Newborn Screening Program for Cystic Fibrosis in Cuba: Three Years' Experience

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Abstract

In Cuba, newborn screening (NBS) for cystic fibrosis (CF) was introduced in January 2019. The results from the first three years of the CF NBS program are presented. An IRT/IRT protocol was followed using a cut-off value of 50 ng/mL. In this period 281,717 neonates were screened, 2,197 samples had increased IRT values, and a second sample was necessary (recall rate=0.78%). In 686 (0.24%) neonates, IRT was still elevated, and they were referred for clinical evaluation. Twenty-one children were confirmed by sweat test and molecular biology. Eighteen newborns presented variant F508del. A false negative case was reported. Demographic data of 32,764 neonates were collected. The average age of sampling was six days with results available at 11 days of life, but 1.7% of the samples were collected 20 days after birth. The mean IRT value was 12.7±11.7 ng/mL (ranging 0–283 ng/mL) with a calculated 98.5 percentile value of 42.4 ng/mL. On average, the samples were processed five days after collection and two days after they were received at the laboratory. Although CF NBS program in Cuba is just beginning, it can be predicted that CF will be one of the most frequent inherited-metabolic diseases in the Cuban population.

Keywords

Cystic fibrosis, IRT, newborn screening, UMELISA®.

Introduction

Cystic fibrosis (CF) is a chronic, progressive, autosomal recessive disorder that affects more than 70,000 individuals worldwide [1] and is the most common life-limiting genetic disease in Caucasian populations with a varying incidence as high as 1:1,353 in Ireland to 1:2,800 in UK, 1:4,500 in France, 1:10,000 in Russia and 1:25,000 in Finland [2]. It is a systemic disease, mostly characterized by recurrent respiratory infections, exocrine pancreatic insufficiency (85 % of patients), and increased electrolyte concentration in sweat, usually presenting in infancy [3].

Since 1979, it has been possible to measure immunoreactive trypsinogen (IRT) as a simple and reliable screening test for CF in neonates [4, 5]. The first newborn screening (NBS) programs for CF were introduced in New Zealand and Australia in 1981 [6].

Numerous studies show that children diagnosed with CF through NBS programs achieve adequate nutrition [7-10], as well as better pancreatic [11, 12] and lung [13] function than those diagnosed by its clinical manifestations. These nutritional, functional, and neurocognitive benefits [11] are sustained during the first few years and are generally prolonged in the long term.

In the last decade, many countries and regions have adopted CF NBS programs, and today it is the most common way to diagnose this disease [14-17].

Estimates suggest that CF affects between 1 in 1,600 and 14,000 live births in Latin America [18], and the incidence in the Cuban population has been estimated to be 1/9**,**862 live

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births as calculated based on the available epidemiological data [19]. However, up to 2018, only 296 CF patients in Cuba (of over 11 million inhabitants) were registered in the Cuban Cystic Fibrosis Registry [20].

In Cuba NBS program has existed for over 35 years using SUMA technology, including five congenital metabolic disorders [21]. From January to June 2018, NBS for CF was introduced as a pilot study in six provinces of our country using UMELISA® TIR NEONATAL [22]. Based on the results obtained with this study, it was decided to extend CF NBS program to cover the whole country starting from January 2019. The aim of this summary is to show the major results obtained during the first three years of Cuban newborn CF NBS program.

Patients and Methods

Study Population

In Cuba, newborn screening is mandatory and offered free of charge. The Cuban NBS program is centrally organised and coordinated by the Ministry of Public Health through its Program for Comprehensive Care for Women and Children (PAMI, Spanish acronyms), which coordinates the actions of the health institutions in charged for the diagnosis, confirmation, control of the cases, treatment, follow-up of patients and attention to families.

The study group included all neonates born between January 2019 and December 2021. A total of 281,717 newborns were screened for CF. Samples were collected in the municipal collection centres between the 5th and 7th days of life.

