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Nested-PCR vs. RT-qPCR: A Sensitivity Comparison in the Detection of Genetic Alterations in Patients with Acute Leukemias

Flávia Melo Cunha de Pinho Pessoa [1], Marcelo Braga de Oliveira [2], Igor Valentim Barreto [1], Anna Karolyna da Costa Machado [1], Deivide Sousa de Oliveira [1,3], Rodrigo Monteiro Ribeiro [3], Jaira Costa Medeiros [3], Aurélia da Rocha Maciel [3], Fabiana Aguiar Carneiro Silva [3], Lívia Andrade Gurgel [3], Kaira Mara Cordeiro de Albuquerque [3], Germison Silva Lopes [4], Ricardo Parente Garcia Vieira [5], Jussara Alencar Arraes [5], Meton Soares de Alencar Filho [5], André Salim Khayat [2], Maria Elisabete Amaral de Moraes [1], Manoel Odorico de Moraes Filho [1] and Caroline Aquino Moreira-Nunes [1,2,6,*]

[1] Department of Medicine, Pharmacogenetics Laboratory, Drug Research and Development Center (NPDM), Federal University of Ceará, Fortaleza 60430-275, Brazil; flaviamelop@outlook.com (F.M.C.d.P.P.); barreto.igorv@gmail.com (I.V.B.); annakcm1.8@gmail.com (A.K.d.C.M.); deividearmorial@gmail.com (D.S.d.O.); betemora@ufc.br (M.E.A.d.M.); odorico@ufc.br (M.O.d.M.F.)
[2] Department of Biological Sciences, Oncology Research Center, Federal University of Pará, Belém 66073-005, Brazil; oliveira.mb23@gmail.com (M.B.d.O.); khayatas@gmail.com (A.S.K.)
[3] Department of Hematology, Fortaleza General Hospital (HGF), Fortaleza 60150-160, Brazil; rmonteiroribeiro@icloud.com (R.M.R.); jairacostamedeiros@hotmail.com (J.C.M.); aureliarocha347@gmail.com (A.d.R.M.); fabianaaguiarcs@gmail.com (F.A.C.S.); livia.hemato@gmail.com (L.A.G.); drakairahematologista@gmail.com (K.M.C.d.A.)
[4] Department of Hematology, César Cals General Hospital, Fortaleza 60015-152, Brazil; germison@gmail.com
[5] Department of Hematology, São Vicente de Paulo Maternity Hospital, Barbalha 63180-000, Brazil; ricardopgv@gmail.com (R.P.G.V.); jussaraexu@hotmail.com (J.A.A.); meton.filho@icloud.com (M.S.d.A.F.)
[6] Clementino Fraga Group, Central Unity, Molecular Biology Laboratory, Fortaleza 60115-170, Brazil
* Correspondence: carolfam@gmail.com

Abstract: The detection of genetic alterations in patients with acute leukemias is essential for the targeting of more specific and effective therapies. Therefore, the aim of this study was to compare the sensitivity of Nested-PCR and RT-qPCR techniques in the detection of genetic alterations in patients with acute leukemias. This study included samples from 117 patients treated at the Fortaleza General Hospital. All samples were submitted to analysis using the Nested-PCR and the RT-qPCR techniques. Acute Myeloid Leukemia (AML) patients' samples were submitted to the analysis of the following alterations: *FLT3-ITD*, *RUNX1::RUNX1T1*, *CBFB::MYH11* and *PML::RARA*; meanwhile, *BCR::ABL1*, *TCF3::PBX1*, *KMT2A::AFF1*, *ETV6::RUNX1*, and *STIL::TAL1* fusions were investigated in the Acute Lymphoblastic Leukemia (ALL) patients' samples. Throughout the study, 77 patients were diagnosed with AML and 40 with ALL. Among the 77 AML patients, *FLT3-ITD*, *RUNX1::RUNX1T1*, *PML::RARA*, and *CBFB::MYH11* were detected in 4, 7, 10 and 8 patients, respectively. Among the 40 ALL patients, the presence of 23 patients with *BCR::ABL1* translocation and 9 patients with *TCF3::PBX1* translocation was observed through the RT-qPCR methodology. Overall, the present study demonstrated that the RT-qPCR technique presented a higher sensitivity when compared to the Nested-PCR technique at the time of diagnosis of the acute leukemia samples studied.

Keywords: biomarkers; molecular diagnostic techniques; polymerase chain reaction; acute myeloid leukemia; acute lymphoblastic leukemia

Citation: Pessoa, F.M.C.d.P.; de Oliveira, M.B.; Barreto, I.V.; Machado, A.K.d.C.; Oliveira, D.S.d.; Ribeiro, R.M.; Medeiros, J.C.; Maciel, A.d.R.; Silva, F.A.C.; Gurgel, L.A.; et al. Nested-PCR vs. RT-qPCR: A Sensitivity Comparison in the Detection of Genetic Alterations in Patients with Acute Leukemias. *DNA* **2024**, *4*, 285–299. https://doi.org/10.3390/dna4030019

Academic Editor: Darren Griffin

Received: 12 July 2024
Revised: 28 August 2024
Accepted: 3 September 2024
Published: 6 September 2024

1. Introduction

The diagnosis of hematological cancers still represents a great challenge. The various stages of normal hematopoietic differentiation give rise to a series of biologically and clinically distinct cancers [1–3]. Following the World Health Organization (WHO) and European Leukemia Net (ELN) of 2022 risk stratification guidelines, the detection of

cytogenetic and molecular changes in leukemias has demonstrated extreme importance at diagnosis [4,5].

The diagnosis of leukemias in the northeastern Brazilian states, especially in Ceará, is still based on morphological and immunophenotyping tests, which only consider the identification of blast cells and their membrane markers. This may induce stratification errors of the various subtypes of leukemias, impairing prognosis, as well as the choice of the most indicated molecular therapy, which increases the chances of survival and quality of life of patients during treatment [6–9].

