Development and Validation of a One-Step RT-qPCR Assay for Identifying Common Fusion Gene Transcripts Associated with the Prognosis of Mexican Children with B-Lineage Acute Lymphoblastic Leukemia

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Abstract

The aims of the present study were to optimize the detection of common fusion gene transcripts associated with childhood B-ALL prognosis through the development and validation of a one-step RT-qPCR assay and to identify the prevalence of these genes in a cohort of pediatric patients attended in public hospitals in Mexico City. The one-step RT-qPCR assay was sensitive, specific, easy to use, fast and cost efficient. The standard curves were linear, with amplification efficiencies between 97-107%; coefficients of variation were 2.1-3.0% in reproducibility and 2.6-3.8% in repeatability, with agreement between two different laboratories. The prevalence of fusion gene transcripts was *ETV6-RUNX1*, 10.3%; *TCF3-PBX1*, 7.5%; *BCR-ABL1p*^{190}, 3.6%; and *KMT2A-AFF1*, 2.8%. The prevalence of the *ETV6-RUNX1* fusion was low, consistent with the findings in Hispanics. The *KMT2A-AFF1* fusion was not exclusively present in infants, as we also found it in patients 4-16 years of age, in whom it was related to a poor prognosis. *TCF3-PBX1* patients were associated with an intermediate outcome. The present study highlights the importance of the detection of common genetic fusions in Mexican children with ALL, taking into account that some have molecular subtypes associated with a poor prognosis.

1. Introduction

B-lineage acute lymphoblastic leukemia (B-ALL) is a malignant transformation of B-lymphoid cell precursors characterized by uncontrolled proliferation and accumulation of leukemia blasts in the bone marrow [1]. B-ALL is the most frequent malignancy diagnosed in children and adolescents worldwide [2]. In the Mexican population, it is the second leading cause of death in the group aged between 5–14 years, with an estimated incidence of 49.5 cases per million children under 15 years of age [3]. Previous studies conducted in Mexico City have pointed out the importance of continuing with the refinement of diagnosis and prognostic stratification schemes to increase the survival rates in our population [4, 5]. Fusion gene transcript detection has contributed to improved survival rates in children from other populations, mainly in developed countries [6].

The presence of translocations in cancer reflects the genomic instability of cells and may characterize specific pathways involved in malignancy; however, the mechanisms that cause these translocations are poorly understood. To date, some mechanisms have been proposed to explain chromosomal rearrangements, such as illegitimate V(D)J recombinase activity or switch recombination, as well as nonhomologous end joining (NHEJ) after double-stranded break DNA (DSB) occurs on two different chromosomes [7, 8]. Translocations, often associated with hematological malignancy, have been postulated as one of the main causes of oncogenic transformations associated with leukemogenesis during fetal development [9, 10]. However, they are not sufficient by themselves to trigger the leukemic process. Additional cooperating mutations are usually required [1, 11]. Different studies have reported the detection of t(8;21) and t(12;21) translocations in newborns at a rate of ~ 1%. These translocations have been associated with an increase in the risk of developing B-ALL [11, 12].

Genes involved in translocations that fuse to the preferred partner or other less frequent genes may result in the deregulation of one of the genes involved (underexpression of proto-oncogenes or inhibition of the tumor suppressor gene) or in the expression of oncogenic proteins such as *BCR/ABL1* [9]. The study of these translocations has allowed the characterization of four common subtypes in children with B-ALL: t (4; 11) *KMT2A-AFF1*, t (12; 21) *ETV6-RUNX1*, and t (1; 19) *TCF3-PBX1*, which alter gene expression, and t (9; 22) *BCR- ABL1*, which disturbs signaling pathways.

For these common subtypes, the *BCR/ABL1* fusion gene produces a hybrid transcript that encodes a protein with tyrosine kinase activity leading to transformation. Both *MLL* (*KMT2A*) and *ETV6* (*TEL*) have been labeled "promiscuous" genes because they are rearranged with more than 80 and 160 different partner genes, respectively [13]. MLL (*KMT2A*) is mainly rearranged with AF4 in B-ALL pediatric patients. *KMT2A-AFF1* fusion is associated with childhood de novo leukemias originating in utero, and patients tend to have a poor prognosis. Another translocation encoding a protein with transformative properties is t(1;19)(q23;p13), creating a *TCF3-PBX1* fusion associated with the pre-B immunophenotype mainly associated with isolated central nervous system relapse that is present in approximately 5% of children with B-ALL [14].

