LABORATORY NOTE

Development and validation of a screening test for 12 common mutations of the cystic fibrosis CFTR gene

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ABSTRACT: The results obtained using deoxyribonucleic acid (DNA) amplification-based tests must be accurate and reproducible. One such test that simultaneously detects any of 12 of the most common mutations of the cystic fibrosis transmembrane conductance regulator gene is presented in this report.

An investigation was conducted into how changes of primer, DNA template and *Taq* DNA polymerase concentrations and of polymerase chain reaction annealing temperatures affect the test. A total of 383 DNA samples obtained from different laboratories was then examined.

The preliminary studies defined the conditions under which accurate results are obtained even if the test is performed under suboptimal conditions. Subsequently, 377 (98.4%) of the DNA samples analysed were in full agreement with DNA typing results derived by other methods. The remaining 1.6% of samples were not mistyped, rather they were not scored owing to failure to detect control DNA sequences. These were also archival DNA preparations rather than freshly prepared samples from venous blood.

Careful primer design and optimization of reaction conditions are important in the development of multiplex deoxyribonucleic acid amplification-based diagnostic tests. Providing the recommended protocols are followed, the test described here is simple to carry out, gives accurate results and works well if performed within defined operational windows for each reaction variable.

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Cystic fibrosis (CF) is the most common fatal autosomal recessive disease affecting Caucasian populations. CF has an incidence of 1 in 2,000–3,000 births depending on the population group [1] and this indicates a carrier frequency of around 1 in 25. The prognosis for an affected child with CF is a median life expectancy currently estimated to be 40 yrs [2]. Since the discovery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene [3] over 600 CF associated mutations have been identified.

The Amplification Refractory Mutation System (ARMS™) is an established technique that enhances the utility of the polymerase chain reaction (PCR) for the detection of mutations and other polymorphisms in deoxyribonucleic acid (DNA) [4]. ARMS also provides the means to distinguish between homozygotes and heterozygotes for any given allelic variation. The principle of the method is that under appropriate conditions an oligonucleotide which is not matched to target (genomic) DNA sequence at its 3'-end will not be extended by *Taq* DNA polymerase. The 3'-end of an ARMS primer therefore confers its allele specificity. Hence, an ARMS product is only generated if the primer

is complementary to its target at the 3'-end. The present authors [5] and others [6, 7] have described the use of ARMS to test for $\Delta F508$, the most common mutation of the CFTR gene [3]. It has also been shown that four ARMS analyses can be performed simultaneously by multiplexing allele-specific primers [8] and these observations have been confirmed by FORTINA *et al.* [9].

Here, the development and validation are described of a two-tube multiplex ARMS test, the CF(12)m test, which detects 12 of the most prevalent CF mutations [10]. The CFTR gene mutations that are detected by the test are 1717-1G>A, G542X, W1282X, N1303K, $\Delta F508$, 3849+10kbC>T, 621+1G>T, R553X, G551D, R117H, R1162X and R334W, which are described by KAZAZIAN [10] and papers cited therein. The test also distinguishes between CF $\Delta F508$ heterozygotes and homozygotes. The CF Genetic Analysis Consortium data [10] allow the minimum detection capability of the test to be calculated by country, as shown in table 1. Similarly calculated continental values are shown in table 2. A further observation from these data is the very high minimum detection capability for Ashkenazic Jews, which is calculated to be 95.4%.

Table 1. – Minimum detection capabilities of the CF (12)m Amplification Refractory Mutation System test

Country	CF chromosomes %
Albania	75.0
Austria	72.2
Belgium	84.5
Bulgaria	67.1
Czech Republic	80.6
Denmark	91.0
France	78.8
Germany	78.6
Hungary	73.0
Greece	68.7
Ireland	79.1
Italy	60.3
Netherlands	83.5
Poland	65.0
Portugal	48.4
Russia	53.4
Slovenia	69.1
Spain	63.2
Sweden	56.0
Switzerland	80.3
UK	81.3

CF: cystic fibrosis.

Materials and methods

DNA samples

The panel of samples was selected to include normal DNA, DNA from CF $\Delta F508$ homozygotes and several examples of each mutation that the test was designed to detect. All DNA samples were prepared from EDTA-treated blood. DNA samples from external sources were prepared and typed independently using standard, recognized procedures and typed by the methods outlined in table 3. DNA samples from CF unaffected individuals were prepared as described previously [8].