The polymerase chain reaction (PCR) has dramatically altered how molecular studies are conducted, as well as the improvement of investigations and diagnosis of many different types of diseases. This technique was firstly introduced in the 1980s by Kary Mullis and it is capable of synthesizing millions of copies of a specific DNA sequence in a simple reaction [10–15]. Over the years, the PCR methodology has evolved, and several variations of the technique have been developed in order to improve the sensitivity, specificity, and speed of the method [16–19].

The work of van Dongen et al. (1999) established Nested-PCR as the gold standard methodology in the detection of gene fusions in acute leukemias at diagnosis and in the investigation of minimal residual disease (MRD) [20–22].

The Nested-PCR is a technique that consists of a double amplification of a DNA or cDNA template that uses the product obtained in the first amplification as a model for the second. In this way, the sensitivity and specificity of the analysis are improved. The alteration detection is confirmed by performing an agarose gel electrophoresis [23–26].

Real-time quantitative PCR (RT-qPCR), in particular, has revolutionized the diagnosis and follow-up of MRD, as it enables the highly sensitive detection of residual leukemic cells [23,27–30]. This technique basically consists of the exponential amplification of a specific region of nucleic acids. In RT-qPCR, the amplification and detection of nucleic acid fragments occur simultaneously, providing greater speed and sensitivity to the method. Through this technique, it is possible to analyze the gene expression profile of several genes and/or chromosomal fusions, so it can be used both for the diagnosis and for the monitoring of the disease and detection of MRD [1,28,30–37].

Despite the fact that Nested-PCR is still considered the gold standard method for detecting cytogenetic changes in patients with leukemias, it is a very time-consuming technique whose last standardization was performed in 1999. Given this, most Brazilian public health services use the karyotype method to carry out this type of diagnosis, which, however, is also a very time-consuming technique and sometimes does not generate results for patients [38,39]. Currently, with the RT-qPCR technology available and widely disseminated worldwide, this methodology seems to be more sensitive and effective in detecting these same alterations [23,28,40,41]. Therefore, this study aimed to perform a sensitivity comparison in the detection of genetic alterations in patients with acute leukemias.

2. Materials and Methods

2.1. Ethical Aspects

This project was submitted and approved by the Research Ethics Committee (CEP) of the Federal University of Ceará, under registration number 4339719. The participants of this study were patients with suspicion/diagnosis of acute leukemias of the myeloid or lymphoid type treated at the Fortaleza General Hospital. The patients or their guardians were submitted to readings and analysis of the Free and Informed Consent Form (FICF) and only participated in the research after acceptance of the above and signature.

The collection of information from medical records and blood and bone marrow samples from patients with suspected or diagnosed acute leukemias were carried out from July 2021 to May 2023 at the Fortaleza General Hospital, totaling 175 patients. After applying the exclusion criteria, the study had the participation of 117 patients, due to the fact that the remaining 58 patients who sought medical attention for suspected acute leukemias were diagnosed with other hematological diseases, such as chronic lymphoid leukemia

(CLL), multiple myeloma (MM), myelodysplastic syndrome (MDS), and myeloproliferative syndrome (MPS).

2.2. Samples for Molecular Study

All 117 research participants were directed to peripheral blood collection for genetic evaluation, regardless of the percentage of circulating blasts. Patients' samples were collected in EDTA collection tubes at the time of diagnosis and were packed in a thermal case at 2–4 °C for transport to the laboratory for latter processing.

This material was collected at the health service where the patient was receiving care by means of peripheral venipuncture by trained personnel. A total of 5 mL of peripheral blood was collected for RNA extraction in an EDTA tube. Bone marrow collection was possible in only 84 patients out of 117 study participants. A total of 2–3 mL of bone marrow was collected in an EDTA tube. These samples were collected at the time of myelogram examination at diagnosis, without causing any additional inconvenience to the patients. The collected samples did not show lysis and were processed by separating the buffy coat through centrifugation at 5000 rpm for 5 min. The cytogenetics study was made by the state blood center and their results were obtained through the system integrated into the medical records.

2.3. RNA Extraction and Quantification

RNA from the patients' peripheral blood and bone marrow samples was extracted from the buffy coat with commercial TRIzol Reagent® (Applied Biosystems, Foster City, CA, USA) kit according to the manufacturer's instructions.

The quantification was performed using a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, SUA) following the protocol designated by the company. Our group established that all samples should be standardized to a concentration of 20 ng/μL. RNA quality was determined by the 260/280 nm ratio provided by the NanoDrop2000 spectrophotometer (Thermo Scientific) used, where the samples with ratios between 1.8 and 2.0 were considered to have a good degree of purity.

2.4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

From 20 μL of RNA, a reverse transcriptase polymerase chain reaction (RT-PCR) was performed for cDNA synthesis. The conversion was performed with the aid of the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), according to the manufacturer's protocol. This step was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA). After this step, the samples were stored in a freezer at −20 °C until use for analysis.

2.5. Identification of Genetic Biomarkers

Initially, a panel of 9 genetic biomarkers (*BCR::ABL1 p(190), TCF3::PBX1, KMT2A::AFF1, ETV6::RUNX1, STIL::TAL1, FLT3-ITD, PML::RARA, CBFB::MYH11,* and *RUNX1::RUNX1T1*), described in the literature as the most frequent in acute leukemias, was used to screen patients seen in the health service using both PCR techniques in both peripheral blood and bone marrow samples.

2.5.1. Nested Polymerase Chain Reaction (Nested-PCR)

The gene detection by Nested-PCR was made using Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, EUA) and commercial kit Invitrogen™ Platinum™ SuperFi™ II PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, SUA).

The primers used in this technique were based on the consensus established for the diagnosis of acute leukemias in the article on the standardization of the technique published by van Dongen et al. (1999) [20]. In addition, the *GAPDH* and *HPRT* genes were used as reference/positive control genes, as they were used in van Dongen et al.'s (1999) [20] study.

