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DNA Analysis of Cystic Fibrosis in Brazil by Direct PCR Amplification From Guthrie Cards

S. Raskin, J.A. Phillips III, M.R.S. Krishnamani, C. Vnencak-Jones, R.A. Parker, T. Rozov, J.M. Cardieri, P. Marostica, F. Abreu, R. Giugliani, F. Reis, N.A. Rosario, N. Ludwig, and R.F. Pilotto

Vanderbilt University, Nashville, Tennessee (S.R., J.A.P., M.R.S.K., C.V.-J., R.A.P.); Universidade de São Paulo, São Paulo (T.R., J.M.C.), Universidade Federal do Rio Grande do Sul, Porto Alegre (P.M., F.A., R.G.), Universidade Federal de Minas Gerais, Belo Horizonte (F.R.), Universidade Federal do Parana, Curitiba (S.R., N.A.R., R.F.P.), and Hospital Joana de Gusmao, Florianopolis (N.L.), Brazil

A 3 bp deletion of codon 508 (phenylalanine) of the cystic fibrosis (CF) gene constitutes the mutation of most CF chromosomes. The frequency of this mutation (referred to as ΔF508), varies considerably between populations, ranging from 26% of the CF mutations in Turkey to 88% in Denmark. To determine the frequency of the ΔF508 mutation in Brazilian Caucasoid CF patients, we used direct polymerase chain reaction (PCR) amplification of DNA obtained from dried blood spots on Guthrie cards, followed by ethidium bromide staining of gels. Although the overall frequency of the ΔF508 mutation was 47% of 380 CF chromosomes from Brazilian Caucasoids born in five different states, significant interstate differences were observed, ranging from a ΔF508 frequency of 27% to 53%. While our method could be used to screen patients and their relatives for carrier testing and prenatal diagnosis, the efficacy of screening only for the ΔF508 mutation would be low, and would vary from state to state. Screening for a panel of local mutations will be needed to increase the mutation detection rate and optimize genetic counseling.

KEY WORDS: Brazil, cystic fibrosis, ΔF508 deletion, PCR, Guthrie cards

INTRODUCTION

A worldwide survey found that 9,027/13,291 (68%) of CF chromosomes have the ΔF508 mutation, a 3 bp deletion causing the loss of phenylalanine residue at position 508 in its tenth exon [Riordan et al., 1989; Rommens et al., 1989; Kerem et al., 1989]. Interestingly, the frequency of the ΔF508 mutation differs between populations, ranging from as low as 26% of the CF alleles in Turkey to as high as 88% in Denmark [Tsui, 1990]. Since local and regional differences in the frequency of ΔF508 affect the false negative rates that arise when ΔF508 analysis is used for the diagnosis of CF and CF carrier testing, such data should be derived for each country.

Brazil has a population of approximately 146 million people [Brazilian Census, 1991], making it the sixth most populous country in the world. The five large regions of Brazil (North, Northeast, Southeast, South, and Center-West) vary in racial background, which includes European, Black, and Amerindian groups. Caucasoids make up 54% of the total population, mixed 39%, blacks 6%, and Asian 1%. Brazil was conquered and colonized by Portugal in 1500, but the major European migration flow started in 1802 and decreased dramatically after 1950. Eighty percent of the Caucasoid immigrants came from Portugal, Italy, Spain, and Germany. Although this racial group is ethnically heterogeneous and race admixture was widespread, European descendants relatively free from admixture are found primarily in the Southeast and South [Salzano and Freire-Maia, 1967; Salzano and Pena, 1987]. To determine the frequency of the ΔF508 mutation in these 5 states of Southeast and Southern Brazil, 190 Brazilian CF patients were genotyped by direct PCR amplification of DNA obtained from dried blood spots on Guthrie cards.

MATERIALS AND METHODS

Subject Population

Mutation analyses were done on samples from 190 nonrelated Caucasoid CF subjects from 5 different states of Southeast and Southern Brazil [60 subjects were born in Rio Grande do Sul (RS), 24 in Santa Catarina (SC), 17 in Parana (PR), 58 in São Paulo (SP), and 31 in Minas Gerais (MG)] (Table I). The subjects were 54% male and the mean subject age was 6.7 years, ranging from 2 months to 32 years. All patients and almost all parents were born in Brazil and most are members of the first four generations of immigrants.