Primer design

In designing the ARMS primers it was important to ensure that a false result could not arise through other DNA sequence variations at the same site or in the vicinity of the mutations for which tests were being conducted. For example, $\Delta F508$ and non- $\Delta F508$ alleles should not be confused with either the mutant $\Delta I507$ or the benign F508C allele. This discrimination was achieved after particular consideration of the orientation of each primer with respect to the direction of transcription of the CFTR gene. Careful attention was also given to the inclusion of additional base-pair mismatches between each primer and the genomic DNA sequence, as well as the length and concentration of each primer, as described previously [4, 8]. The nucleotide sequence for each of the primers in the CF(12)m kit is described elsewhere [11]. In combining primers for multiplex analysis, many primers are included in one reaction mix. It was, therefore, necessary to minimize any primer interactions that might affect the test performance.

Table 2. – Minimum detection capabilities of the CF(12)m Amplification Refractory Mutation System test

	Total CF chromosomes screened	R117H	621+1G>T	R334W	$\Delta F508$	1717-1G>A	G542X	G551D	R553X	R1162X	3849+10kb C>T	W1282X	N1303K	Minimum detection capability %
Europe, North	21154	62	97	18	14866	160	439	356	165	36	23	120	209	78.2
Europe, South	7281	3	37	21	4007	65	259	37	44	68	8	43	179	65.5
America, North	10438	61	154	12	6900	44	234	206	96	19	57	245	130	78.2
America, South/ Central	758				342		38	1	5				11	52.4
Australasia	3095	7	27	2	2309	12	56	117	11	2		6	23	83.1
Asia, mainly	608	0			173	3	27	0	0			120	29	60.5
Middle East														
Africa	515				351		9		1		16	2	8	72.0
Totals	43849	133	315	53	28948	284	1062	717	322	125	104	536	589	75.7

Analysis by continent was derived from data in [10]. Empty cells indicate that not all centres screened for each mutation detected by the test; therefore, the actual detection capabilities for some mutations may be higher. CF: cystic fibrosis.

Table 3. – Analysis of the 754 chromosomes tested

Mutation	Independent typing method*	Totals
1717-1G>A	ASO	16
G542X	ASO	10
W1282X	ASO	16
N1303K	ASO	12
ΔF508	Electrophoresis	89
3849+10kbC>T	Digest (<i>Hph</i> I)	11
621+1G>T	Digest (<i>Mse</i> I)	7
R553X	Digest (<i>Hinc</i> II)	15
G551D	Digest (<i>Nde</i> I)	16
R117H	ASO	13
R1162X	Digest (<i>Dde</i> I)	11
R334W	Digest (<i>Msp</i> I)	6
Other/none		532
Number of samples		377
Total number of chromosomes		754

*: Confirmatory typing as detailed in references cited within [10]. ASO: allele-specific oligonucleotide hybridization.

Test design

The CF(12)m test consists of two tubes, the A-tube and the B-tube. The A-tube contains ARMS primers specific for the 1717-1G>A, G542X, W1282X, N1303K, ΔF508 and 3849+10kb C>T mutations. The B-tube contains ARMS primers specific for the 621+1 G>T, R553X, G551D, R117H, R1162X and R334W mutations. The B-tube also contains an ARMS primer specific for the normal ΔF508 allele. There are also two control reactions in each tube. One amplifies a region of the apolipoprotein (apo)B gene, while the other amplifies a region of the ornithine decarboxylase (ODC) gene. The apoB reaction is designed to amplify less efficiently than the ARMS reactions, ensuring that this control reaction will fail preferentially if PCR variables are not within the defined tolerances of the test. The apoB control, therefore, safeguards against a false-negative result if the test is performed under sub-optimal conditions. The concentrations of the component ARMS primers in each tube were adjusted to amplify their respective targets with equal efficiency. The remaining constituents of each tube comprise ARMS buffer; 10 mM Tris-HCl, (pH 8.3) 1.2 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin and deoxynucleoside triphosphates (dNTPs) (100 μM each). Genomic DNA and *Taq* DNA polymerase (Ortho Clinical Diagnostics) are added at the start of the test.

Test method and analysis

The test was performed by adding genomic DNA prepared from EDTA-treated blood to each tube, which was then heated at 94°C for 5 min. *Taq* DNA polymerase (2.5 units) was added to each tube and thermal cycling (35 cycles of 94°C for 1 min, 58°C for 2 min and 72°C for 1 min, with a final extension at 72°C for 10 min) was initiated. The amplification products were separated by agarose gel electrophoresis against a 50 base-pair (bp) DNA ladder marker (Pharmacia Biotech, St. Albans, UK) and visualized by ultraviolet (UV) transillumination. All procedures generally accepted for avoiding PCR carryover

Fig. 1. – Diagrammatic representation of electrophoretic mobilities of the Amplification Refractory Mutation System (ARMS), control and size marker gel bands. The respective ARMS and control bands are labelled and all band sizes (base pairs (bp)) are shown in parentheses. The 250 bp marker band (■) is more intense to provide a reference position on the gels. In samples that do not contain any of the mutations detected by the test, only the apolipoprotein (apo)B and ornithine decarboxylase (ODC) control bands (both tubes) and ΔF508 normal band (B-tube) will be visible (■).

contamination [12] were employed during the development and validation of the test and are recommended when using the test. The presence of the control products and a specific ARMS product, defined by electrophoretic mobility, is diagnostic of the presence of the respective mutant allele and/or normal ΔF508 allele. The output of the CF(12)m test is shown in figure 1.