Therefore, accurate detection of fusion genes in patients with B-ALL has prognostic implications favoring chemotherapy decisions, improving the therapeutic effect in terms of decreasing treatment-related toxicity and improving patient survival. Despite its undeniable importance, in Mexico City, detecting a wide range of fusion genes that may be associated with B-ALL prognosis can be complicated in terms of time and resources. Various molecular biology and cytogenetic techniques, such as banding analysis (karyotyping), fluorescent in situ hybridization (FISH), reverse transcription polymerase chain reaction (RT-PCR) and, more recently, second-generation sequencing techniques [15], have been used for fusion gene transcript detection.

Real-time qPCR has facilitated the diagnosis of a variety of fusion genes because it can quantify and control very small amounts of nucleic acids while amplification occurs. The primary objective of this study was to optimize the detection of rearrangements associated with B-ALL prognosis using one-step RT-qPCR in which the synthesis of cDNA and qPCR are performed in a single reaction, improving the sensitivity and the time for obtaining valid results to timely diagnose and stratify a patient with leukemia. In the present study, it was also possible to identify the prevalence of the most common fusion gene transcripts associated with the prognosis of B-ALL in a cohort of pediatric patients attended in public hospitals in Mexico City over a two-year period.

2. Methodology

We conducted the present study in two stages: A) development of the method and B) analysis of clinical samples. An overview of the research described here is presented in Fig. 1.

A) Development of the method

2.1 Primer and probe design

RT-qPCR for each fusion gene transcript that is clinically relevant in pediatric leukemia (*ETV6-RUNX1, TCF3-PBX1, BCR-ABL1p190, KMT2A-AFF1)* and an endogenous control transcript (*GAPDH*) were performed using specific primers and TaqMan probes. Gene sequences of each fusion transcript and *GAPDH* were obtained from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) for alignment and design procedures. The specific primers and probes were designed using the website Primer3Plus software program, and a BLAST search was performed to ensure specificity to the fusion target region. The melting temperature (Tm) should be 60 ± 3°C and 70 ± 3°C for primers and probes, respectively, and hairpin formation should not occur. Expected amplicons should be between 80–150 pb. The position of the primers is shown in Supplementary Fig. 1. At this moment, the sequence of primers and probes is protected for the reason of a patent claim.

2.2 Positive RNA controls

To evaluate the primer sets, we used total RNAs derived from Reh (ATCC® CRL-8286™), SUP-B15 (ATCC® CRL-1929™) and RS4;11 (ATCC® CRL-1873™) leukemia cell lines for ETV-6-RUNX1, BCR-ABL1, and *KMT2A-AFF1* fusion, respectively. For *TCF3-PBX1* fusion, we used RNA derived at diagnosis from a positive patient (P300). Cell lines were maintained in RPMI-1640 medium (Gibco BRL) or Dulbecco's medium supplemented with 10% fetal calf serum, 1 nM glutamine, and 50 mg penicillin/streptomycin in a saturated humidified atmosphere with 5% CO_2 at 37°C.

2.3 Selection of optimal one-step RT-qPCR TaqMan system for testing fusion genes

To select an appropriate real-time one-step RT-qPCR kit for this standardization, we compared the efficiency of different commercially available technologies: a) QuantiTect Probe RT-PCR (Qiagen, Venlo, Netherlands), b) AgPath-ID One-Step RT-PCR Reagents (Thermo Fisher Scientific), c) Superscript III One-Step RT-PCR (Invitrogen), and d) FastGene® PROBE One Step (NIPPON Genetics Europe, Germany). Each method was performed according to the manufacturer's instructions on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) using positive RNA controls, probes and primers.

2.4 Primer, probe and total RNA concentration

As total RNA was added directly to the RT-qPCR mixture, we determined the maximum (optimal) amount of total RNA that could be added without an inhibitory effect. We used different concentrations of positive RNA controls, which were diluted in series to obtain the following final amounts: 50, 100, 200, 300, and 500 ng/reaction. Different gradients of temperature alignment (56–64°C), primer concentrations (200, 250 and 300 nM), and probe concentrations (50, 125, 250 and 500 nM) were used to optimize PCR conditions for each common fusion gene.

