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Genetic Testing for Familial Mediterranean Fever in Austria by Means of Reverse-Hybridization Teststrips, *Christian Oberkanins,[1*] Andreas Weinhäusel,[2] Gernot Kriegshäuser,[1] Anne Moritz,[1] Fritz Kury,[1] and Oskar A. Haas[2]* ([1] ViennaLab Labordiagnostika GmbH, Am Kanal 27, A-1110 Vienna, Austria; [2] CCRI, St. Anna Children's Hospital, A-1090 Vienna, Austria; * author for correspondence: fax 43-1-74040-199, e-mail oberkanins@viennalab.co.at)

Familial Mediterranean fever (FMF) is an autosomal-recessive, inflammatory disorder characterized by short, recurrent attacks of fever, accompanied by pain in the abdomen, chest, or joints and erysipelas-like erythema. Its most severe complication is progressive amyloidosis, leading to end-stage renal failure. FMF predominantly affects Turks, Arabs, Armenians, and Sephardic Jews, with carrier rates reported as high as 1 in 5, but it has been observed in lower frequencies throughout the Mediterranean area *(1)*. It is caused by several mutations within the marenostrin/pyrin-encoding gene *MEFV* on chromosome 16p13.3, which differently affect the severity of the disease phenotype and the risk of developing renal amyloidosis *(2–4)*. Although established clinical criteria for FMF exist *(5)*, many patients remain undiagnosed because of rather nonspecific symptoms; therefore, molecular genetic analysis could substantially improve early and correct diagnosis of FMF and allow initiation of lifelong prophylactic treatment of affected individuals with colchicine *(6)*.

Aiming at a simple but powerful first-line screening tool for FMF genotyping, we have set up a reverse-hybridization, teststrip-based assay (FMF StripAssay) for the simultaneous detection of 12 *MEFV* mutations: E148Q in exon 2, P369S in exon 3, F479L in exon 5, and M680I (G/C), M680I (G/A), I692del (2076–2078), M694V, M694I, K695R, V726A, A744S, and R761H in exon 10. For this purpose, we collected DNA samples from individuals who had previously been typed positive for one of these mutations and used them to generate recombinant plasmid clones for the 12 mutant alleles (TOPO TA Cloning Kit; Invitrogen). After confirming the presence of mutations by DNA sequencing, we used these plasmid clones as homozygous reference samples to determine suitable reverse-hybridization probes.

We synthesized a series of candidate 15- to 25mer oligonucleotides, selected from the FMF databank sequence (GenBank accession no. AF111163) and encoding all wild-type or mutant alleles, and immobilized them via 3′-poly(dT) tails on nitrocellulose membrane as an array of parallel lines *(7)*. We then amplified the 12 mutant plasmids, as well as a wild-type genomic DNA (obtained from a nonsymptomatic individual, and mutation-negative by DNA sequencing) by multiplex PCR, using four sets of biotinylated primers specific for *MEFV* exons 2, 3, 5, and 10 *(3, 8)*. Biotinylated amplification products were then hybridized under controlled stringency (45 ± 0.5 °C) to our preliminary probe arrays, and specifically bound fragments were identified at room temperature by use of streptavidin-alkaline phosphatase conjugate and enzymatic color reaction *(7)*.

After selecting the oligonucleotides that could differentiate best between wild-type and mutant alleles, we prepared a final probe array, which also included a 5′-biotinylated control oligonucleotide to allow performance control of the detection system. For two particular *MEFV* gene regions (codons 680 and 692–695), where more than one mutation is located within the standard length of our hybridization probes, a common oligonucleotide represented the wild-type sequence. The membrane-bound array was finally sliced into 3-mm, ready-to-use teststrips.

We evaluated the specificity of the FMF StripAssay by analyzing a series of amplification products obtained from mutant plasmid clones, as well as wild-type, heterozygous, or homozygous mutant genomic DNA samples (Fig. 1). Reverse-hybridization and enzymatic color detection were carried out either manually, using thin-walled plastic incubation trays (Bio-Rad) and a shaking waterbath (GFL) set to 45 °C, or in a fully automated device (profiBlot IIT; TECAN AG). We observed no difference in staining patterns between manual and automated teststrip processing (data not shown). Our results demonstrated that the assay will specifically detect the presence of any of the 12 *MEFV* mutations as well as correctly identify homozygotes by the absence of the corresponding wild-type signal (Fig. 1, strips 1–12). For amplified wild-type DNA, none of the mutant signals was visible (Fig. 1, strip 26), and for a negative PCR product obtained with water used in place of DNA, only the biotinylated control probe, which is expected to produce color irrespective of the presence of hybridizing DNA fragments, stained positively (Fig. 1, strip 27).