Results

A series of experiments was performed to determine the tolerance of the CF(12)m ARMS test system to variation in primer concentrations, annealing temperatures, *Taq* DNA polymerase concentration, input DNA amount, and the method of its extraction. This served to demonstrate the permissible limits of each test variable and to confirm that a nonresult or test failure, rather than an incorrect result, was observed should any variable fall outside defined tolerances. The CF(12)m test was functional at ±2°C of the standard annealing temperature (58°C). Under sub-optimal conditions (3°C above the standard temperature) all diagnostic product bands were visible but the upper control product band was absent. This feature of the test prevents the occurrence of false-negative results due to operation at higher temperatures. The results demonstrate that the test remains functional over a broad range of annealing temperatures (fig. 2). The effects of the primer, *Taq* DNA polymerase and DNA concentrations were assessed in an analogous manner. This defined the respective

Fig. 2. – Effect of annealing temperature on the CF(12)m test function in a) A-tube and b) B-tube reactions performed at 56, 58 (standard), 60 and 61°C. 1: 1717-1G>A/+; 2: G542X/+; 3: W1282X/+; 4: N1303K/+; 5: Δ F508/+; 6: 3849+10kbC>T/+; 7: +/+; 8: +/+; 9: Δ F508/ Δ F508; 10: 621+1G>T/+; 11: R553X/+; 12: G551D/+; 13: R117H/+; 14: R1162X/+; 15: R334W/+; 16: +/+; 17: +/+; 18: Δ F508/ Δ F508; 19: Δ F508/+.

windows within which both of the control bands and each mutation-specific band were appropriately generated. Typical test results are shown in figure 3.

DNA samples ($n=383$) prepared from EDTA-treated blood were analysed using the CF(12)m test, of which 377 gave results in agreement with those obtained independently. Six samples (1.6%) were not scored due to the absence of one or both control bands. These were all archival DNA preparations known to be low concentration, and degradation of these samples could not be eliminated. Where rare non- Δ F508 compound heterozygotes

have been obtained (3849+10kbC>T/W1282X; 3849+10kbC>T/G542X; G542X/N1303K; G542X/W1282X; G551D/R553X; N1303K/1717-1G>A; G542X/17171G>A; N1303K/W1282X; R553X/R334W) and analysed, both mutations were correctly identified. Similarly, all Δ F508 compound heterozygotes ($n=9$) were correctly typed. There was no mistyping when Δ I507, 1717-2A>G, R1283M, R117C, 3617G/T, 621+2T>C or F508C alleles were present. These data demonstrate that, for the clinical material available, one allele did not fail to amplify when combined with another in the same ARMS tube and one allele did not give rise to artifactual products derived from alternative allele-specific primers. Table 3 provides an analysis of the 754 chromosomes typed using the CF(12)m ARMS test.

Discussion

Early diagnosis followed by expert management has resulted in an improved prognosis for CF patients. This has led to the evaluation of neonatal screening protocols for CF. There is also a common objective of identifying carriers and ultimately those couples that are at risk of having a child affected with CF, which is shared by "Active cascade" [13], "Couple" [14–17] and "Two-step" or "Stepwise" [15, 17] screening protocols. Couple screening has been identified as medically the screening method of choice [17] and it has recently been established that CF carrier testing is acceptable to patients and can be successfully integrated into the antenatal booking appointment in general practice [18].

The risks associated with *in vitro* fertilization have also been recognized. Up to ten offspring may be fathered by one sperm donor. If a donor is a CF carrier there will be a higher than normal chance of at least one of the offspring being affected by the disease. For this reason it was recommended that sperm donors should be tested for mutations in the CFTR gene [19].

Fig. 3. – a) Typical CF(12)m test results showing all mutations detected. 1: +/+; 2: 1717-1G>A/+; 3: G542X/+; 4: W1282X/+; 5: N1303K/+; 6: Δ F508/+; 7: 3849+10kbC>T/+; 8: 621+1G>T/+; 9: R553X/+; 10: G551D/+; 11: R117H/+; 12: R1162X/+; 13: Δ F508/ Δ F508; 14: R334W/+. b) Typical results obtained from 11 of the 383 of the CF(12)m test trial samples. 15: +/+; 16: +/+; 17: R553X/+; 18: +/+; 19: Δ F508/+; 20: +/+; 21: +/+; 22: R117H/ Δ F508; 23: Δ F508/ Δ F508; 24: +/+; 25: G542X/N1303K; 26: no deoxyribonucleic acid (DNA) control. M: a 50 base-pair DNA ladder marker.