These five states well represent the Brazilian Cau-

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Address reprint requests to Salmo Raskin, M.D., Rua Martin Afonso, 1200, ap. 9, Curitiba, Parana, Brazil 80430-100.

TABLE I. ΔF508 Mutation in CF Subjects From Different Populations

	Chromosomes	ΔF508 (%)	CF Genotypes		
			ΔF508/ΔF508	ΔF508/Non-ΔF508	Non-F508/Non-ΔF508
State					
RS	120	49	.32	.35	.33
SC	48	27	.08	.38	.54
PR	34	44	.18	.53	.29
SP	116	52	.24	.55	.21
MG	62	53	.35	.35	.30
Total	380	47	.26	.43	.31
Country					
Brazil[a]	380	47			
U.S.A.[b]	439	76			
England[c]	224	81			
Portugal[c]	84	53			
Spain[c]	466	51			
Italy[c]	122	47			
Argentina[d]	92	62			

[a] Data from this study.
[b] Lemna et al. [1990].
[c] Tsui [1990].
[d] Pivetta and Olek [1992].

casoid population because demographic data [Brazilian Census 1989 and 1991] show that: (1) they comprise almost 50% (70 million) of the Brazilian population, (2) their white population comprises about 65% of all Caucasoids in Brazil, and (3) in all but one (MG) > 75% of the population is comprised of European descendants relatively free from admixture.

Each of these states are served by well-established CF regional centers. The 190 CF subjects studied comprise around 80% of all living patients with CF referred to regional reference centers in the entire country [Macri et al., 1992].

Criteria for Diagnosis of CF

Criteria for diagnosis included clinical findings of chronic pulmonary disease and positive iontophoresis. Patients were characterized as pancreatic sufficient if the stool fat content was normal without oral enzyme replacement. The presence or absence of meconium ileus (MI) was determined by review of medical records and questionnaires. The clinical evaluation was carried out from 1990 to 1992. Case reports of each patient were evaluated carefully for presence or absence of MI, sweat chloride concentration values, age of diagnosis, age of onset of first symptoms, and current age. Age at onset was defined as the age at the start of characteristic symptoms of CF (mainly respiratory and gastrointestinal, including MI), as evaluated by careful retrospective analysis of the case report; this analysis was done without prior knowledge of the DNA typing results.

Blood Samples

Blood samples were collected by fingerstick on Guthrie cards. Care was taken to avoid contamination of samples by use of gloves and plastic bags to separate samples during collection, mailing and analysis.

DNA Analysis

Following receipt of filter papers, a 1 mm^2 piece was cut from each filter and added to a 50 μl reaction mixture containing 200 pM of each oligonucleotide primer (5′GTTTTCCTGGATTATGCCTGGCAC3′ 5′GTTGGCATGCTTTGATGACGCTTC-3′) [Kerem et al., 1989] 10 mM Tris (pH 8.0); 50 mM KCl, 1.5 mM $MgCl_2$; 0.001% gelatin and 200 mM of each deoxynucleotide triphosphate [Saiki et al., 1988]. The contents of the reaction tube were denatured at 96°C for 3 minutes and annealed at 64°C for 3 minutes 3 times in a Perkin Elmer Cetus Thermocycler. Two units of Taq polymerase were then added to each tube, and one extension cycle was done at 72°C for 1 minute. Next, 33 cycles of PCR amplification consisting of 94°C for 60s, 64°C for 30s and 72°C for 60s were done followed by a 10 minute extension period at 72°C and the products were cooled on ice. Next, 30 μl aliquots of each reaction mixture were subjected to electrophoresis in 8% polyacrylamide gels in 1 × TBE (Tris-Borate-EDTA, pH 8.0) for 2 hours at 400 mV. The PCR products were then visualized by ethidium bromide staining and exposure to u.v. light [Raskin et al., 1992a,b] (Fig. 2).

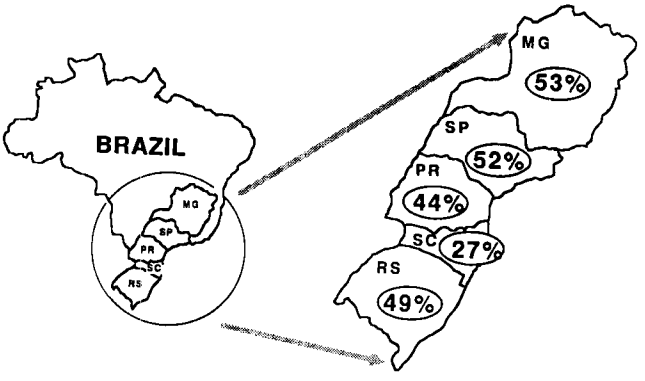

Fig. 1. ΔF508 in different states of Brazil. MG, Minas Gerais; SP, Sao Paulo; PR, Parana; SC, Santa Catarina; and RS, Rio Grande do Sul.