Ultra-pure water was used as a negative control. The information on the chosen primers for the study is summarized in Table S1 in the Supplementary Material.

The reaction protocol used 4.25 µL of deionized water, 6.25 µL of 2X Platinum SuperFi II PCR Master Mix, 0.5 µL of forward primer (F), 0.5 µL of reverse primer (R), and 1 µL of cDNA, totaling a reaction of 12.5 µL. The protocol for reaction amplification consisted of 35 cycles as follows: 95 °C/3 min, 94 °C/2 min, 65 °C/1 min, 70 °C/2 min, and 70 °C/30 min.

After the last Nested-PCR, an agarose gel electrophoresis was performed to reveal whether or not there was an amplification of the target region. UltraPure™ Agarose (Invitrogen, Waltham, Massachusetts, EUA) was used to make a 100 mL gel with a 1.5 concentration. The 1 KB Plus DNA Ladder kit (Thermo Fisher Scientific) was added along with the Nested-PCR products in each well. Electrophoresis was performed with 100 V, 400 mA and for 60 min. Then, the gel was visualized through the IBright 1500 (Thermo Fisher Scientific).

2.5.2. Real-Time Polymerase Chain Reaction (RT-qPCR)

The expression detection of genetic alterations by quantitative real-time PCR (RT-qPCR) was performed using the QuantStudio 5 device (Applied Biosystems) and commercial kit TaqMan® Expression Master Mix (Thermo Fisher Scientific). In addition, the expressions of the endogenous genes *ACTB* and *ABL1* were analyzed in all samples as positive controls, as is recommended by Pessoa et al. (2024) [21]. Ultra-pure water was used as negative control. Information on the genes and probes chosen for the study is summarized in Table S2 in the Supplementary Material.

About the protocol, for each sample, the following were used: 1 µL of cDNA, 0.5 µL of each primer/probe, 5 µL of TaqMan® Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA), and 3.5 µL of ultra-pure water. Applied Biosystems MicroAmp® Optical 96-Well Reaction Plates were used and each sample was analyzed in triplicate for experimental and technique validation, according to the international standards for evaluation of gene expression by real-time PCR. The protocol of amplification of the reactions consisted of the following cycling: 50 °C/2 min, 95 °C/10 min, and 50 cycles of 95 °C/15 s and 60 °C/1 min.

After amplification, the fragments were quantified by fluorescent data analysis using software version 1.1 in the QuantStudio 5 (Applied Biosystems). All the RT-qPCR tests followed Minimum Information for Publication of Quantitative Real-Time PCR Experiments MIQE Guidelines requirements [42].

Although detection based on an RT-qPCR-based threshold value (Cq) is feasible and widely used, it is important to note that the Cq value can vary based on various non-technical factors. For example, improper pipetting can change the initial amount of loading.

2.6. Statistical Analysis

The fusion gene detections were analyzed in terms of frequency. The data were analyzed using the Chi-square test and described in a contingency table, using the GraphPad Prism 8.0 program, adopting a significance level of $p < 0.05$.

3. Results

Throughout the study, 77 (65.8%) patients were diagnosed with Acute Myeloid Leukemia (AML) and 40 (34.2%) with Acute Lymphoblastic Leukemia (ALL), of which 35 (87.5%) corresponded to type B and 5 (12.5%) to type T.

Among all 77 AML patients, 4 patients with the *FLT3-ITD* mutation, 7 patients with the *RUNX1::RUNX1T1* translocation, 10 patients with *PML::RARA* translocation, and 8 patients with *CBFB::MYH11* translocation were detected by RT-qPCR. Among the 40 ALL patients, there were 23 with the *BCR::ABL1* translocation and 9 with the *TCF3::PBX1* translocation (Figure 1).

Through the RT-qPCR technique, it was observed that, of all 77 patients, *PML::RARA* was detected in 13%, *RUNX1*::*RUNX1T1* in 9%, *FLT3-ITD* in 5.2%, and *CBFB::MYH11* in

10.4% of all 77 AML patients (Figure 2). The analysis through Nested-PCR of this same group of patients was not able to identify the presence of any researched alteration.

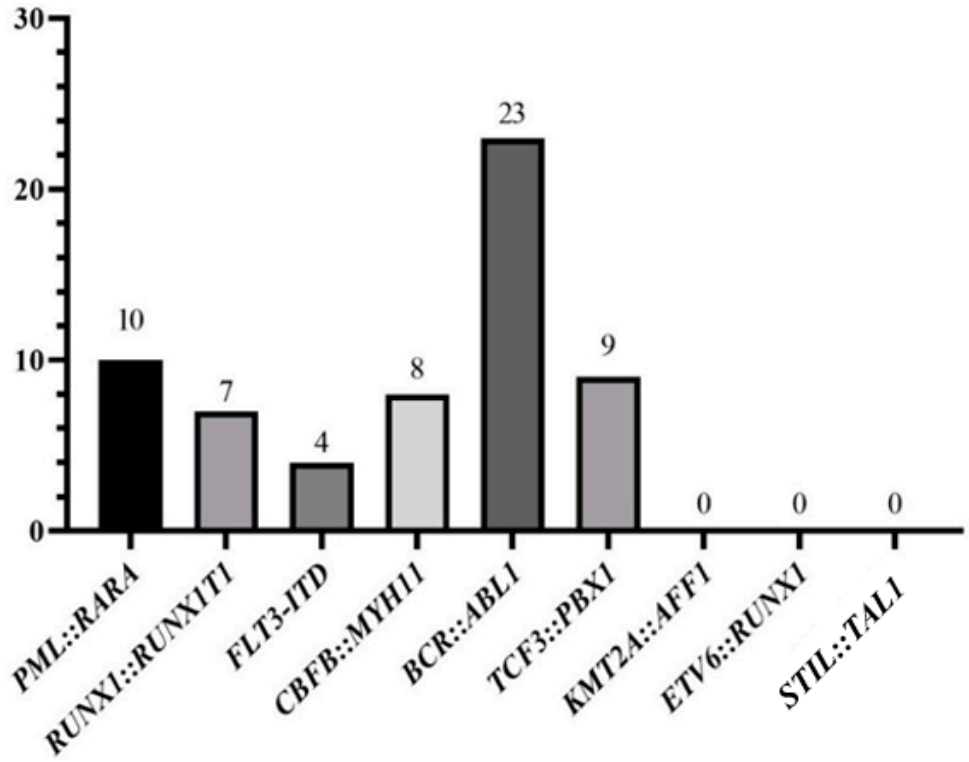

Figure 1. Molecular alteration frequency in research participants. This figure illustrates how many times each fusion was detected in the analyzed patients.