The primary application of the CF(12)m test is for screening individuals who may be carriers of CF and so can be applied in meeting the requirements outlined above. The CF(12)m test screens for the CF mutations 1717-1G>A, G542X, W1282X, N1303K, Δ F508, 3849+10kbC>T, 621+1G>T, R553X, G551D, R117H, R1162X and R334W, the most common CF mutations in Caucasians and Ashkenazi Jews. In addition, the kit provides genotypic information for the Δ F508 mutation. In Europe, CF individuals who are non- Δ F508 CF compound heterozygotes are rare. As a consequence it has not been possible to analyse for all mutations in combination in the same test. However, nine compound heterozygotes have been sourced and analysed. In each of these cases and in all Δ F508 CF compound heterozygotes both mutations were correctly identified, clearly demonstrating the diagnostic value of the test.

There is a clear difficulty in distinguishing particular alleles in the routine genetic screening laboratory [20]. CUPPENS and CASSIMAN [20] demonstrated that 12.5% of laboratories mistyped the F508C polymorphism as a true mutation and that 12.5% confused the Δ I507 mutation for Δ F508. One conclusion of that study was that the accuracy of CFTR typing should be improved [20]. The CF (12)m test was designed such that a false result does not arise because of other DNA sequence variations in the vicinity of the mutations tested. This was achieved through the design and choice of transcriptional orientation of each respective ARMS primer. The CF(12)m test discriminates accurately between Δ F508 and non- Δ F508 alleles which, in turn, are distinguished from the mutant Δ I507 and the benign F508C alleles, although the mutant Δ I507 allele is not detected using the kit. This addresses the problems associated with typing particular alleles identified by CUPPENS and CASSIMAN [20]. Furthermore, the finding from another study [15], which compared multiplex ARMS screening for the Δ F508, G551D, G542X and 621+1G>T alleles [8] with alternative routine procedures for the same alleles, was that multiplex ARMS was the preferred method.

Standardization of all test procedures and implementation of appropriate quality-control measures can control for some test variables and it is important to understand which ones are likely to affect test performance. The annealing temperature is one key parameter that can affect the performance of any PCR or ARMS reaction. If the reaction temperature falls below the required annealing temperature it is likely that reaction specificity will be affected. Similarly, if the annealing temperature is higher than that required the efficiency of the ARMS reaction may be reduced, resulting in PCR failure. There is expected to be some degree of variability between the temperature accuracy and uniformity exhibited by different thermal cyclers. Therefore, the temperature range within which all the component reactions of the test would function (at least $\pm 2^\circ\text{C}$) was established. All thermal cyclers currently available commercially are claimed by their manufacturer to have a temperature accuracy of $\pm 1^\circ\text{C}$ or better. Similarly, the range within which other parameters can vary without adversely affecting test function was determined and, thus the tolerances within which the kit must be assembled have been established. The two key variables that can be influenced by the kit user are the annealing temperature of the ARMS reaction and the amount of the *Taq* DNA polymerase added to individual reaction

mixes. The function of the test when the upper and lower limits of these variables are applied in concert was addressed. From these studies, even with low enzyme and high annealing temperature and *vice versa*, the control amplifications were produced and the test did not give false results using the present panel of DNAs.

It can, therefore, be concluded that the CF(12)m test is reliable and robust and is unaffected by external variations so long as the kit instructions and recommendations are adhered to. If a reaction should fail for any other reason that is not addressed by the parameters investigated herein, a misdiagnosis would still not occur since a control amplification would be eliminated prior to any Amplification Refractory Mutation System amplification. This feature emphasizes the importance of the internal controls and their role in avoiding misdiagnoses. Six archival deoxyribonucleic acid samples failed to produce one or both control bands; these may have been degraded and were not scored. Because the CF(12)m test is primarily a screening test, by implication deoxyribonucleic acid used in the test will be freshly prepared. The age and degradation of a sample should therefore not be a problem during the test's routine application.

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The PCR process is covered by patents held by Hoffmann-La Roche Inc.

ARMS™ is the subject of European Patent No. 0 332 435 (Zeneca Ltd) and corresponding patents worldwide. The CF(12)m test has been developed by Zeneca Diagnostics for Ortho Clinical Diagnostics Ltd and is the subject of UK Patent Application No. 9800536.6 (Zeneca Ltd).

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