We then evaluated the FMF StripAssay for routine diagnostic purposes in a clinical setting (CCRI, St. Anna Children's Hospital, Vienna). A total of 199 patients suspected to suffer from FMF, mainly Austrian immigrants from countries with a high prevalence of the disease (Turkey and Arab countries), have been analyzed to date. Initially, 45 patient samples were tested in parallel by reverse-hybridization and by direct sequencing of PCR products covering *MEFV* exons 2, 3, 5–6, and 10. After we obtained complete concordance of the results for these patients, we analyzed new samples with only the FMF StripAssay. We identified *MEFV* mutations in 109 of 199 patients, 80 of whom were found to carry two mutations (homozygous mutant or compound heterozygous), thus strongly supporting their FMF phenotype. In the remaining 29 cases, only 1 mutation was identified (Table 1A). Direct sequencing of all *MEFV* exons, including flanking intronic regions, is now being performed on these samples to eventually identify other known or novel mutations. Ninety patients tested negative for the 12 *MEFV* mutations, leaving them without immediate genetic explanation for their clinical FMF symptoms. These cases are currently being investigated for other types of periodic fever by direct DNA sequencing of the *MVK* (HIDS) and *TNFRSF1A* (TRAPS) genes. The FMF genotypes and allele frequencies are summarized in Table 1. In addition to the 199 patients referred to our clinic because of FMF-related symptoms, the relatives of 6 mutant index cases were tested. Among these, we found another 3 homozygotes and 13 heterozygotes. To date we have applied the FMF StripAssay in a total of ~220 cases for the molecular genetic diagnosis of clinically suspected patients and for elucidating mutation carriers within families.

Several different methods, among them restriction fragment length polymorphism (RFLP) analysis, amplification refractory mutation system (ARMS), denaturing gradient gel electrophoresis, and DNA sequencing, have been successfully applied to FMF genotyping. However, most of the available techniques are very limited in their usefulness for routine diagnostic purposes. The simpler techniques, such as RFLP analysis or ARMS, are barely capable of identifying more than one mutation in a single run; therefore, comprehensive genetic analysis with these techniques could become rather labor-intensive. On the other hand, highly informative methods, such as DNA sequencing, are time-consuming and costly and are incompatible with high sample throughput. In a recent method-comparison study on cystic fibrosis, Tomaiuolo et al. *(9)* showed that reverse-hybridization represents a particularly efficient first-line diagnostic approach for disorders characterized by a limited number of common mutations in the population of interest. We have for the first time applied the reverse-hybridization technique, which has previously been described for genotyping of cystic fibrosis *(10)*, β-thalassemia *(11)*, hemochromatosis *(7)*, and several other complex hereditary diseases, to analyze 12 different *MEFV* mutations simultaneously in a single experiment. Our panel includes the five most common founder mutations [E148Q, M680I (G/C), M694V, M694I, V726A], which according to Touitou *(4)* should account for an average of ~74% of FMF chromosomes within the four most frequently affected ethnic groups (Turks, Arabs, Armenians, Jews), as well as seven less common mutations, for which reference samples became available to us. Although these rare variants will add another fraction of coverage, it is known that up to 40% of FMF chromosomes may remain negative for

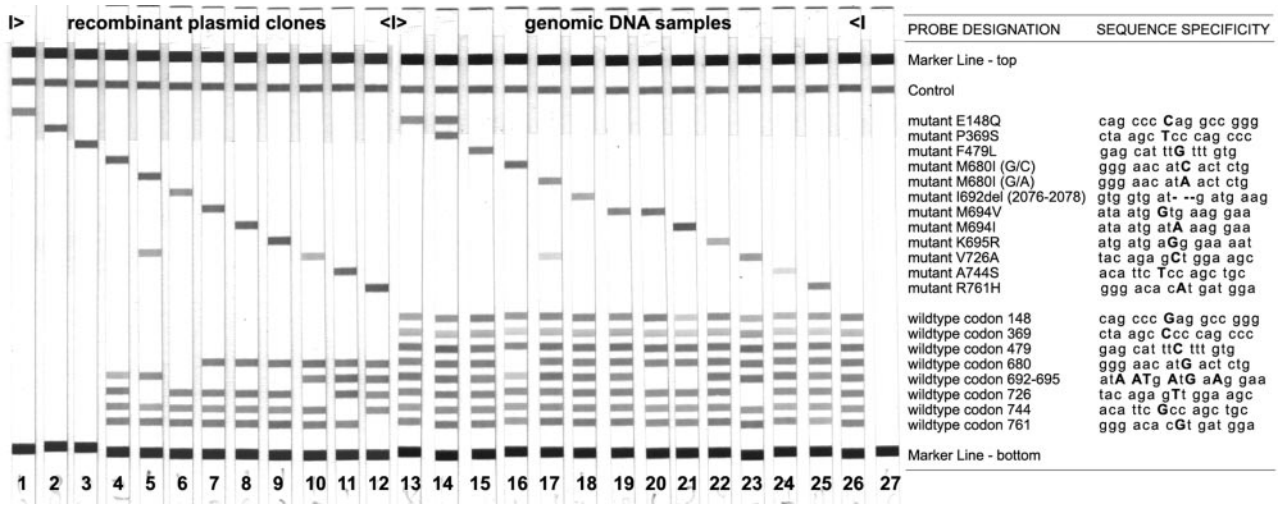

Fig. 1. Identification of *MEFV* mutations by reverse-hybridization.