Screening Strategy

The screening strategy used was the IRT/IRT protocol followed by sweat test and molecular biology testing for further confirmation (Figure 1). Taking into consideration that newborn screening program in Cuba is decentralized and according to previous studies completed in 2017 and 2018 (4406 and 6470 samples each year, respectively) [23, 22], it was established a fixed IRT concentration cut-off value of 50 ng/mL, corresponding to 98.5 percentile of the distribution. Infants with an elevated first IRT (IRT1) value were recalled collecting the second IRT (IRT2) sample at age 2 to 3 weeks. The cut-off value for IRT2 was the same as used for IRT1.

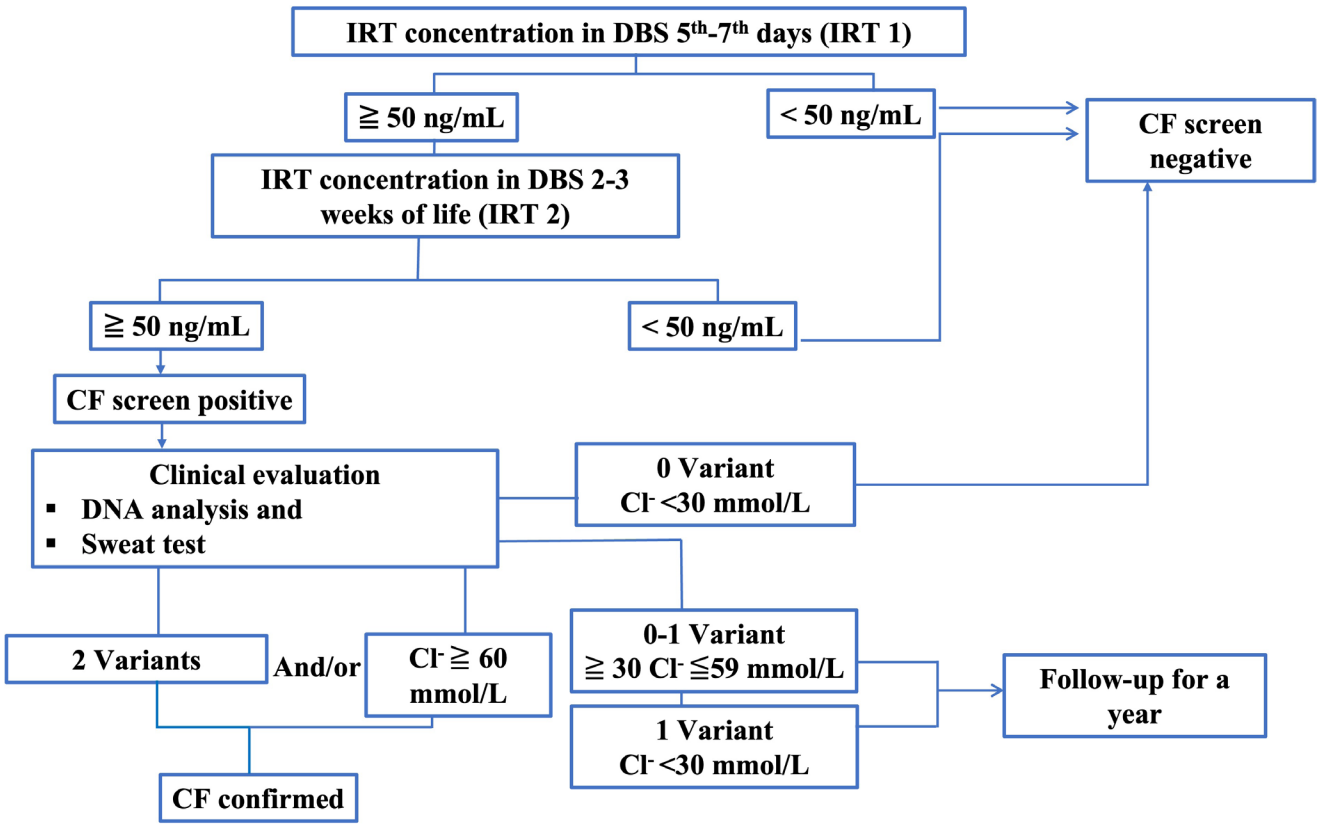

Figure 1. CF Cuban National Newborn Screening Program algorithm. An IRT/IRT strategy was followed, using sweat test and molecular biology testing for further confirmation. All newborns with IRT2 ≥ 50 ng/mL were considered as "possible CF" and were referred to consultation for clinical evaluation, as well as indication of variant analysis and sweat tests for all of them. Those newborns who were detected 0-1 variant and persistent sweat test values ranged between 30-59 mmol/L were followed-up in consultation every three months until they reached the first year of life, as well as those patients with one variant and sweat test values <30 mmol/L, to rule out false-negative results. Afterwards they were followed-up once a year.

All newborns with IRT2 positive results were referred to specialized consultations for CF patients for clinical evaluation. Sweat test and variant analysis were indicated. Those newborns who were detected 0-1 variant and persistent sweat test values ranged between 30-59 mmol/L were followed-up in consultation every three months until they reached the first year of life, as well as those patients with one variant and sweat test values <30 mmol/L. Afterwards they were followed-up once a year. All of them also received genetic counselling.

IRT Analysis Protocol (1st and 2nd Tier)

