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ORIGINAL

High-resolution dissociation analysis for the molecular diagnosis of cystic fibrosis in Cuba

Análisis de disociación de alta resolución para el diagnóstico molecular de fibrosis quística en Cuba

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ABSTRACT

Cystic fibrosis is a common recessive disease in the Caucasian population. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene can cause damage to the protein it encodes. Seven mutations are directly analyzed in Cuba, representing 55,5 % of the pathogenic allelic variants in cystic fibrosis patients. The high allelic heterogeneity of this gene and its varied phenotypic expression make it vitally important to detect a greater number of genetic variants that cause the disease. With this data, patients can receive better genetic counseling and personalized treatment that would improve their quality of life. It is therefore necessary to search for new variants to complete the diagnosis. The objective of this work was to identify new genetic variants in the CFTR gene and confirm the clinical diagnosis of Cystic Fibrosis. Twenty-one patients clinically diagnosed as cystic fibrosis by the National Medical Genetics Network or identified as positive through the Neonatal Screening Program were analyzed. Eleven real-time polymerase chain reaction assays were performed on the isolated DNA, followed by high-resolution dissociation curve analysis. The amplification of these eleven fragments covered the five exons of the gene with the highest number of described mutations and the adjacent intronic regions. Subsequently, the samples that showed changes in the curve pattern were sequenced. Five mutations were detected: one pathogenic, two of uncertain significance, one benign, and one with conflicting clinical significance. Point genetic variants were identified in four of the five exons analyzed. The real-time polymerase chain reaction – high resolution melting curve technique proved useful for searching for point mutations along the CFTR gene sequence, which, in combination with Sanger sequencing, will allow for the identification of new variants in the Cuban population.

Keywords: CFTR; Cystic Fibrosis; HRM; Sequencing.

RESUMEN

La fibrosis quística es una enfermedad recesiva frecuente en la población caucásica. Las mutaciones en el gen regulador de la conductancia transmembrana de la fibrosis quística (CFTR) pueden provocar daños en la proteína que este codifica. En Cuba se analizan directamente siete mutaciones, lo que representan el 55,5 % de las variantes alélicas patogénicas en los pacientes fibroquísticos. La alta heterogeneidad alélica de este gen y su variada expresión fenotípica hacen que resulte de vital importancia la detección de una mayor cantidad de variantes genéticas que causen la enfermedad. Con estos datos los pacientes pueden recibir un mejor asesoramiento genético y obtener un tratamiento personalizado que mejoraría su calidad de vida.

Resulta necesario, por tanto, la búsqueda de nuevas variantes para completar el diagnóstico. El objetivo de este trabajo fue identificar nuevas variantes genéticas en el gen CFTR y confirmar el diagnóstico clínico de Fibrosis Quística. Se analizaron 21 pacientes diagnosticados clínicamente como fibroquísticos por la Red Nacional de Genética Médica o identificados como positivos a través del Programa de pesquisa neonatal. A partir del ADN aislado se realizaron once ensayos de reacción en cadena de la polimerasa en tiempo real seguidos del análisis de curvas de disociación de alta resolución. La amplificación de estos once fragmentos abarcó los cinco exones del gen con mayor cantidad de mutaciones descritas y las regiones intrónicas adyacentes a ellos. Posteriormente, las muestras que presentaron cambios en el patrón de la curva se secuenciaron. Fueron detectadas cinco mutaciones, de ellas: una patogénica, dos de significado incierto, una benigna y una con conflicto en su significación clínica. Se identificaron variantes genéticas puntuales en cuatro de los cinco exones analizados. La técnica de reacción en cadena de la polimerasa en tiempo real - curva de disociación de alta resolución demostró su utilidad para la búsqueda de mutaciones puntuales a lo largo de la secuencia del gen CFTR, que en combinación con la secuenciación Sanger permitirá identificar nuevas variantes en la población cubana.