Teststrips obtained after hybridization to various PCR products and enzymatic color development are shown. *Strips 1–12* (*left*) show recombinant plasmid clones encoding mutations E148Q, P369S, F479L, M680I (G/C), M680I (G/A) + V726A, I692del (2076–2078), M694V, M694I, K695R, V726A, A744S, and R761H, respectively. [Except for cloned I692del (2076–2078), which starts after codon 680, all inserts contain the entire sequence of the respective exons 2, 3, 5, or 10]. Strips on the *right* show genomic DNA samples heterozygous for E148Q (*strip 13*), F479L (*strip 15*), I692del (2076–2078) (*strip 18*), M694V (*strip 19*), K695R (*strip 22*), A744S (*strip 24*), and R761H (*strip 25*); compound heterozygous for E148Q + P369S (*strip 14*) and M680I (G/A) + V726A (*strip 17*); and homozygous mutant for M680I (G/C) (*strip 16*), as well as the mutations M694V (*strip 20*), M694I (*strip 21*), and V726A (*strip 23*); wild-type DNA (*strip 26*); and negative PCR control (*strip 27*).

any of the 40 functional *MEFV* mutations currently listed in the INFEVERS (http://fmf.igh.cnrs.fr/infevers/) registry *(4, 12)*.

One of the known limitations of reverse-hybridization is the difficulty to correctly identify and distinguish between two closely spaced mutations. The *MEFV* gene contains at least three hotspots (codons 148, 680, and 694) with more than one mutation known for each, some of which are included in our present assay. Taking two codon 148 mutations (the common E148Q and the rare E148V) as an example, the FMF StripAssay will correctly identify heterozygous and homozygous E148Q but will fail to identify heterozygous E148V, showing a wild-type staining pattern, as with any other mutation not covered. A patient compound heterozygous for E148Q/E148V will be typed E148Q homozygous (E148Q mutant signal present, wild-type missing), whereas in the rare incidence of homozygous E148V, no interpretation for this locus will be possible because both codon 148 signals will be missing. As shown here for M680I (G/C) and M680I (G/A), as well as M694V and M694I, technical solutions to this problem exist, but difficulties will increase with the number of closely spaced sequence variants. It is important to keep in mind, however, that any method based on the binding of oligonucleotides or restriction enzymes directly to mutation sites (e.g., ARMS, RFLP, LightCycler, and TaqMan) will be impaired in a similar fashion by the existence of multiple adjacent mutations *(13)*.

The entire FMF StripAssay procedure, starting from blood sampling to reading final results, requires <6 h at costs of approximately €45 (US$45.00) per sample, follows a simple protocol, and relies only on commonly available instrumentation, such as a thermocycler and waterbath. For increased sample throughput, hybridization/detection may be fully automated with use of commercially available instrumentation (e.g., profiBlot IIT). Very small amounts (~50 ng) of DNA are sufficient for comprehensive mutation analysis, and the current type of teststrips can easily be extended to include additional mutations.

In conclusion, the FMF StripAssay provides a fast, easy-to-perform, and reliable firstline screening method to identify common *MEFV* mutations, and if it were used in combination with a second, more comprehensive technique (e.g., DNA sequencing), very efficient genotyping could be performed.

Table 1. *MEFV* genotypes and allele frequencies among 199 Austrian FMF patients.

A. Genotypes

Genotype		
Allele 1	**Allele 2**	**No. of patients**
E148Q	P369S	1
E148Q	M680I (G/C)	2
E148Q	M694I	2
E148Q	M694V	2
E148Q	V726A	1
E148Q	R761H	1
E148Q	wt[a]	11
P369S	wt	1
M680I (G/C)	M680I (G/C)	6
M680I (G/C)	M694V	18
M680I (G/C)	R761H	3
M680I (G/C)	wt	2
M694V	M694V	30
M694V	V726A	11
M694V	R761H	3
M694V	wt	10
V726A	wt	3
R761H	wt	2
wt	wt	90
Total		199

B. Allele frequencies

Mutation	**No. of alleles**	**Frequency**
E148Q	20	0.050
P369S	2	0.005
M680I (G/C)	37	0.093
M694I	2	0.005
M694V	104	0.261
V726A	15	0.038
R761H	9	0.023
Total mutant	189	0.475
Wild type	209	0.525
Total	398	1.000

[a] wt, wild type.

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