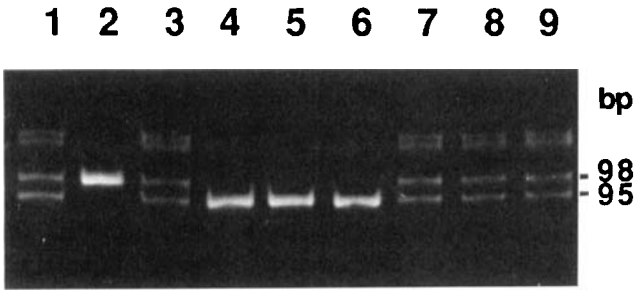

Fig. 2. Ethidium bromide-staining patterns of DNA fragments obtained by PCR amplification of DNA blood spots. Fragment sizes are shown on right. Additional upper fragments seen in heterozygotes are due to heterodimer formation which occurs when different strands of 95 and 98 bp PCR products anneal. 95/95 bp **(lanes 4–6)**: ΔF508/ΔF508. 98/98 bp **(lane 2)**: Non-ΔF508/Non-ΔF508. 98/95 bp **(lanes 1, 3, 7–9)**: ΔF508/Non-ΔF508.

Statistical Methods

We used Chi-square tests for comparing the prevalence of ΔF508 deletions over all states, and Fisher's exact test (two-sided) for comparison between pairs of states, or between the observed prevalence of ΔF508 deletions in Brazil and those of other countries. In order to estimate the overall prevalence of ΔF508 deletion in Brazil, we weighted the observed prevalence in each state by the Caucasoid population of the state based on the 1989 census.

Since continuous variables such as age were not normally distributed, we used nonparametric tests. For comparisons between all three genotypes, we used the Kruskal-Wallis test (the nonparametric equivalent of an analysis of variance), while for comparisons between two genotypes we used the Wilcoxon-Rank Sum test (the nonparametric equivalent of a t-test).

RESULTS

While the ΔF508 deletion was present on 47% (180/380) of all CF alleles examined, its frequency varied (49, 27, 44, 52 and 53%) in patients from RS, SC, PR, SP and MG, respectively (Fig. 1 and Table I). Overall, the rate of ΔF508 deletions in CF cases are significantly different between states ($X^2 = 9.96$ on 4 df, $P = 0.041$). This difference is due to the results in SC. Without SC, the rates in the other four states are not significantly different ($X^2 = 0.89$ on 3 df, $P > 0.8$). The rate of ΔF508 deletion in SC is significantly lower than that in RS, SP, and MG ($P < 0.01$, Fisher's exact test, two-sided, for each comparison). When the prevalence in the individual states is weighted by the white population of the state, the estimated overall prevalence for the five states is 49%. The observed prevalence of ΔF508 deletion is different between Brazil and the U.S. and Brazil and England ($P < 0.0001$ for each), but not between Brazil and Portugal, Spain, Italy, or Argentina ($P > 0.25$ for each comparison).

There were no significant differences between the 3 genotype groups in sweat chloride values at diagnosis, current age, or age of onset of first symptoms, either including or excluding patients with MI. When we excluded patients that presented with MI, there was a significant difference between the mean ages of diagnosis for the three genotypes ($P = 0.023$, Krushal-Willis test). This was due to the difference between the ΔF508 homozygous group (mean = 1.9 years, range 1 month to 4 years) and the group homozygous for the absence of ΔF508 (mean = 4.8 years, range 1 month to 19 years) ($P = 0.011$, Wilcoxon Rank Sum test).

Of the subjects, 28/190 (15%) were pancreatic sufficient (PS) and the remaining 162/190 (85%) were pancreatic insufficient (PI) (Table II). Virtually all of our subjects (98%) who were homozygous for ΔF508 were PI. Twelve of 190 (6%) of the subjects had presented with MI, and all of these 12 were PI. Tabulations of the pancreatic status and meconium ileus and ΔF508 genotypes are shown in Table II.

