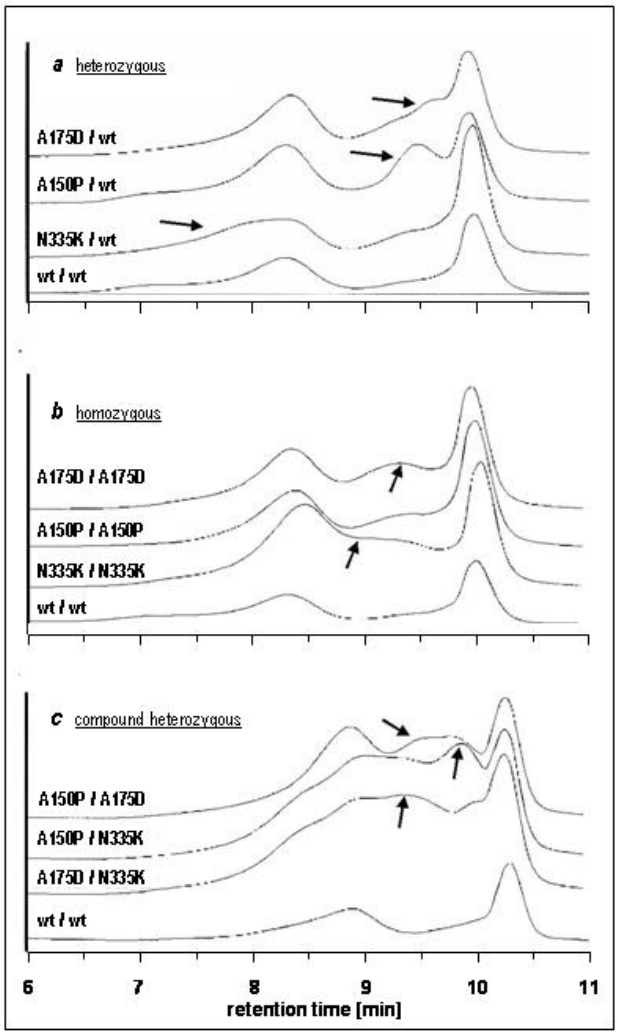


**Figure 1.** DHPLC elution profiles of common *ALDOB* mutations. All profiles represent millivolts over retention time in minutes and are depicted in comparison to a wild type (wt) profile. The most discriminating features are marked by arrows. For clarity reasons, curves have been piled. **A:** Elution profiles of PCR products from heterozygous individuals: all three mutations show a specific retention pattern. **B:** Elution profiles of PCR products from homozygous individuals: p.A175D and p.N335K show a specific retention pattern; p.A150P can only be discriminated from wt after admixture of wt PCR product (*see* panel A). **C:** Elution profiles of PCR products from compound heterozygous individuals: all three combinations show a specific retention pattern.

**Prevalence study**

Twenty-one of 2,000 newborns were found to be heterozygous for one of the three common mutations of the *ALDOB* gene (Table 4) which results in a carrier frequency of 1 : 95. Based on these data, after calculation of homo- and compound heterozygosity for the three most common mutations and correction for less frequent *ALDOB* mutations, the expected frequency of HFI in our area is1: 26,100 ( CI (95%) 1 : 12,600 – 79,000 ).

Furthermore, DHPLC analysis of the 2,000 randomly selected neonates revealed three other new changes, leading to amino acid substitutions, in the ALDOB exon 5. Three individuals were found to be heterozygous for c.400C>A (p.R134S), c.488C>T (p.A163V), and c.538C>A (p.Q180K), respectively.

