

USE OF PCR FOR THE DETERMINATION OF THE FREQUENCY OF THE $\Delta F508$ MUTATION IN BRAZILIAN CYSTIC FIBROSIS PATIENTS

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The $\Delta F508$ mutation in the cystic fibrosis (CF) gene was studied in a population of 18 Brazilian CF patients and their 17 families by use of PCR and differential hybridization with oligonucleotides. In a total of 34 chromosomes considered, 12 (35%) carried the F508 deletion, a frequency much lower than that reported in most other populations. As a consequence, CF in Brazil would be predominantly caused by mutations different from the F508 deletion.

Key words: cystic fibrosis – PCR – $\Delta F508$ – diagnosis

Cystic fibrosis (CF) is the most common recessive inherited lethal disorder among Caucasians, occurring with a frequency of about 1 in 2000 live births (Rommens et al., 1989). The gene involved in cystic fibrosis codes for the CF transmembrane conductance regulator (CFTR), a 168 KD protein of 1480 amino acids (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989). Over 100 distinct CF mutations have been identified and the frequency of the most prominent mutation, $\Delta F508$, present in the majority of CF cases in Caucasian populations, has been analyzed and presented in many reports (Romeo & Devoto, 1990). So far, only preliminary data are available on the frequency of the $\Delta F508$ mutation in Brazilian CF patients. We have analyzed 17 different families comprising 18 CF patients (including two sibs) and 35 relatives with the PCR technique and differential hybridization. Data on the frequency of $\Delta F508$ in Brazilian patients is presented.

MATERIALS AND METHODS

Patient information – All index CF patients were selected by a positive sweat test with the exception of three patients, whose electrolytic

sweat concentration values fell on the borderline of the sweat test. One family had two affected sibs. Patients were of mixed race, descendent from Latin-Europeans, Brazilian natives and negros. The clinical description of each case will be reported elsewhere (manuscript in preparation).

DNA extraction – 10 ml of fresh blood was mixed with 1.7 ml of ACD solution (23 mM citric acid; 45 mM sodium citrate; 50 mM glucose) to prevent coagulation. Erythrocytes were lysed by the addition of 35 ml of lysis buffer (155 mM NH_4Cl ; 10 mM KHCO_3 ; 1 mM EDTA; pH 7.4) and incubation on ice for 30 min. The solution was centrifuged for 10 min at 700xg, the supernatant was discarded and the pellet rinsed with lysis buffer. The pellet was resuspended in 3 ml of nuclei lysis buffer (10 mM Tris-HCl; 400 mM NaCl; 2 mM EDTA; pH 8.2), and 150 μl of 20% SDS and 100 μl of protease type I (20 mg/ml; Sigma Inc., St. Louis, USA) was added. After careful mixing, the resulting solution was further incubated at 37 °C for 16 hr, followed by the addition of 1 ml of saturated NaCl (6M), vigorous shaking for 15 sec and centrifugation at 12000xg for 15 min. This step was repeated until the supernatant was clear. DNA in the supernatant was precipitated with two volumes of ethanol at room temperature and redissolved in 0.4 to 1 ml of Tris-TE buffer (10 mM Tris-HCl; 0.1 mM EDTA; pH 7.0), and incubated for 30 min at 65 °C to eliminate any residual

This work received financial support from CAPES and CNPq.

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Received 6 October 1992.

Accepted 18 February 1993.

DNase activity. DNA was dissolved for 16 h at room temperature and stored at 4 °C.

DNA amplification – Oligonucleotides were synthesized in our laboratory on a DNA synthesizer model 381A (Applied Biosystems, Foster City, USA) using the beta-cyanoethylphosphoramidite chemistry. Primers used for amplification were: A1 (GTTTTTCCTGGATTACCTGGCAC) and A2 (GTTGGA-TGCTTTGACGCTTC) (Kerem et al., 1989). PCR amplification of DNA samples was performed in a DNA Thermal Cycler model 480 (Perkin Elmer Cetus, Norwalk, USA). Hundred to 300 ng of DNA was amplified after the addition of 2.5 U Taq polymerase (Stratagene, La Jolla, USA), 200 ng of each oligonucleotide, 200 µM of each deoxyribonucleotide triphosphate, 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin and 0.1% Triton X-100 (Sigma Inc.) in a total volume of 50 µl. Amplification occurred by initial denaturation at 92 °C for 1 min; followed by 30 cycles of 2 min at 50 °C, 2 min at 72 °C and 1 min at 92 °C and a unique final step of 7 min at 72 °C. One fifth of the amplified material was analyzed on a gel containing 2% Nusieve (FMC BioProducts, Rockland, USA) and 1% agarose (Sigma Inc.).