Palabras clave: CFTR; Fibrosis Quística; HRM; Secuenciación; Variantes.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease common in the Caucasian population. It occurs with an incidence of approximately 1/5 000 live births, and it is estimated that one in every 25 individuals is a healthy carrier of a mutation.[1] In Latin America, the incidence of CF remains difficult to estimate in most countries. This is due to the lack of records and CF screening programs for newborns, as well as the high ethnic mix of the population. It is estimated that CF affects between 1:1 600 and 1:14 000 newborns in Latin America.[2] In Cuba, as in the rest of Latin America, there is great heterogeneity, as is to be expected given the ethnic origin of the population. However, in 2015, a registry of all patients with a known diagnosis of CF was published, which has made it possible to estimate an incidence of approximately 1 in 9 862.[3]

It is a multisystemic and progressive disease with highly variable expressivity. This condition is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a transmembrane protein involved in chloride (Cl) transport. The loss of its function causes a defect in electrolyte transport in the apical membrane of epithelial cells, altering secretory function in different organs and tissues.[4] In Cuba, the National Cystic Fibrosis Commission (CCFQ) was created to, among other functions, provide the best care for patients and their families.[5] In the 1980s, molecular diagnosis of CF began in the molecular biology laboratory of the National Center for Medical Genetics (CNGM).[6]

Due to the large number of CF mutations and the variability observed in phenotypic expression, researchers have attempted to establish genotype-phenotype relationships. It has been possible to predict with high accuracy the severity of the phenotype at the organ level based on the genotype with respect to the sweat duct, pancreas, and reproductive system. This has led to the notion that both environmental-pulmonary interactions and the individual's genetic background contribute substantially to the severity of lung disease in CF.[7] The clinical manifestations of CF caused by a particular combination of mutations may vary, perhaps due to the effects of genetic modifiers.[8]

This disease is diagnosed in many countries through neonatal screening. However, there are places where diagnosis is based solely on a group of clinical manifestations, elevated sweat chloride concentrations, or detection of mutations in the CFTR gene.[9]

In Cuba, in order to detect the disease early, a nationwide neonatal screening program was developed in 2019, which measures the concentration of the enzyme immunoreactive trypsin in newborns. Currently, at the CNGM, it is possible to detect seven mutations (F508del, G542X, R1162X, R553X, R334W, 3120+1G>A, G85E) by performing allele-specific PCR and PCR-RFLP (restriction fragment length polymorphisms). These studies allow the detection of 55,5 % of pathogenic allelic variants in Cuban cystic fibrosis patients.[10] The high allelic heterogeneity of this gene and its varied phenotypic expression make it vitally important to detect a greater number of genetic variants that cause the disease. With this data, patients can receive comprehensive genetic counseling and obtain personalized treatment that would improve their quality of life. For this reason, the CNGM molecular biology laboratory recently standardized 11 real-time polymerase chain reaction assays—high-resolution melting curve (qPCR-HRM)—to search for mutations in the coding regions of the CFTR gene, as well as in the intronic regions adjacent to them. With these assays available, the search for genetic variants in the CFTR gene began. The objective was to identify new genetic variants in the CFTR gene and confirm the clinical diagnosis of cystic fibrosis.

METHOD

The study population consisted of 21 Cuban patients seen by geneticists from the National Medical Genetics Network and specialists from the National Cystic Fibrosis Commission during 2019. The research was approved by the Ethics Committee of the National Center for Medical Genetics, and informed consent was obtained from the patients and family members who participated in the study after the objective of the study was explained and its confidential nature was understood.

DNA isolation, quantification, and normalization

The extraction was based on 2 ml of peripheral blood, to which ethylenediaminetetraacetic acid (EDTA) was added as an anticoagulant. Genomic DNA was isolated and purified using the magnetic bead method on the QIAsymphony SP automated equipment. It was eluted in a total volume of 200 µL and stored in the DNA bank at -80 °C. The samples were then quantified using a Nanodrop 1000 and normalized to a concentration of 10 ng/µL.