**Table 3. Observed *ALDOB* Genotype Distribution and Allele Frequencies in Patients with HFI (*See Table 1 for approved DNA mutation nomenclature.*)**

| First allele | | | | | | | | | | | | | | | | Second allele | | 2n | allele frequency |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IVS 2 -1 g>a | c.178 C>T (R60X) | c.345-72 del 28 | c.360-3 del CAAA | c.448 G>C (A150P) | c.524 C>A (A175D) | c.532 T>C (C178R) | c.612 T>A (Y204X) | c.612 T>G (Y204X) | c.841-2 del AC | IVS 7 +2 t>a | c.851 T>C (L284P) | c.865 del C | c.1005 C>G (N335K) | c.1044-9 del TTCTCG ins ACACT | n.d. | | | | |
| --- | --- | --- | --- | 1 | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | IVS 2 -1 g>a | | 1 | 0.007 |
| | --- | --- | --- | 2 | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | c.178 C>T | (R60X) | 2 | 0.014 |
| | | --- | --- | 1 | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | c.345-72 *del* 28 | | 1 | 0.007 |
| | | | 1 | 1 | --- | --- | --- | --- | --- | --- | --- | 1 | --- | --- | --- | c.360-3 *del* CAAA | | 4 | 0.028 |
| | | | | 36 | 8 | 1 | --- | 1 | --- | --- | --- | 1 | --- | 1 | 4 | c.448 G>C | (A150P) | 93 | 0.646 |
| | | | | | 2 | --- | 1 | --- | --- | --- | 1 | --- | 1 | --- | 1 | c.524 C>A | (A175D) | 16 | 0.111 |
| | | | | | | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | c.532 T>C | (C178R) | 1 | 0.007 |
| | | | | | | | --- | --- | --- | --- | --- | --- | --- | --- | --- | c.612 T>A | (Y204X) | 1 | 0.007 |
| | | | | | | | | --- | --- | --- | --- | --- | --- | --- | --- | c.612 T>G | (Y204X) | 1 | 0.007 |
| | | | | | | | | | 1 | --- | --- | --- | --- | --- | --- | c.841-2 *del* AC | | 2 | 0.014 |
| | | | | | | | | | | 1 | --- | --- | --- | --- | --- | IVS 7 +2 t>a | | 2 | 0.014 |
| | | | | | | | | | | | --- | --- | --- | --- | --- | c.851 T>C | (L284P) | 1 | 0.007 |
| | | | | | | | | | | | | --- | --- | --- | --- | c.865 *del* C | | 2 | 0.014 |
| | | | | | | | | | | | | | 5 | --- | --- | c.1005 C>G | (N335K) | 11 | 0.076 |
| | | | | | | | | | | | | | | --- | --- | c.1044-9 *del* 6 *ins* 5 | | 1 | 0.007 |
| | | | | | | | | | | | | | | | --- | *n.d.* | | 5 | 0.035 |
| | | | | | | | | | | | | | | | | Σ | | 144 | 1.000 |

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## DISCUSSION

Compared to conventional diagnostic procedures, the molecular genetic diagnosis of HFI has many advantages and has become the method of choice. Our results emphasise the reliability of the method. A screening for only the most common mutations p.A150P, p.A175D and p.N335K confirmed the diagnosis in 52 of 72 index patients (72%) with HFI. Furthermore, all but 4 index patients were at least heterozygous for 1 of these 3 mutations which means that in 68 out of 72 HFI families (94%) at least one of the 3 most frequent mutations was found.

Besides c.360_363delCAAA, p.R60X, c.865delC and p.Y204X (c.612T>A) which have been described previously (*see* Ali et al., 1998 *for review*), an additional 8 novel mutations were identified in 8 different HFI families. Another 3 novel sequence aberrations were detected in the heterozygous state when screening neonatal testcards (Table 2). These 11 deviations from the *ALDOB* wild type sequence can be regarded as private defects. The causative role of 6 of these 11 sequence abberrations can be inferred from their nature, since they predict aberrant and truncated translation products in all cases. For most of the missense mutations it is very likely that they are disease-causing since most of them affect amino acid residues that are highly conserved in aldolase B enzymes of other species and of aldolase A and C isoenzymes. However, the *ALDOB* sequence is generally highly conserved and, as demonstrated for p.A150P, also mutations within non-conserved areas may play a very important role. Therefore, the pathogenicity of these mutations will have to be ultimately proven by expression studies.