DISCUSSION

Our observed frequency of the ΔF508 mutation (47%) among Brazilian Caucasoid subjects with CF is lower than that reported for Northern European CF subjects and similar to that seen in Southern European countries and Argentina (Table I) [Tsui, 1990]. These findings agree with the colonization of Brazil through South European immigration.

Our findings of low ΔF508 frequencies compared with Northern European countries and United States, and disparate haplotype distributions [Raskin et al., 1992b, 1993a,b] suggest that significant heterogeneity exists in the Brazilian CF gene pool. This heterogeneity would have important consequences for both genetic counseling and population screening in the Brazilian population. Since only 47% of all CF chromosomes were ΔF508, only 22% of all CF carrier couples would be identified and the utility of screening would be limited. Moreover, there were significant differences between states, with patients born in one state (SC) having only a 27% frequency of ΔF508 among their CF chromosomes. Therefore, further studies to determine what other mutations constitute the Brazilian CF pool are needed to increase the mutation detection rate. Despite this heterogeneity, linkage analysis in families known to be at risk would be

TABLE II. Comparison of Pancreatic Insufficiency (PI), Meconium Ileus (MI), and CF Genotype Percentages

		Genotype (%)		
Subjects	Number	ΔF508/ΔF508	ΔF508/Non-ΔF508	Non-ΔF508/Non-ΔF508
All	190	26	43	31
PI	162	30	44	26
PS	28	3	36	61
MI	12	25	50	25

useful in cases in which direct mutation detection is not feasible.

Interestingly, the frequency of the ΔF508 mutation differed significantly between CF subjects born in Santa Catarina (SC) state as compared to the other states ($P = 0.041$). Several factors could be responsible for this finding: (1) genetic drift; (2) founder effect; (3) internal migration flow; (4) different origin of ancestors; (5) the small size of the sample of chromosomes analyzed from this state; or (6) a combination of these factors. Racial admixture with the nonwhite population is an unlikely explanation of this finding, since SC, having the lowest frequency of ΔF508, has the highest white population percentage (91%) of the entire country as opposed to MG, where only 57% of the population is considered white [Brazilian Census, 1989].

The incidence of CF in Brazil and Latin American is unknown. Considering the heterogeneity of the Brazilian population, and given the different ethnic composition of the North, Northeast, and Center-West populations, one might expect that the frequency of ΔF508 and other CF mutations, as well as the incidence of CF, will vary between different regions of Brazil, as well as in Latin America, due to different patterns of admixture, external and internal migration, and selection pressures. Based on the results of this study, we are now able to determine the frequency of ΔF508 in the general population, and estimate the incidence of CF in Brazil and its different regions. While the heterogeneity demonstrated by this study and on haplotype data indicates that a large and varied number of non-ΔF508 alleles are expected in the CF population, screening close relatives of probands having the ΔF508 mutation should be useful for certain families.

The correlation previously reported between the CF genotype and the pancreatic function phenotype (PS or PI) [Kerem et al., 1990; Borgo et al., 1990] is supported by our finding that 98% of homozygous ΔF508 subjects were PI. The only exception is a 6-year-old boy, who was diagnosed at age one year with pulmonary disease and sweat test of 90 meq/l, but remains PS although being homozygous for ΔF508. This exception may belong to a group of PS patients whose pancreatic function deteriorates over a period of time [Waters et al., 1990] or his PS may result from other genetics and environmental factors. The frequency of MI in our subjects (6%) is similar to that found in other studies [Kerem et al., 1989].

Diagnosis of CF was made almost 3 years earlier in subjects homozygous for ΔF508 than in subjects homozygous for the absence of ΔF508. This confirms previous findings suggesting that the ΔF508 mutation is associated with a more severe CF phenotype [Johansen et al., 1991; Kerem et al., 1990].

The rapid and relatively inexpensive techniques used in this study to determine the ΔF508 genotype utilize dried blood spots from subjects that could not be conveniently sampled otherwise [Raskin et al., 1992]. Further application of these methods could determine the frequency of different mutations and haplotype distributions, estimating the incidence of CF, and screening to identify at risk individuals. Knowledge gained regarding these parameters in Brazil and Latin America would undoubtedly lead to more efficient approaches to diagnosis and counseling of CF.