IRT was measured in dried blood spot samples from neonates (aged 5–7 days). To determine an IRT concentration (ng/mL), the UMELISA® TIR NEONATAL (TecnoSUMA, Havana, Cuba) was used [23]. Three mm blood discs of standards, controls and samples were punched out of the filter paper and placed into each well of the elution microplates, followed by the addition of 70 µL of the diluted anti-IRT Mab-alkaline phosphatase conjugate in 0.05 mol/L Tris buffer, pH 8.0 containing 0.15 mol/L of NaCl, 3 mmol/L of NaN_3, 2.9 mmol/L of $MgCl_2$, 2.5 mmol/L of $ZnCl_2$, 0.75 mol/L of BSA and 1.1 mmol/L Tween-20. They were incubated in a humid chamber for 16-18 hours at room temperature (20-25°C). Afterwards, 10 µL of eluate were transferred into the well of the reaction opaque polyestirene ultramicroplates coated with the specific anti-IRT Mabs. The immunological reaction occurred for two hours at room temperature in a humid chamber and then, the plates were washed six times with 0.37 mol/L of Tris-HCl solution, pH 8.0 containing 3.76 mol/L of NaCl, 1.1 mmol/L of Tween 20 and 76.9 mmol/L of NaN_3. The fluorogenic reaction was performed by adding 10 µL of the substrate solution, pH 9.6 containing 5.07 mM of 4-methylumbelliferil phosphate, 0.92 mol/L of diethanolamine-HCl, 0.7 mmol/L of $MgCl_2$ and 7 mmol/L of NaN_3. The ultramicroplates remained at room temperature in a humid chamber for 30 minutes. Finally, the fluorescence was automatically measured in the fluorimeter-photometer reader. Automatic validation and interpretation of the results were done using a specific-assay software. Analytical sensitivity of the assay is approximately 4.8 ng/mL blood. Intra and inter-assay coefficients of variation for the clinically relevant area are less than 7.5 %.

Variant Analysis

The genetic reference laboratory at the National Center of Medical Genetics, Havana, Cuba performed diagnostic genetic testing [24]. Genomic DNA was extracted from peripheral blood cells using standard procedures. Eight CF variants were analyzed in all patients (p.F508del, p.G542X, p.R1162X, p.I507del, p.R334W, p.R553X, p.G85E y c.3120 +1G > A).

Amplification Refractory of Mutations Specific [25] was carried out to detect three variants: p.F508del, p.G542X, p.R1162X. The remaining five variants were directly investigated by enzymatic digestion of the corresponding PCR products [24].