All samples were pre-screened for seven CFTR gene mutations (F508del, G542X, R1162X, R553X, R334W, 3120+1G>A, G85E) using the refractory amplification of specific mutations method [11] and polymerase chain reaction and enzymatic digestion.[12,13,14] Exons 4, 8, 11, 14, and 20 of the CFTR gene were selected for PCR-HRM analysis, as they have the highest number of described mutations. The largest exon, exon 14, was divided into four fragments. Exons 4, 8, and 20 were divided into two amplicons. Exon 11 was not divided.

qPCR-HRM

Reagents from the commercial Type-it HRM PCR kit were used to perform the eleven qPCR-HRM assays. The reaction mixture was prepared in a final volume of 25 µL containing: 5 µL of template DNA, 12,5 µL of 2x HRM PCR Master Mix, 0,5 µL of each primer at a final concentration of 0,4 mM, and nuclease-free water to complete the reaction volume. The Rotor-Gene Q real-time thermocycler was used with the following running conditions: five minutes of denaturation at 95°C, followed by 40 cycles at 95°C for 10 seconds, 30 seconds at 58°C, and 10 seconds at 72 °C with acquisition of the fluorescent signal in the green channel at this step. Once the cycles were complete, the following was performed: 30 seconds at 95 °C, 30 seconds at 50°C, and finally a ramp from 65 °C to 95 °C, with temperature increments of 0,1 °C and acquisition of the fluorescent signal in the HRM channel.

The HRM analysis was performed using the equipment's software, where the normalization regions for the eleven amplified fragments were established. Samples that showed changes in Tm and/or curve pattern were selected for further sequencing.

DNA sequencing

The PCR product was purified with the commercial QIAquick PCR Purification kit (Qiagen) to remove oligonucleotides and free deoxynucleotide triphosphates (dNTPs) that interfere with the technique. The commercial GenomeLab Dye Terminator Cycle Sequencing – Quick Start kit (BeckmanCoulter) was used for bidirectional sequencing by the Sanger method. The DNA sequencing reaction was performed in a final volume of 20 µL containing: 5 µL of purified PCR product, 8 µL of DTCS – Quick Start Master Mix, and 1 µL of oligonucleotide (forward or reverse) at a final concentration of 0,2 mM. The amplification program used was the one generally recommended by the manufacturer: 30 cycles of denaturation at 96 °C for 20 seconds, hybridization at 50 °C for 20 seconds, and polymerization at 60 °C for 4 minutes, with final incubation sustained at 4 °C. The sequencing reaction product was purified by alcohol precipitation, as suggested by the manufacturer. Electrophoresis of the sequencing reaction was performed on the GenomeLab GeXP genetic analysis system (BeckmanCoulter).

Analysis of the DNA sequences obtained

The data obtained were processed using the Sequence Analysis module of the equipment's software, GenomeLab Genetic Analysis System (version 11.0.24). The sequences were compared with the reference sequence of the human CFTR gene (GenBank: NG_016465.4). The following databases were used: ClinVar (Genomic variation as it relates to human health), dbSNP (Short Genetic Variations), CFTR1, CFTR2 (The Clinical and Functional TRanslation of CFTR), CFTR-France Database, and CYSMA (Cystic Fibrosis Missense Analysis Website).