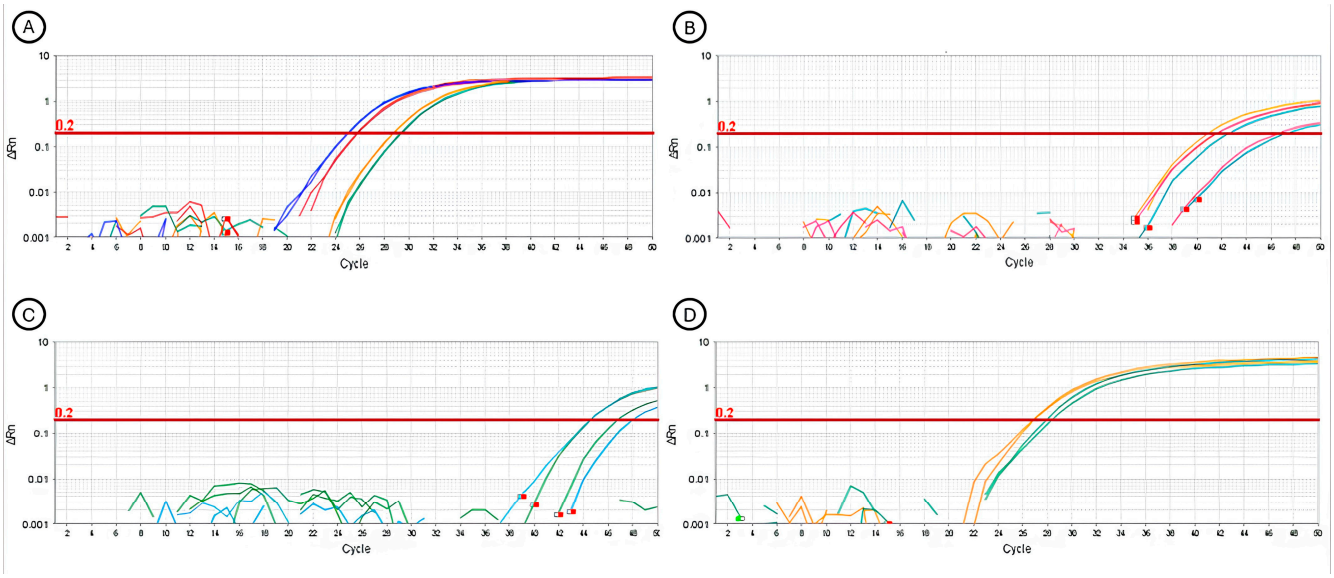

Figure 2. Amplification plots of Acute Myeloid Leukemia patients. This figure illustrates the AML amplification plots in which the researched alterations were detected. (**A**) Amplification plots indicating the detection of *FLT3-ITD* (blue and red curves are from BM samples and the orange and green ones are from PB samples). (**B**) Amplification plots indicating the detection of *PML::RARA* (pink and orange curves are from BM samples and the green and blue ones are from PB samples). (**C**) Amplification plots indicating the detection of *CBFB::MYH11* (blue curves are from BM samples and the green ones are from PB samples). (**D**) Amplification plots indicating the detection of *RUNX1::RUNX1T1* (orange curves are from BM samples and the green ones are from PB samples).

PML::RARA was predominantly reported in male patients, with a mean age of 40.5 years. Only five patients had the characteristic translocation t(15;17)(q24;q21.3) in the karyotypes analyzed. Of the 10 *PML::RARA* AML patients, 5 have died (Table 1).

Table 1. Clinical and epidemiological characteristics of patients with identified molecular alterations.

	N° of Patients	Gender	Age (Mean)	Hb	WBC	Blasts in PB	Karyotype	Deaths
AML								
PML::RARA	10	Male: 8 Female: 2	40.5 years	Hb < 10: 9 Hb > 10: 1	WBC < 10,000: 7 WBC > 10,000: 3	Yes: 5 No: 5	Complex: 1 Classic translocation: 5 Normal: 1 NR: 3	5
RUNX1::RUNX1T1	7	Male: 4 Female: 3	31 years	Hb < 10: 6 Hb > 10: 1	WBC < 10,000: 7	Yes: 3 No: 4	Complex: 2 Classic translocation: 3 Normal: 1 Other alterations: 1	2
FLT3-ITD	4	Male: 2 Female: 2	50.2 years	Hb < 10: 4	WBC < 10,000: 2 WBC > 10,000: 2	Yes: 2 No: 2	Normal: 3 Other alterations: 1	4
CBFB::MYH11	8	Male: 6 Female: 2	41.9 years	Hb < 10: 8	WBC < 10,000: 2 WBC > 10,000: 6	Yes: 6 No: 2	Complex: 1 Classic translocation: 1 Normal: 2 Other alterations: 3 NR: 1	5
ALL								
BCR::ABL1	23	Male: 14 Female: 9	42.2 years	Hb < 10: 19 Hb > 10: 4	WBC < 10,000: 10 WBC > 10,000: 13	Yes: 11 No: 12	Complex: 5 Normal: 7 Other alterations: 1 NR: 10	8
TCF3::PBX1	9	Male: 5 Female: 4	36.9 years	Hb < 10: 8 Hb > 10: 1	WBC < 10,000: 6 WBC > 10,000: 3	Yes: 4 No: 5	Complex: 4 Normal: 3 NR: 2	3

Hb: Hemoglobin; WBC: White Blood Cell Count; PB: Peripheral Blood; AML: Acute Myeloid Leukemia; ALL: Acute Lymphoblastic Leukemia; NR: Not Reported.