Sweat Tests

In Cuba exists ten laboratories for sweat test determinations located in different regions of our country. Sweat chloride measurements were performed in all patients who had two positive IRT values. Quantitative pilocarpine iontophoresis technique was used, collecting sweat in filter paper as described by Gibson and Cooke (26). Cut-off levels were based on the current international reference values [27]. Normal value for sweat chloride was < 30 mmol/L; values between 30-59 mmol/L were considered intermediate and required retesting because of the possible false negative results. Chloride concentration ≥ 60 mmol/L was consistent with CF positive screening.

Statistical Analysis

The mean, median, variance, minimum, maximum and percentiles of the distribution were calculated. The graphics were made with the Microsoft Excel 2003 for Windows spreadsheet. The statistical analysis of the data was done through the Statistica for Windows program, version 4.5 from StatSoft. Nonparametric tests (Mann-Whitney U Test and the Mean Test for independent variables) were used taking as significance level p <0.05.

Results

Cuban CF Newborn Screening Program Results

From January 2019 to December 2021 a total of 281,717 newborn samples were studied for CF covering 98.5% of all neonates born in Cuba during that period. Among neonates screened, 2,197 samples had values ≥ 50 ng/mL and IRT was measured again by a second heel prick in all of them (recall rate=0.78%). In 686 (0.24%) of these children, IRT was still ≥ 50 ng/mL, and they were referred to specialist CF centres for clinical evaluation.

Twenty-one children with the disease were confirmed by sweat test and when evaluated by molecular biology technique, variants causing CF were detected in 20 cases (Table 1). The mean age of diagnosis was 4.0 ± 1.5 months (Range between 2-7 months). Eighty-five percent of all newborns diagnosed were carriers of variant F508del, either in a homozygous or heterozygous state. One of the samples corresponded to a baby with meconium ileus and two were from twins. A false negative case was reported from the province of Guantánamo, with an IRT1 value of 46 ng/mL, which was referred to a genetics consultation for presenting symptoms consistent with the disease and a history of CF in his family, being subsequently evaluated by molecular biology (F508del / R334W) and sweat test. The age of diagnosis was seven months. Including the infant with meconium ileus, the sensitivity, specificity, and positive predictive values (PPV) were 95.45 %, 99.76 % and 3.1 %, respectively.

The distribution by provinces of newborns screened during the first six months of the program showed that Havana was the province with the highest number of neonates studied,

Table 1. CF-positive samples. Mean age of diagnosis was 4.0 ± 1.5 months, ranging between 2-7 months.

Sample	Gender	Variant analysis	Sweat test results (mmol/L)	Province
1	M	F508del/3120+1G>A	133	Santiago de Cuba
2	M	F508del/3120+1G>A	100	Camagüey
3	M	F508del/F508del	120	Santiago de Cuba
4	F	F508del/R334W	110	Santiago de Cuba
5	F	F508del/F508del	97	Holguín
6	M	F508del/F508del	90	Holguín
7	M	F508del/?	84	Camagüey
8	F	F508del/G85E	89	Pinar del Río
9	M	F508del/G85E	108	Cienfuegos
10	M	F508del/?	111	La Habana
11	F	F508/G542X	102	Camagüey
12	M	No variants detected	82	Guantánamo
13	F	R334W/R334W	81	Las Tunas
14*[a]	F	F508del/F508del	42.5	Camagüey
15*[b]	M	F508del/R334W	55	Sancti Spíritus
16*[b]	M	F508del/R334W	125	Sancti Spíritus
17	M	F508del/?	109	Santiago de Cuba
18	F	F508del/F508del	114	La Habana
19	F	G85E/?	135	La Habana
20	M	F508del/?	68	Granma
21	F	F508del/R1162X	85	Granma

14*[a] sample from a baby with meconium ileus,
15*[b] and 16*[b] samples from twins

however, it was Santiago de Cuba the one that reported the highest number of elevated results (n=7,916, 0.2% high IRT2 *vs* n=5,027, 0.44% high IRT2).