Our data on the relative distribution of the most common HFI mutations are similar to published results from other European populations (Ali et al., 1998; Cross and Cox 1990). The high prevalence of p.A150P and the significant contribution of p.A175D and p.N335K to the manifestation of HFI in the central European population (including patients from Italy, former Yugoslavia and Turkey) offer the possibility for a rational mutation screening in patients with suspected HFI, or even for a screening of neonates.

**Table 4. Results of Mutation Testing in 2,000 Randomly Selected Newborns**

| | **Neonatal screening** | |
|---|---|---|
| | ***n*** | ***%*** |
| **p.A150P / *wt*** | **17** | ***0.85*** |
| **p.A175D / *wt*** | **3** | ***0.15*** |
| **p.N335K / *wt*** | **1** | ***0.05*** |
| p.A150P / p.A150P | 0 | *0* |
| p.A175D / p.A175D | 0 | *0* |
| p.N335K / p.N335K | 0 | *0* |
| p.A150P / p.A175D | 0 | *0* |
| p.A150P / p.N335K | 0 | *0* |
| p.A175D / p.N335K | 0 | *0* |
| **total** (newborns carrying common *ALDOB* mutations) | **21** | ***1.05*** |
| **total** (newborns tested) | **2,000** | ***100*** |
| **Heterozygosity for common alleles** | **1 : 95** | |
| Calculated homo- / compound heterozygosity for common alleles | 1 : 36,300 ( CI 1 : 17,500 - 1 : 110,000 ) | |
| **Estimated HFI prevalence when correcting for non-common alleles** | **1 : 26,100** ( CI 1 : 12,600 - 1 : 79,000 ) | |

In this regard, a further aspect of this investigation is of particular interest. For a subset of patients and for the prevalence study in neonates, a screening method using DHPLC for mutation detection was used. This method turned out to be very convenient and efficient. In patients, it had a 100% detection rate with no false negative and false positive results when compared to direct sequencing (*data not shown*). Although the purchase of the analyzer is expensive, this methods provides several advantages over other methods like the automated procedure, the short analysis time, the possibility not only to detect mutations but also to discriminate them due to specific elution profiles, and the option to collect eluted DNA for further analyses. These positive aspects have already been used for the detection of common mutations in other inborn errors of metabolism (Santer et al., 2000). The use of DHPLC could be one step towards a general screening for HFI in neonates, however, it might be necessary for a wider acceptance that also other steps such as the extraction of DNA and the PCR will be automated.

The importance to screen for an inborn error of metabolism depends on the prevalence of a disease. To date, only a rough estimate has been reported from Switzerland where HFI has been estimated to occur in 1 out of 20,000 newborns (Gitzelmann and Baerlocher, 1973). The only systematic study has been reported from England where 1 out of 23,000 neonates was calculated to be homozygous for the *ALDOB* p.A150P mutation (James et al., 1996) and where the prevalence rate of HFI was calculated to be 1 : 18,000 (Cox 2002). From our results it seems that HFI is slightly less common in our area when compared to England (Table 4) although there is a significant overlap of confidence intervals.

Molecular genetic screening of diseases generally focusses on common mutations. If we applied our DHPLC method and screened the German population with approx. 715,000 newborns per year for the common *ALDOB* exon 5 and exon 9 mutations, we would have detected about 20 ( CI (95%) 6 – 41 ) newborns every year. Probably, as demonstrated during this study, the number would be higher since other mutations within these exons can easily be detected by their unusual elution patterns. On the other hand, rare and private mutations are of special interest, since an elevated cumulative frequency will complicate screening projects. Our report on 8 novel mutations contributes to a more heterogeneous picture of the molecular genetics of HFI and emphasises the impact of rare or private mutations on genetic testing in mixed populations.

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