It was observed that *RUNX1::RUNX1T1* was also more frequent in male patients, with a mean age of 31 years. All patients with *RUNX1::RUNX1T1* had a leukocyte count below 10,000/mm^3 at the time of diagnosis. Only three patients had the characteristic translocation t(8;21)(q22;q22) in the analyzed karyotypes. Only one patient of the seven diagnosed have died (Table 1).

It was possible to identify that the *FLT3-ITD* mutation was predominantly detected in patients with a mean age of 50.2 years. Of the four *FLT3-ITD* AML patients, three had normal karyotypes and all four have died (Table 1).

Furthermore, our data reported that *CBFB::MYH11* was mostly detected in male patients with a mean age of 41.9 years. Of eight patients with this diagnosis, five had abnormal karyotypes (presence of deletions, additions, and translocations), but only one had the characteristic translocation t(16;16)(p13.1;q22). In addition, five of the eight *CBFB::MYH11* AML patients have died, and all had abnormal karyotypes (Table 1).

Regarding the 40 ALL patients, *BCR::ABL1* was detected in 57.5% and *TCF3::PBX1* in 22.5% by the RT-qPCR technique (Figure 3).

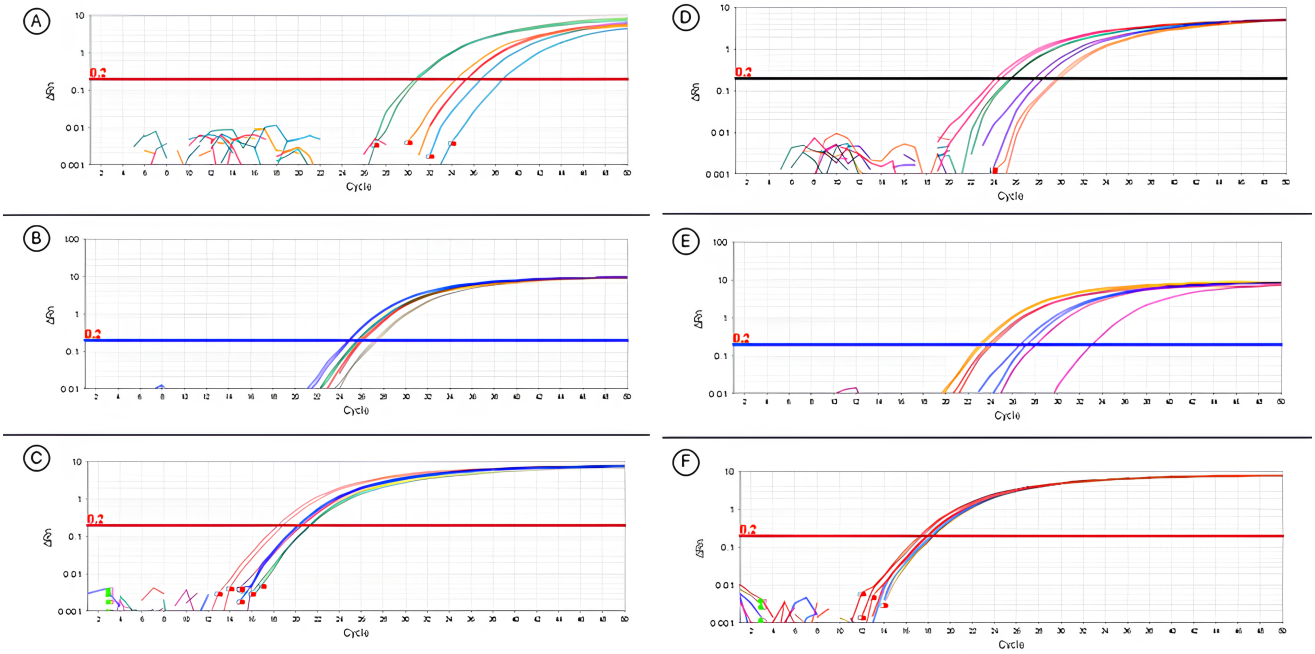

Figure 3. Amplification plots of Acute Lymphoblastic Leukemia samples. All amplification plots in this figure show the results of two samples in duplicate (bone marrow and peripheral blood) of two ALL patients. The figure's left side shows the results of two patients samples in which *BCR::ABL1* alteration was detected; meanwhile, the figure's right side demonstrates the amplification plots of two patients' samples in which *TCF3::PBX1* was detected. (**A**) *BCR::ABL1* amplification plots (green and orange curves are from BM samples and the pink and blue ones are from PB samples). (**B**) *ABL1* amplification plots of two *BCR::ABL1* patients (blue and green curves are from BM samples and the red and gray ones are from PB samples). (**C**) *ACTB* amplification plots of two *BCR::ABL1* patients (pink and purple curves are from BM samples and the green and blue ones are from PB samples). (**D**) *TCF3::PBX1* amplification plots (green and pink curves are from BM samples and the purple and orange ones are from PB samples). (**E**) *ABL1* amplification plots of two *TCF3::PBX1* patients (pink and orange curves are from BM samples and the purple and blue ones are from PB samples). (**F**) *ACTB* amplification plots of two *TCF3::PBX1* patients (pink and purple curves are from BM samples and the orange and blue ones are from PB samples).