Demographic data were collected from the first 32,764 samples corresponding to three western provinces of the country (Havana, Mayabeque and Matanzas), as well as the special municipality of Isla de la Juventud. The average age of sampling was six days with results available at 11 days of life. 1.7% of the samples were collected 20 days after the child was born and 0.84% of them corresponded to samples taken after 30 days.

The mean IRT value was 11.8 ± 11.0 ng/mL (ranging between 0–344 ng/mL) and the median was 9.8 (IQR 5.2–15.2) ng/mL. The 98.5, 99.0, and 99.5 percentile values were 41.2, 44.0, and 52.2 ng/mL, respectively. Figure 2 shows the frequency distribution obtained for this group of samples in comparison with the results of the pilot study. On average, the samples were processed five days after collection and two days after they were received at the laboratory.

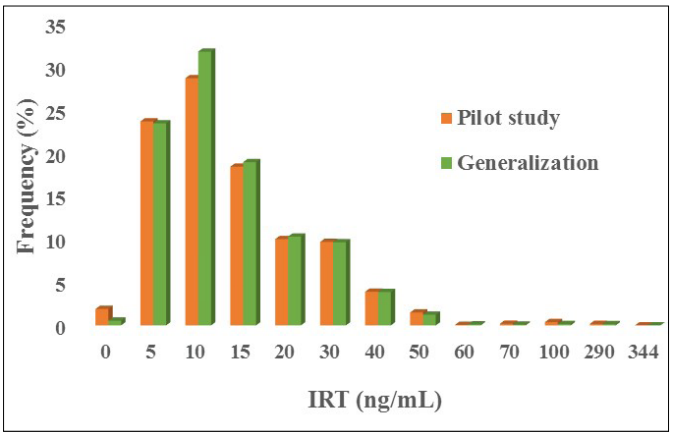

Figure 2. Comparison of the distribution of IRT concentrations in Cuban newborns using UMELISA® TIR NEONATAL during the pilot study and generalization phase. Demographic data were collected from the first 32,764 samples corresponding to three western provinces of the country.

Discussion

Newborn screening programs for CF, based on the determination of IRT levels in dried blood samples on filter paper, allow early identification of newborns affected with this pathology. In accordance with the Cuban registry data of CF patients, up to 2019 the mean age of diagnosis was two years (20). With the introduction of the newborn screening program this parameter decreased to four months. Even though this result should be improved, it represents a great achievement of the Cuban health system giving the opportunity to children with classic CF to receive early specialized medical treatment to maintain normal development and delay the onset of lung damage, greatly improving the quality of life of these patients.

Although the IRT assay is a valid instrument for NBS, even the most effective programs in the world are not capable of detecting 100% of patients affected with the disease, however, studies carried out in the Netherlands and Canada have shown a positive cost-benefit ratio for screened populations respecting the non-screened ones, regardless of the screening strategy chosen in each case [28, 29]. When the costs associated with treatment are reduced by 5% because of early diagnosis, screening will result in long-term savings [28].

Specificity and sensitivity obtained during the first three years of NBS for CF in Cuba are like those reported in other populations [14, 30]. PPV, even though is comparable with similar studies in our region [31, 32], is low and needs to be improved. To date there is no consensus on which is the ideal strategy for CF NBS, however, the reported specificity for all algorithms (regardless of the marker used as the second sample) is greater than 99% [14]. Regarding sensitivity, calculated percentages differ from one program to another according to the strategy followed in each case. In the US, for example, each state performs a different algorithm for CF NBS, and the reported sensitivity ranges 85-95% [14].

In our study, a false negative case was reported with an IRT1 value lower than the cut-off level used in our NBS program. Its diagnosis was confirmed by sweat test and molecular biology technique: compound heterozygous for F508del and R334W, two of the most common variants in our population [33]. Even though F508del is the most frequent and severe variants, there are several population studies where either heterozygous or homozygous newborns have been found whose IRT1 levels were much lower than the cut-off value established by these programs [9, 34-37], which indicates that, regardless of the established cut-off, there is always the possibility of obtaining true physiological false negatives [38].