RESULTS

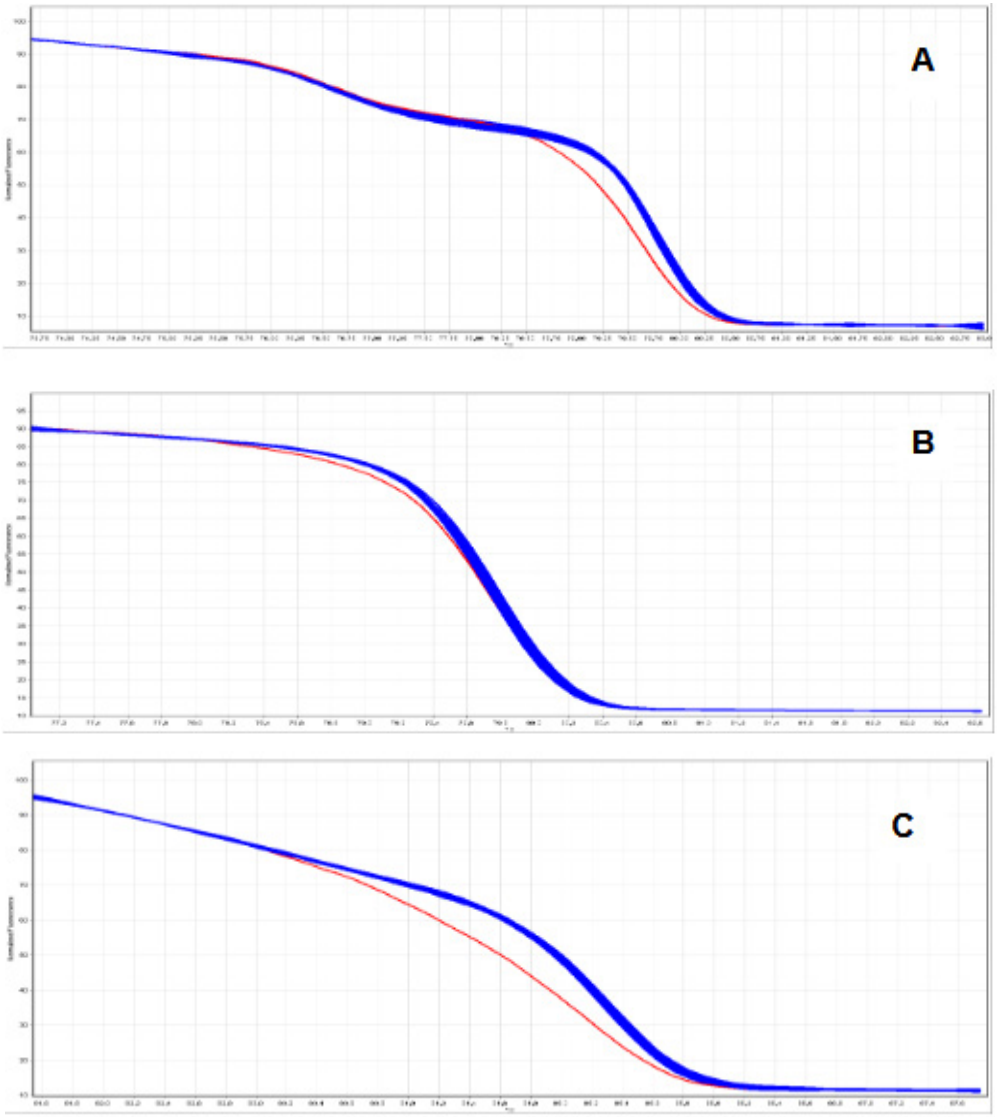
The patients came from several provinces, representing the west, center, and east of the island. Twelve of them had a clinical diagnosis of the disease and nine were positive in neonatal screening.

Table 1. Number of patients studied by province

Province	Number of patients
Havana	2
Matanzas	3
Cienfuegos	1
Villa Clara	1
Las Tunas	4
Camagüey	3
Holguín	3
Santiago de Cuba	3
Guantánamo	1

Analysis of the F508del, G542X, R1162X, R553X, R334W, 3120+1G>A, and G85E mutations revealed that five of the cases studied were heterozygous for the F508del mutation and one presented 3120+1G>A.

Eleven qPCR-HRM assays were performed for each sample. No changes in the curve pattern were detected in six of the eleven fragments (4-1, 4-2, 8-2, 14-1, 14-4, and 20-2). In the analysis of fragments 8-1 (figure 1A), 14-2, 14-3 (figure 1B and C), and 20-1 (figure 1D), a sample was detected in each case where the curve shape and Tm varied from the analyzed group. In the fragment corresponding to exon 11, the samples were grouped into four different curve patterns (figure 1E). One of these patterns coincided with the five heterozygous samples for F508del. From the other three patterns, one sample from each was selected for sequencing and identification of the variant present.