Transference to membrane and hybridization – NaOH was added to one fifth of the amplified DNA (10 µl) to a final concentration of 0.4 N; samples were heated for 5 min at 95 °C and put on ice. Samples were applied in duplicate to nylon membranes (Bio-Rad, Richmond, USA) using a DOTBLOT apparatus (Minifold, Schleicher and Schuell, Dassel, Germany). After application of the DNA, membranes were rinsed with 2X SSC (0.03 M sodium citrate; 0.3 M NaCl) and dried at 80 °C for 30 min. After incubation for 3 hr at 37 °C in 20 ml of pre-hybridization solution (6X SSC; 1 mM EDTA; 1X Denhardt), an oligonucleotide H1 (AACACAATGATATTTT-CCTTT) complementary to the normal gene, and an oligonucleotide H2 (CACCAAAGAT-GATATTTTC) complementary to the gene carrying the F508 deletion (Kerem et al., 1989) were each hybridized to the amplified DNA on a separate membrane in pre-hybridization

solution for 16 hr at 37 °C. Oligonucleotides were radiolabeled by kination with gamma-32p-ATP (ICN Inc., Irvine, USA) to a specific activity of 1.5 x 10⁸ cpm/µg. Approximately 10⁶ cpm/ml were added for hybridization. After this, membranes were washed twice with 5X SSC containing 0.1% SDS at room temperature, followed by two washings with 2X SSC containing 0.1% SDS at 39 °C. Hybridization was visualized by autoradiography.