Obtaining false negative results for CF is more common than for other genetic disorders included in NBS panels, mainly due to the cut-offs used in NBS programs that use IRT as a marker, seasonal variations associated with temperature and analytical variables related to the performance of the reagent kits [30]. Additionally, the characteristics of the disease itself determine that individual biological variability is relevant because not all variants in the CFTR gene result in high levels of IRT [39], which may cause false negative results. In the present study we obtained that the 98.5 percentile of the distribution corresponded to 41 ng/mL, a value below the cut-off level established for the program, that is why we would highly recommend adjusting a new cut-off value to decrease false negative results as low as possible.

Among the variants found during the first three years of the program, F508del was present in most patients diagnosed with the disease, which is consistent with that reported by Armas et al. in 2018 about the frequency of appearance of this variant in the Cuban population [33]. DNA analysis detects both classic and mild forms of the disease, as well as variant carriers [40, 41]. There is disagreement about the effects caused by the identification of CF carriers due to the possible psychological involvement of patients and their families [40, 41], although we think that their identification may contribute to reducing the incidence of the disease, through adequate genetic counseling.

A greater number of samples repeatedly raised for IRT2 were detected in the province of Santiago de Cuba with respect to the rest of the country. This could be associated with handling errors during the performance of the technique, since most of the repeatedly raised results to the investigation were obtained in the first two months after the generalization of the program began.

Another possible explanation for this phenomenon could be associated with skin color. Several studies report higher IRT levels in Afro-descendant newborns in relation to the rest of the population groups [42-44]. In the French population, Cheillan et al. found that the mean IRT values in newborns from North African families (21.17 ng/mL, n=8,817) were statistically higher than in newborns of Caucasian origin (19.74 ng/mL, n=26,310). Such difference influenced on percentiles, doubling the percentage of results with IRT values above the cut-off established for the test, without being later confirmed as CF [43]. For four years Giusti et al. analyzed the results of the CF NBS program in New York; it was shown that the risk of having high IRT values doubled in African American newborns regarding the rest of the population groups [44]. Although the reason why higher IRT levels are observed in these neonates is not exactly known, the fact that it occurs in these two groups (North Africans and African Americans) raises the possibility that there is a common genetic-based explanation [44].

According to data from the National Office of Statistics and Information of the Republic of Cuba, the color of the skin of the population varies remarkably by territory, being Santiago de Cuba one of the Cuban provinces with the highest percentage of population that is considered to have "black" and "mixed race" skin color [45]. In a study carried out by Marcheco et al. in 2015, it was shown that the population of Santiago de Cuba and Guantanamo had the highest percentage of African ancestry genes (39% and 40%, respectively) and that the individuals who were reported with skin color "black" presented 65.5% African origin genes. It was also found that in these provinces the percentage of African ancestry is slightly higher in those individuals who were reported with "white" skin color [46].

According to the national registry data of CF patients, the prevalence of the disease in Cuba is similar in all regions of the country [20]. However, 81% of newborns diagnosed with CF, after the implementation of the NBS program correspond to children from the center and eastern part of the nation, which could indicate that there has been an underdiagnosis of this pathology in that area throughout the years.

Two samples had intermediate sweat test results (between 30-59 mmol/L). One of them corresponded to a baby with meconium ileus who died at three months of age and the other was from one of the twins. Sweat testing is technically very challenging with many hands-on steps. Numerous errors may occur including inadequate number of sweat tests performed annually, improper collection time of sweat, and improper analysis of quantity-not-sufficient sweat samples [38], which may have cause sweat test result of sample 14. Unfortunately, since this baby died at three months of age, it was impossible to perform a second sweat determination. Twins had variant R334W which has been reported to present intermediate sweat chloride values [38]. Besides, a study carried out by Collaco et al. in 2016 revealed that that most variation in sweat chloride measurements is related to CFTR genotype and subjects with the same CFTR genotype (F508del homozygotes) still exhibit considerable variation in sweat chloride measurements [47].