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REFERENCES

Borgo G, Mastella G, Gasparini P, Zorzanello A, Doro R, Pignatti PF (1990): Pancreatic function and gene deletion ΔF508 in cystic fibrosis. J Med Genet 27:665–669.

Brazilian Census (1989): Instituto Brasileiro de Geografia e Estatistica, Rio de Janeiro, Brasil. Pesquisa Nacional por Amostra de Domicilios, capitulo 22-cor.

Brazilian Census (1991): Instituto Brasileiro de Geografia e Estatistica, Rio de Janeiro, Brasil. X Recenseamento Geral do Brasil-1990-Resultados preliminares. 1:35.

Johansen KH, Nir M, Hoiby N, Koch C, Schwartz M (1991): Severity of cystic fibrosis in patients homozygous and heterozygous for ΔF508 mutation. The Lancet 337:631–634.

Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC (1989): Identification of the cystic fibrosis gene: Genetic analysis. Science 245:1073–1080.

Kerem E, Corey M, Kerem BS, Rommens J, Markiewicz D, Levison H, Tsui LC, Durie P (1990): The relation between genotype and phenotype in cystic fibrosis-analysis of the most common mutation (ΔF508). N Engl J Med 323:1517–1522.

Lemna W, Feldman G, Kerem BS, Fernbach S, Zevkovich E, O'Brien W, Riordan J, Collins F, Tsui LC, Beaudet A (1990): Mutation analysis for heterozygote detection and the prenatal diagnosis of cystic fibrosis. N Engl J Med 322:291–296.

Macri C, Gentile A, Pivetta OH (1992): Registro Latino Americano de Fibrosis Quistica (REGLAFQ).

Pivetta OH, Olek K (1992): Relacion entre la delecion ΔF508 e la expression clinica em pacientes fibroquisticos. Publicacion Oficial del IV Congresso Latinoamericano de Fibrosis Quistica, Uruguay, Montevideu.

Raskin S, Phillips JA, Dawson E, Kaplan G, McClure M, Vnencak-Jones C (1992a): Utility of internal markers to improve the accuracy of cystic fibrosis genotype analysis. Biotechniques 13:2–3.

Raskin S, Phillips JA III, Kaplan G, McClure M, Vnencak-Jones C (1992b): Cystic fibrosis genotyping by direct PCR analysis of Guthrie blood spots: PCR methods and applications 2:154–156.

Raskin S, Phillips JA, Krishnamani MRS, Vnencak-Jones C, Parker RA, Rozov T, Cardieri JM, Marostica P, Abreu F, Giugliani R, Reis F, Rosario N, Ludwig N, Pilotto RF (1993a): Regional distribution of cystic fibrosis linked DNA haplotypes in Brazil, a multicenter study. (Submitted).

Raskin S, Phillips JA, Krishnamani MRS, Vnencak-Jones C, Parker RA, Rozov T, Cardieri JM, Marostica P, Abreu F, Giugliani R, Reis F, Rosario N, Ludwig N, Pilotto RF (1993b): Cystic fibrosis in the Brazilian population: F508 mutation and KM-19/XV-2C haplotype distribution. (Submitted).

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczack Z, Zielenski J, Lok S, Plavsic N, Chou JL (1989): Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. Science 245:1066–1073.

Rommens JM, Iannuzi MC, Kerem B, Drumm ML, Melmer G, Dean M, Rozmahel R, Cole JL, Kennedy D, Hidaka N (1989): Identification of the cystic fibrosis gene: Chromosome walking and jumping. Science 245:1059–1065.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Elich HA (1988): Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.

Salzano FM, Freire-Maia N (1967): "Populacoes Brasileiras: Aspectos Demograficos, Geneticos e Antropologicos." Sao Paulo, Brasil: Companhia Editora Nacional, pp 1–44.

Salzano FM, Pena SDJ (1987): Ethics and Medical Genetics in Brazil. In Wertz D and Fletcher JC (eds): "Ethics and Human Genetics." New York, U.S.A. Springer Verlag, pp 100–118.

Tsui LC (1990): Worldwide survey of the ΔF508 mutation. Amer J Hum Genet 47:354–359.

Waters DL, Dorney SF, Gaskin KJ, Gruca MA, O'Halloran M, Wilcken B (1990): Pancreatic function in infants identified as having cystic fibrosis in a neonatal screening program. N Engl J Med 322: 303–308.