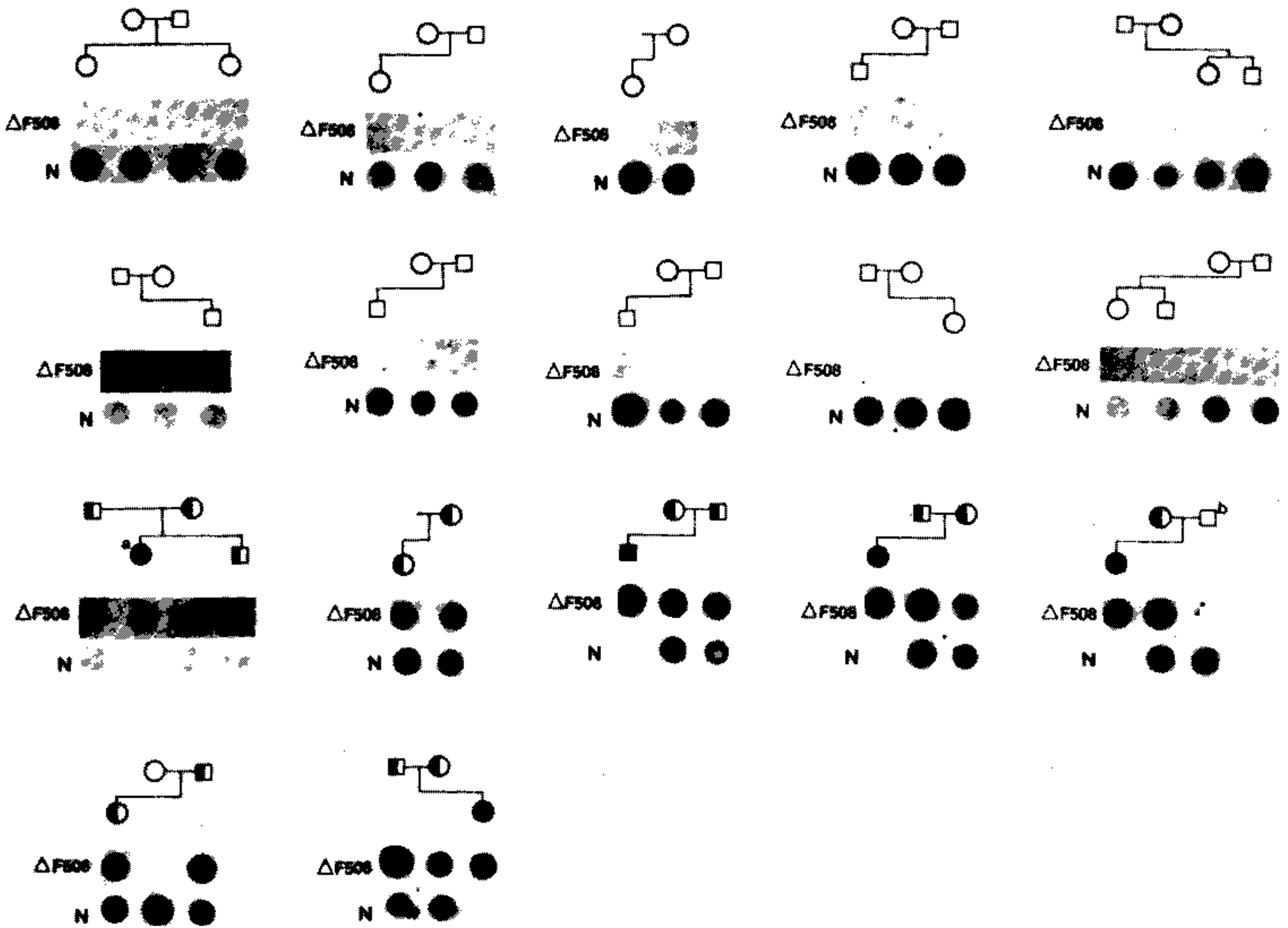
RESULTS AND DISCUSSION

DNA samples from 53 individuals from 17 CF families were studied by PCR to verify the frequency of the ΔF508 mutation. The figure shows a graphical representation of the segregation of ΔF508 and non-ΔF508 alleles in these families, corresponding to the dot-blot hybridization results of oligonucleotides H1 and H2 to amplified DNA.

The frequency of ΔF508 was calculated from its presence in 17 CF patients. Five patients (29%) were homozygous for ΔF508, two (12%) were heterozygous and 10 patients (59%) did not have the F508 deletion. From a total of 34 chromosomes analyzed, 12 carried and 22 did not carry the ΔF508 mutation. The frequency of the ΔF508 mutation per chromosome 7 was therefore 0.35, with a variance of 0.0067, a standard deviation of 0.08, a related error of 0.16 and a minimal and maximal frequency of respectively 0.27 and 0.43.

Our results suggest that, at least in the region of Rio de Janeiro, with a quite heterogeneous population, the ΔF508 mutation is present only in 35% of the chromosomes of the CF patients. This frequency is much lower than that found in most countries (Romeo & Devoto, 1990), frequencies in southern Europe being approximate those found in our study*. Only Israeli and Turkish CF patients show frequencies of ΔF508 that are lower than those found in our population (32 and 27% respectively; Lerer et al., 1990; Hundrieser et al., 1990). Preliminary results have suggested some geographic differences in the frequency of the F508 deletion in Brazilian CF alleles (Raskin et al., 1991). Altogether, this means that in Brazil, non-ΔF508 mutations are more frequently responsible for the manifestation of CF. Considering the fact that the ΔF508 mutation is recessive, and that no dominant CF mutation has been found so far, the total frequency of non-ΔF508 mutations should be at

*European Working Group on CF Genetics, 1990. Gradient of distribution in Europe of the major CF mutation and its associated haplotypes. *Hum. Genet.*, 85: 436-445.



Graphical representation of the segregation of 17 CF families. One family contained two affected sibs^a; male and female subjects are represented respectively by a circle and a square. DNA was applied on a membrane in duplicate and the membranes were hybridized either with a probe specific for the gene carrying the mutation ($\Delta F508$), or with a probe hybridizing with the gene that is not carrying the F508 deletion (N). Individuals, homozygous for $\Delta F508$ or for N are represented by a black or white symbol, respectively, while heterozygous persons are represented by a half-filled symbol. The mutation inheritance in one family had no straightforward explanation^b.

least 65%. We can not exclude that in Brazilian CF patients other unknown (eventually dominant) mutations will be found. This complicates prenatal diagnosis with PCR, as family informativity by analysis of $\Delta F508$ becomes low. An analytical approach, using PCR-mediated detection of non- $\Delta F508$ mutations has been reported recently (Estivill et al., 1991), and can be used to improve genetic counseling in couples from regions with low $\Delta F508$ profiles.

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