The results obtained from the analysis of the demographic data of the samples corresponding to the western region of the country are very similar to those found during the pilot study [22], which demonstrates the reproducibility of the quantification of IRT levels using different UMELISA® TIR NEONATAL reagent lots. A small percentage of samples that were collected after 30 days of birth were processed, an indicator that should be improved as the program progresses to prevent false negative results, since IRT is not an effective marker after 30 days of life [15].

Limitations of the Study

Although the results shown in this study are relevant since CF has been included in the Cuban NSP, the study has some limitations. Even though the screening protocol takes into consideration those infants with intermediate or low sweat tests and 0-1 variant, there is no available data from these patients' follow-up at specialized consultations. It would be very useful to gather all the information related to these children to improve the quality of the NSP. The management of these patients should be done very carefully because a small proportion of them may eventually develop clinical features consistent with a diagnosis of CF and others may convert to a CF diagnosis if their sweat chloride concentration increases into the CF diagnostic range. Therefore, regular review by clinicians with an interest in CF should be undertaken so that opportunities to provide the appropriate treatment are not missed.

During the study, we only obtained a false negative result, but the possibility of a higher number of false negative cases is not ruled out. For example, when analyzing demographic data, it was observed that 1.7% of samples had been taken after 20 days of life, which might be a cause of false negatives results since IRT levels decreases after this time [30]. Besides it would be very useful to obtained demographic data from all the regions of our country to evaluate the performance of the screening program. It is for these reasons that primary health care personnel play a fundamental role in identifying those children who present symptoms consistent with CF, even if the results of the investigation have been negative, with the aim of indicating the corresponding diagnostic studies.

Mutation panels available for CF NBS should be adapted to the genetics of the population studied and it is of great importance including variants found in confirmed clinical cases. Recently, the development of a new allele-specific PCR and high-resolution melting analysis (HRMA) trial has been reported for the detection of 18 CFTR gene variants that cause CF in Cuba and Latin America [48]. It incorporates 10 new variants to the panel of variants that are currently detected in Cuban patients with suspected CF [24]. This new test has the advantage that uses dried blood samples on filter paper, which would be very beneficial to improve the performance of the protocol and even assess the possibility of applying another of the strategies used worldwide, if it is considered that the variant analysis can be carried out directly in the same sample collected between the 5^{th} – 7^{th} days of birth for IRT1. The combination of this methodology with the use of a floating cut-off for IRT determinations could greatly contribute to an improvement in the sensitivity of the program.

Conclusions

Although the CF NBS program in Cuba is just beginning and a greater number of newborns should be studied to define the real incidence of this disease in our country, it can be predicted that CF, according to the results obtained to date, will be one of the most frequent inherited-metabolic diseases in the Cuban population.

Abbreviations

CF: cystic fibrosis
IRT: immunoreactive trypsin
CFTR: cystic fibrosis transmembrane conductance regulator
NBS: newborn screening
SUMA: Ultra Micro Analytical System
PAMI: Program for Comprehensive Care for Women and Children (PAMI, Spanish acronyms)
IRT1: IRT concentration in DBS between 5^{th} -7^{th} days
IRT2: IRT concentration in DBS 2-3 weeks of life
ELISA: Enzyme-linked immunosorbent assay
UMELISA: Ultramicro enzyme-linked immunosorbent assay
PCR: polymerase chain reaction
HRMA: high resolution melting analysis
PPV: Positive Predictive Value

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Authors' Contributions

EMC, AS, AF and ECG conceived and administered the newborn screening part of the generalization project. Resources (monoclonal antibodies) were provided by GM. YM, ES, NO, TL, ALA and FR performed data curation. EMC, AF, TC, LDR, OM, ME, CA, PLP and ZN oversaw investigation and validation of results. EMC interpreted the data and drafted the manuscript. AM together with ECG, supervised EMC and AS, interpreted the data, reviewed, and edited the draft and provided critical input to the manuscript. All authors read and approved the final manuscript. EMC is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of Conflicting Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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