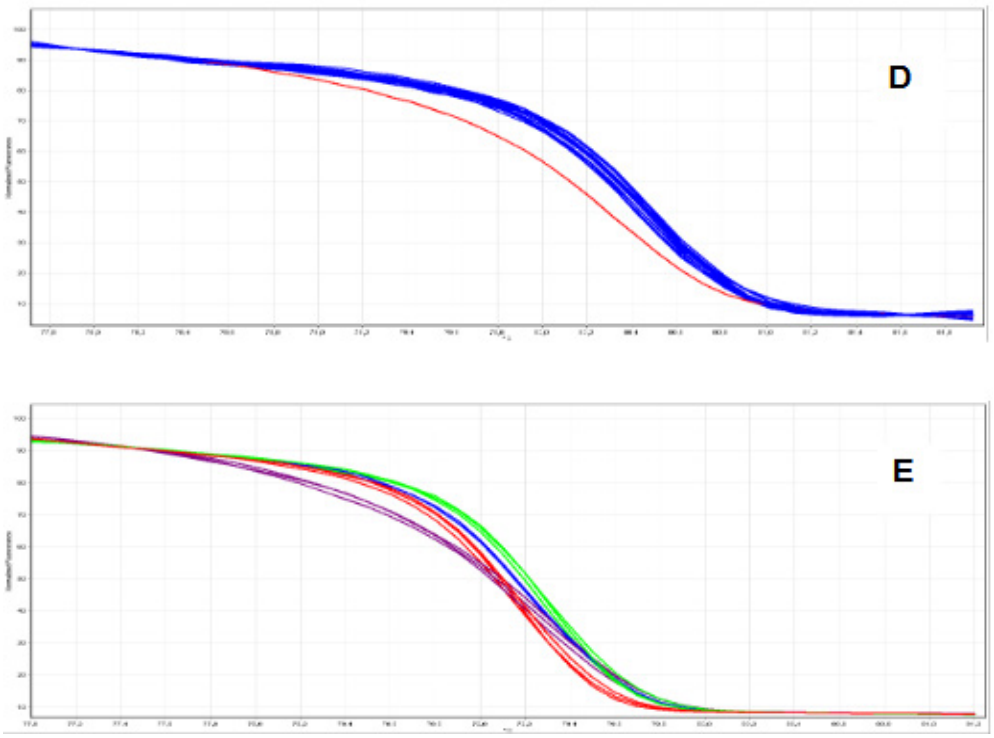


Figure 1. Normalized curves. A: fragment 8-1. B: fragment 14-2. C: fragment 14-3. D: fragment 20-1. E: fragment 11.

Analysis of the sequencing of the seven selected samples, based on the results obtained from the qPCR-HRM assays, allowed five genetic variants to be identified (table 2). All variants were found in a heterozygous state, with the exception of p.Val470Met, where the three possible genotypes were found

Table 2. Genetic variants found

Genetic variant	Change at the protein level	Location	Clinical classification*
c.890G>A	p.Arg297Gln	Exon 8	Meaning uncertain
c.1408G>A	p.Val470Met	Exon 11	Benign
c.2057C>A	p.Ser686Tyr	Exon 14	Meaning uncertain
c.2260A>G	p.Val754Met	Exon 14	Meaning uncertain / Probably benign / Benign
c.3197G>A	p.Arg1066His	Exon 20	Pathogenic

Note: * According to the ClinVar database

DISCUSSION

The c.890G>A (p.Arg297Gln) variant is located in exon 8 and causes a change from guanine to adenine. This results in a change from arginine to glutamic acid. According to the CYSMA database, the p.Arg297 residue belongs to domain 1 that crosses the membrane (MSD1), composed of six transmembrane helices (TM1-TM6). This is not a highly conserved region within the DNA, which may indicate that it is not essential for the proper functionality of the protein. The substitution of Q297 may not change the three-dimensional structure of the protein, as 3D structures predict that both R297 and Q297 form an α-helix.[15] CFTR1 data confirm that this variant does not cause the disease. According to dbSNP data, it occurs in the global population with a frequency of 0,1 %.[16] The patient presenting this variant was referred because he tested positive in the neonatal screening.

According to CFTR1,[16] Val470Met is a very common variant present in approximately 50 % of the population. Many healthy individuals in the population have two copies of Val470Met. These findings have allowed this variant to be classified as a polymorphism. This variant is found in exon 11 and causes a change from adenine to guanine. This causes valine to be replaced by methionine. According to the CYSMA database, the p.Val470 residue belongs to the NBD1 domain.[15] This region is not highly conserved in DNA (CYSMA). By combining the sequencing results with those of qPCR-HRM, we conclude that nine of the samples analyzed are heterozygous and three are homozygous for this polymorphism.