The Nested-PCR technique was able to detect *BCR::ABL1* and *TCF3::PBX1* in ALL patients samples. Nonetheless, *TCF3::PBX1* was only detected in patients' bone marrow samples, even if they presented ≥20% of circulating blasts in peripheral blood, which may indicate that this technique's sensitivity is not great (Figure 4).

The presence of *BCR::ABL1* was predominantly reported in male patients, with a mean age of 42.2 years. Among the karyotypes analyzed, it was observed that 6 were complex, 7 were normal, and 10 did not have karyotype results. Of the 23 patients diagnosed, 8 have died, of which 3 had a normal karyotype, 3 had a complex karyotype, and 2 did not have karyotype results (Table 1).

It was also observed that 22.5% of the ALL patients had the *TCF3::PBX1* translocation detected by RT-qPCR. The presence of this mutation appeared to be associated with male patients, with a mean age of 36.9 years. It was possible to identify that four patients had a complex karyotype, three patients had a normal karyotype, and two patients did not have karyotype results. In addition, of the nine patients diagnosed, three died, of which two had a complex karyotype and one had a normal karyotype (Table 1).

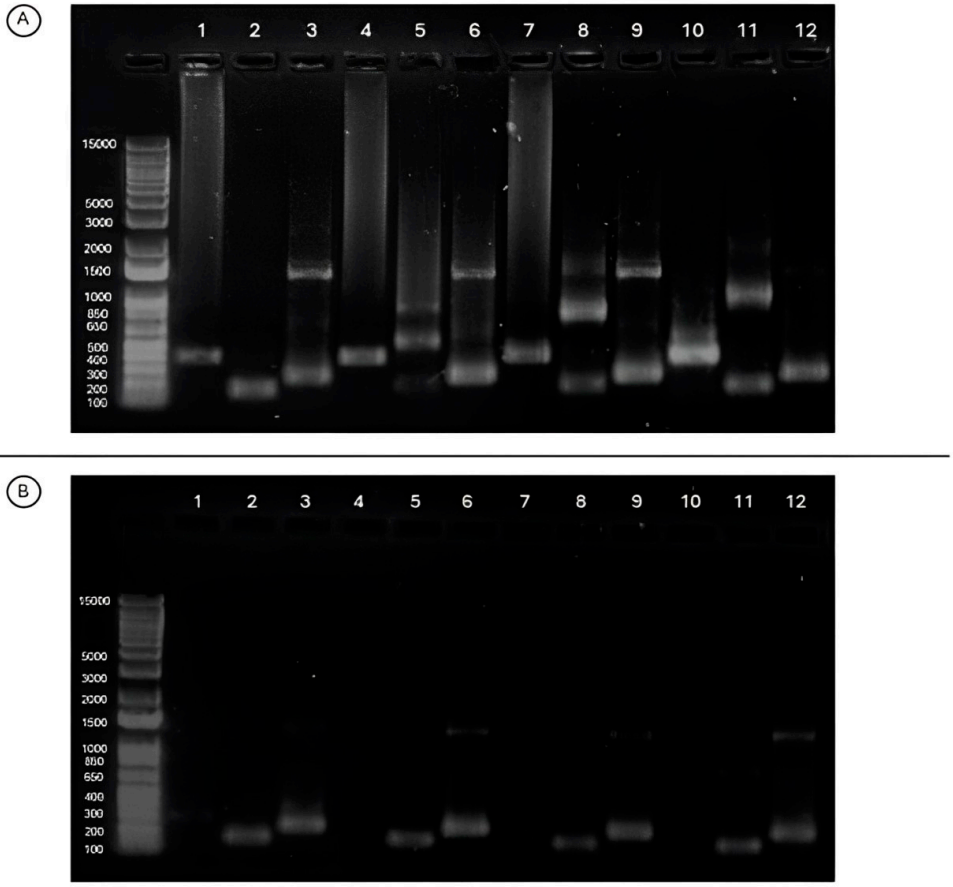

Figure 4. Nested-PCR results of Acute Lymphoblastic Leukemia samples. These Nested-PCR results are from the same patient's samples analyzed in the amplification plot figure. (**A**) Shows the *BCR::ABL1* (1 and 7—bone marrow; 4 and 10—peripheral blood), *GAPDH* (2 and 8—bone marrow; 5 and 11—peripheral blood), and *HPRT* (3 and 9—bone marrow; 6 and 12—peripheral blood) detection in ALL patients. (**B**) Shows the TCF3::PBX1 (1 and 7—bone marrow; 4 and 10—peripheral blood), *GAPDH* (2 and 8—bone marrow; 5 and 11—peripheral blood), and *HPRT* (3 and 9—bone marrow; 6 and 12—peripheral blood) detection in ALL patients.

Moreover, it was possible to observe that 62.3% of the AML patients did not present any of the cytogenetic alterations investigated in this study, which is not surprising since adult AML is mainly driven by point gene mutations [43–46]. This profile predominated in male patients, with a mean age of 55.5 years. Regarding clinical characteristics, these patients presented Hb < 10 g/dL, a leukocyte count of >10,000/mm^3, and circulating blasts in peripheral blood. Of the 48 patients, 14 had no karyotype results, 16 had normal karyotypes, and 18 had karyotypes with alterations such as translocations, additions, and inversions. In total, 23 patients died in 2 years (Table 2).

Only eight ALL patients did not have any of the cytogenetic alterations investigated in the study. Most of these patients were male, with a mean age of 41.4 years. The analysis of karyotype tests identified two complex karyotypes, two normal, one with the presence of translocations, and three patients had no results. In total, five patients died (Table 2).

Table 3 is a contingency table that shows the comparison between the detection capacities of karyotype, Nested-PCR, and RT-qPCR techniques. In this table, only samples from patients submitted to the three tests (karyotype, Nested-PCR, and RT-qPCR) were analyzed, so the sample number analyzed is not so expressive. The karyotype technique was performed only on bone marrow samples, while the Nested-PCR technique was carried out on samples from patients in whom some of the genetic alterations had already been detected. Overall, it was possible to observe that the karyotype presented low sensitivity

in the detection of genetic alterations when compared to the molecular methods. This is probably due to the low number of metaphases analyzed per study and, often, to the difficulty in collecting satisfactory samples for the test. In addition, the Nested-PCR technique also demonstrated low sensitivity, considering that in certain cases, such as in patients with *TCF3::PBX1*, detection was only possible in bone marrow samples. The RT-qPCR technique, on the other hand, observed that, in general, the sensitivity is satisfactory both in bone marrow samples and in peripheral blood samples. No statistical significance was observed in the Chi-square tests, as shown in Table 4.

Table 2. Clinical and epidemiological characteristics of patients without identified molecular alterations.

	N° of Patients	Gender	Age (Mean)	Hb	WBC	Blasts in PB	Karyotype	Deaths
AML	48	Male: 26 Female: 22	55.5 years	Hb < 10: 47 Hb > 10: 1	WBC < 10,000: 22 WBC > 10,000: 26	Yes: 30 No: 18	Complex: 5 Normal: 16 Other alterations: 13 NR: 14	23
ALL	8	Male: 5 Female: 3	41.4 years	Hb < 10: 7 Hb > 10: 1	WBC < 10,000: 3 WBC > 10,000: 5	Yes: 4 No: 4	Complex: 2 Normal: 2 Other alterations: 1 NR: 3	5

Hb: Hemoglobin; WBC: White Blood Cell Count; PB: Peripheral Blood; AML: Acute Myeloid Leukemia; ALL: Acute Lymphoblastic Leukemia; NR: Not Reported.

Table 3. Contingency table of number of detections by Karyotype, Nested-PCR, and RT-qPCR techniques.

		ALL Genetic Alterations			AML Genetic Alterations				
Sample Type	**Test Type**	***BCR::ABL1***	***TCF3::PBX1***	**Total**	***CBFB::MYH11***	***FLT3-ITD***	***PML::RARA***	***RUNX1::RUNX1T1***	**Total**
BM									
	Karyotype	4	3	7	1	0	6	6	13
	Nested-PCR †	6	2	8	0	0	0	0	0
	RT-qPCR	14	4	18	1	4	3	6	14
	Total	24	9	33	2	4	9	12	27
PB									
	Karyotype *	0	0	0	0	0	0	0	0
	Nested-PCR †	10	1	11	0	0	0	0	0
	RT-qPCR	22	7	29	7	4	7	7	25
	Total	32	8	40	7	4	7	7	25
Total									
	Karyotype *	4	3	7	1	0	6	6	13
	Nested-PCR †	16	3	19	0	0	0	0	0
	RT-qPCR	36	11	47	7	8	10	13	38
	Total	56	17	73	8	8	16	19	51

AML: Acute Myeloid Leukemia; ALL: Acute Lymphoblastic Leukemia; BM: Bone Marrow; PB: Peripheral Blood. * The karyotype technique is performed only with bone marrow samples. † The Nested-PCR technique was performed only on samples from patients in whom some of the genetic alterations had already been detected.

Regarding the Nested-PCR methodology, of the five genetic alterations related to ALL patients studied, only two were detected, being *BCR*::*ABL1* and *TCF3::PBX1*. It was not possible to detect any of the four genetic alterations investigated regarding AML patients through this technique.

After all the analyses, it was possible to establish that the RT-qPCR methodology presented high sensitivity for detecting molecular changes in both bone marrow and peripheral blood samples from patients with acute leukemias. The opposite was observed in the Nested-PCR analysis, where the detection of changes occurred mostly in bone marrow samples, even if the patients had high leukometry and a number of circulating blasts over 20%.

Table 4. Chi-squared results.

	Chi-Square AML Patients			Chi-Square ALL Patients		
Sample Type		**Value**	***p***		**Value**	***p***
BM	X^2	4.970	0.174	X^2	1.109	0.574
	N	27		N	33	
PB	X^2	-	*	X^2	-	*
	N	25		N	40	
Total	X^2	5.365	0.147	X^2	2.099	0.350
	N	52		N	73	

AML: Acute Myeloid Leukemia; ALL: Acute Lymphoblastic Leukemia; BM: Bone Marrow; PB: Peripheral Blood. * The X^2 could not be calculated—at least one row or column contains all zeros. This table aims to compare the detection sensitivity in bone marrow and peripheral blood samples by RT-qPCR.

4. Discussion

According to WHO, morphological analysis, immunohistochemistry, and flow cytometry techniques still play a fundamental role in the diagnosis of acute leukemias. And, in addition to these techniques, there are others that are of great help to the diagnostic process, such as karyotyping and molecular biology techniques such as RT-qPCR and FISH. FISH is a molecular cytogenetics technique that allows the analysis of chromosomal rearrangements [4,5,47].

The *PML::RARA* fusion was detected in 13% of the participating AML patients, corroborating the findings of the literature that established that this alteration is found in approximately 5–20% of all cases of the disease [48–50].

However, the other cytogenetic abnormalities observed in AML were detected at lower frequencies compared to other studies. In this study, 9% of the patients had the *RUNX1::RUNX1T1* translocation, 10.4% had the *CBFB::MYH11* fusion, and 5.2% had the *FLT3-ITD* mutation. However, the frequencies usually described in the literature are about 15%, 5–7%, and 20–25%, respectively [51–59].

The opposite was observed in ALL patients. This study showed incidences of 57.5% of *BCR::ABL1* translocation and 22.5% of *TCF3::PBX1* fusion. These frequencies are higher than what is normally reported in the literature, where *BCR::ABL1* and *TCF3::PBX1* represent 50% and 5% of adult ALL cases, respectively [60–66].

In addition, this study was able to compare the efficiency of Nested-PCR and RT-qPCR techniques in the diagnosis of cytogenetic changes in acute leukemia patients, demonstrating that the real-time PCR method has a considerably higher sensitivity than Nested-PCR.