The c.2057C>A (p.Ser686Tyr) variant is located in exon 14 and is caused by a change from cytosine to adenine, resulting in a change from serine to threonine. According to the CYSMA database, this genetic alteration affects the regulatory domain of the CFTR protein. The serine residue (p.S686) is phosphorylated by protein kinase C (PKC) and plays an important role in chloride channel activity. It is a highly conserved region in the DNA sequence (CYSMA).[15] This variant is classified in ClinVar as of uncertain significance. The mutated allele has an overall frequency of approximately 0,01 %, according to dbSNP. According to the CFTR1 database, it was first described in an Italian man with asthenospermia.[16] In the present study, it was found in a sample from a 10-month-old male patient referred for being positive in the neonatal screening. In the future, fertility studies should be performed on this Cuban patient, as he may present the same clinical manifestation, thus providing appropriate genetic counseling.

In exon 14, the c.2260G>A (p.Val754Met) variant was detected, causing a change from guanine to adenine, which results in a change from valine to methionine. In vivo/vitro functional studies showed that Cl conductance is not altered in channels formed by the CFTR-Val754Met protein, suggesting that the variant does not affect channel function.[17] Despite this, there are conflicts in the classification of its pathogenicity. In ClinVar, different researchers have registered it as: of uncertain significance, probably benign, or benign. This conflict may be due to the fact that in 20 % of patients, Val754Met forms a complex allele with another pathogenic variant. Its global frequency is 0,02 %.[18] In Mexico, this variant has a frequency of 0,5 %,[19] while in Algeria it is 1,4 %.[20] In Cuba, according to Martínez's 2017 research, its frequency is 1,9 %.[21] This mutation was found in a sample from a 4-year-old female patient with a clinical diagnosis who also has the pathogenic variant F508del. It is necessary to perform analyses of large rearrangements in the CFTR gene to determine whether the Cuban patient has the complex allele that could be causing her symptoms.

The c.3197G>A (p.Arg1066His) variant is located in exon 20 and causes a change from guanine to adenine, which results in a variation in the encoded amino acid, arginine to histidine. The CFTR1 database suggests that this mutation causes the disease, which could be because the affected region is 100 % conserved in the gene's orthologs.[22] According to the CYSMA database, the p.Arg1066 residue belongs to the MSD2 domain and forms a salt bridge with E474 (MSD1). When affected, this bridge cannot be established.[15] According to dbSNP, it has a global frequency of 0,006 %. According to CFTR2, the combinations of tezacaftor and ivacaftor (Symdeko or Symkevi) and elexacaftor, tezacaftor, and ivacaftor (Trikafta or Kaftrio) have been determined to be effective in treating patients with this variant.[18] The patient presenting this variant was referred after testing positive in the neonatal screening.

Point genetic variants were identified in four of the five exons analyzed, one of which was pathogenic. The qPCR-HRM technique proved useful for searching for point mutations along the CFTR gene sequence. In combination with Sanger sequencing, it will allow to identify new variants in the Cuban population. It is necessary to continue the analysis of the remaining exons to complete the study of the patients and incorporate the analysis of large rearrangements of the CFTR gene using other molecular biology techniques.

Molecular data from Latin America indicate, on the one hand, a lower incidence of CF in these populations and, on the other, high heterogeneity as a result of the mixing of very diverse ethnic groups.

In addition to the CFTR gene, which is the source of genetic variability, there is also the action of modifier genes, mainly genes that modulate the immune response due to their importance in terms of infections and inflammatory response in the respiratory tract, the main condition that compromises the lives of these patients,[15] environmental factors, and the effectiveness of medical treatments. The interaction between this group of factors further complicates the diagnosis and prognosis of CF. It is important to note at this point that medical criteria are the determining factor in the decision to diagnose and treat a patient.

Studies conducted on Cuban cystic fibrosis patients demonstrate the high heterogeneity of our population. The Argentine population, like those in southern Europe, was found to be highly heterogeneous for *CFTR* gene mutations. The study of F508del and nine of the most frequently reported mutations detected 67 % of the mutated alleles;[23] consequently, only 51 % of patients were identified as having both mutated alleles (complete genotype). In summary, reports compiled from different populations show that there are significant differences in the distribution of CFTR mutations in different populations, with detection of the 24 most common mutations ranging from 50 % to 97 %.[24]

CONCLUSIONS

Point genetic variants were identified in four of the five exons analyzed, one of which was pathogenic. The qPCR-HRM technique proved useful for searching for point mutations throughout the CFTR gene sequence. In combination with Sanger sequencing, it will allow new variants to be identified in the Cuban population.

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The authors declare that they have no conflicts of interest.

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