In the literature, controversial results are observed regarding the Nested-PCR technique. Some works, such as those by Lin et al. (2019), Strom et al. (1998), Grote et al. (2002), and Lan et al. (1994) determined this methodology as a reliable method with high sensitivity for the diagnosis of several diseases [67–70]. However, studies by Alvarez-Martínez et al. (2006), Hafez et al. (2005), and Kortela et al. (2021) corroborate the data of the present research that indicates the use of more efficient methods such as RT-qPCR [71–73]. In general, the use of the Nested-PCR technique has been indicated in cases where diagnosis by simple conventional PCR is not sufficient [74–76].

Although the RT-qPCR technique has some disadvantages such as high equipment cost, high necessity of technical ability, and increased risk of false-negative results due to human error, it is still considered one of the best techniques for the rapid and effective diagnosis of various diseases [71,77]. It is undeniable that RT-qPCR has revolutionized

the molecular diagnosis of several diseases and that, after the COVID-19 pandemic, it is widespread in many of the laboratories specialized in diagnosis around the world. This is an effective, fast and more sensitive technique when compared to other PCR methods and other molecular techniques [78–81].

The present study demonstrated that the RT-qPCR technique presented a higher sensitivity compared to the Nested-PCR technique at the time of diagnosis of the acute leukemia samples studied. This was also seen in the works of Alvarez-Martínez et al. (2006), da Costa Lima et al. (2013), and Hafez et al. (2005) who demonstrated that the RT-qPCR methodology is more sensitive and fast in the detection of several diseases when compared to Nested-PCR. RT-qPCR proved to be very efficient for the rapid and sensitive diagnosis of genetic alterations in both types of samples analyzed in this study. Although Nested-PCR is still considered the gold standard methodology for the diagnosis of genetic alterations in acute leukemias, it is a methodology that requires a lot of standardization and a lot of time, becoming disadvantageous when compared to RT-qPCR [71,72,82].

As reported in Table 3, the diagnosis of cytogenetic alterations through classical cytogenetics, that is, through karyotype examination, is insufficient in many cases. Nordkamp et al. (2009) conducted a study that demonstrated that, despite ensuring reliable results, the karyotype test had low sensitivity in the detection of cytogenetic changes. This is probably due to the analysis of a few metaphases and the low quality of the sample collected. Karyotype examination requires bone marrow collection. However, in many cases, the material's collection is unsatisfactory or impossible due to such infiltration of leukocytes in the bone marrow. In addition, another issue of this test, at least in Brazil, is the delay in the delivery of results, which often makes it impossible for patients to receive the most appropriate therapeutic intervention quickly. However, the use of karyotype examination is still very useful in several other cases. Given this, the WHO suggests the use of molecular biology techniques as complementary tests, considering that they are fast and efficient techniques [4,83].

The proposal of this study to include the RT-qPCR technique in the list of tests for the diagnosis and monitoring of acute leukemias aims precisely to allow the rapid and reliable detection of genetic changes that can influence the prognosis of these patients, ensuring that they obtain target-directed treatments and, consequently, lower mortality rates due to therapeutic toxicity and better quality of life [3,84].

5. Conclusions

This work has demonstrated the importance of developing more sensitive molecular biology techniques that can integrate the panel of tests for the diagnosis and monitoring of acute leukemias. It was possible to detect the four genetic alterations associated with AML (*PML::RARA, RUNX1::RUNX1T1, CBFB::MYH11,* and *FLT3-ITD*) in the population studied. In the ALL cases, of the five alterations that were investigated, only two were detected (*BCR::ABL1* and *TCF3::PBX1*).

In addition, the study demonstrated that the RT-qPCR and Nested-PCR techniques presented good sensitivity in the detection of molecular abnormalities in acute leukemia samples. Nonetheless, in our experience, the RT-qPCR technique demonstrated higher sensitivity compared to the Nested-PCR method at the time of diagnosis of the acute leukemia samples studied, taking less time and using a smaller amount of reagents. With this in mind, RT-qPCR allows the diagnosis and monitoring of the disease status in patients quickly and reliably and can also be carried out using peripheral blood samples, which is very useful in cases where bone marrow collection is insufficient or unsuccessful.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dna4030019/s1, Table S1. Nested-PCR primers sequences. In this table, the primers sequences that were used in the Nested-PCR for the genetic alteration detection are listed. Table S2. RT-qPCR probe identification. In this table, the assays from Thermo Fisher that were used in the RT-qPCR for the genetic alteration detection are listed.

Author Contributions: F.M.C.d.P.P., M.B.d.O., A.S.K. and C.A.M.-N. conceived and the study; F.M.C.d.P.P., M.B.d.O., I.V.B., A.K.d.C.M., R.M.R., D.S.d.O., K.M.C.d.A., L.A.G., F.A.C.S., G.S.L., R.P.G.V., J.A.A., J.C.M., A.d.R.M., M.S.d.A.F., M.O.d.M.F., M.E.A.d.M., A.S.K. and C.A.M.-N. analyzed the data; F.M.C.d.P.P. and C.A.M.-N. wrote the manuscript; F.M.C.d.P.P., M.B.d.O., A.S.K. and C.A.M.-N. collected the data; M.O.d.M.F. and C.A.M.-N. obtained the funding. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by Brazilian funding agencies: National Council of Technological and Scientific Development (CNPq grant number 404213/2021-9 to CAM-N; and Productivity in Research PQ scholarships to MOdMF, MEAdM, ASK, and CAM-N), and Cearense Foundation of Scientific and Technological Support (FUNCAP grant number P20-0171-00078.01.00/20 to FMCdPP, MOdMF and to CAM-N). The Authors would like to thank PROPESP/UFPA for the publication payment.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of CEARÁ FEDERAL UNIVERSITY (4339719, approved on 15 October 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or data interpretation; in the writing of the manuscript, or in the decision to publish the results.

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