2.5 Cloning of fusion gene transcripts, preparation of standard curves and efficiency determination

Plasmids containing the *GAPHD, ETV6-RUNX1, KMT2A-AFF1, BCR-ABL1p*^{190} and *TCF3-PBX1* cDNA transcripts were generated by cloning the PCR products amplified from RNA extracted from cell lines and one patient (*TCF3-PBX1)*, as described in Pakakasama et al. (2008) [16], into the pJET1.2 vector using the CloneJET™ PCR Cloning Kit (Thermo Scientific™). Then, plasmids were transformed and propagated in *Escherichia coli* JM109, extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific™) and sequenced using an ABI3500xL genetic analyzer (Applied Biosystems, Waltham, MA, USA). Plasmid DNA concentration was calculated using a Qubit dsDNA BR assay (Life Technologies Co., Carlsbad, CA, USA) to improve the accuracy of quantification. We converted DNA plasmid size to copy numbers by multiplying the number of base pairs of each plasmid (*ETV-6-RUNX1, BCR-ABL1, KMT2A-AFF1, TCF3-PBX1*) by the average molecular mass of one base pair (~ 660 g/mol) to obtain the approximate mass and then divided by Avogadro´s constant: N_A= 6.022 x 10^{23} molecules. The plasmids were serially diluted as follows: 1.0 × 10^2, 1.0 × 10^3, 1.0 × 10^4, 1.0 × 10^5, and 1.0 × 10^6 copies/reaction with each dilution, in quadruplicate. Linear regression was performed between the cycle threshold value (Ct-value) and the log10 of the copy number. The amplification efficiency for each fusion reaction was calculated using the equation E = 10(-1/slope) and converted to E% by (E − 1) × 100.

2.6 Efficiency and specificity assays

Plasmids with the *TCF3-PBX1, ETV6-RUNX1, BCR-ABL1*^{190}, *KMT2A-AFF1, EP300-ZNF384, CREBBP-SRGAP2B, DNAH14-IKZF1, ETV6-SNUPN*, and *ETV6-NUFIP1* fusion genes were used to test the existence of cross-reactions. A mixture of all plasmids was prepared at 300,000 copies/reaction and tested for each *TCF3-PBX1, ETV6-RUNX1, BCR-AB1P*^{190}, and *KMT2A-AFF1* fusion in one real-time PCR protocol, including the positive RNA and water controls. Additionally, a set of total RNA samples of clinical isolates with positive and negative results for different fusion genes were used.

2.7 Blind comparisons using QuanDx

The reliability and sensitivity of the one-step RT-qPCR assay developed in this study was compared with the QuanDx Kit, a qualitative in vitro diagnostic test for the screening of 30 fusion genes involved in chronic and acute leukemia, at a separate laboratory (Hospital Juarez de Mexico). Forty-five clinical samples were blindly analyzed at that hospital using the QuanDX Kit.

2.8 Repeatability and reproducibility

To evaluate the repeatability and reproducibility of the one-step RT-qPCR assay, we used positive RNA controls for each transcript. Regarding the assessment of repeatability, the detection of each transcript was independently tested four times on five days by the same operator, while for the reproducibility, each fusion was analyzed four times by three different operators on three different days for all fusion genes and the internal control *GAPDH*.

B) Analysis of clinical samples

2.9 Patients

This study was conducted from January 2018 to December 2019 and included bone marrow available samples obtained from 252 patients below the age of 19 years newly diagnosed with ALL from eight public hospitals in Mexico City. The study was approved by the National Ethics and Scientific Committees (R-2015-785-121). Written informed consent was obtained from the child´s parents to participate in the study. The diagnosis of leukemia was based on the morphology of leukemic cells and immunophenotyping. Table 2 contains relevant information of patients.

2.10 RNA isolation from bone marrow samples

Total RNA was extracted from white blood cells (WBCs) of bone marrow using Direct-zol MiniPrep (Zymo Research, USA) according to the manufacturer's protocol. The RNA was suspended in a final volume of 30 µl elution buffer and stored at -80°C until use. Concentrations of RNAs were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA).

2.11 Clinical samples: the quality of total RNA

The quality of RNA was examined using 100–150 ng of total RNA by analysis of glyceraldehyde-3´-phosphate dehydrogenase (*GAPDH)* transcript copy numbers determined by plasmid DNA standard curves. The copy number needed to be greater than 2.5 X 10^5, which is considered a suitable RNA quantity for RT-qPCR screening purposes, as has been previously reported [17]. Only samples and RNA cell lines with sufficient quality were analyzed.

2.12 Reverse transcription–polymerase chain reaction (RT-PCR) for fusion gene detection

The reproducibility and repeatability tests were carried out with the QuantiTect Probe RT-PCR Kit with 5 µl of 2x QuantiTect Probe RT-PCR Master Mix containing ROX™ as passive reference dye and HotStarTaq® DNA Polymerase, 0.1 µl QuantiTect RT Mix, 200 nM each primer, and 500 nM each TaqMan probe in a final volume of 50 µl. Thermal cycling conditions were as follows: 30 min at 50°C, 15 min at 95°C, followed by 38 cycles of 94°C for 15 sec and 60°C for 1 min. Amplification reactions contained 50–100 ng of the total RNA. GAPDH was used as an internal gene control for each sample.

2.13 Statistical analysis

Statistical analyses were performed using SPSS IBM (Statistical Package for the Social Sciences, Inc., Version 21, Chicago, IL, USA. [18] The prevalence of the four most frequent gene rearrangements in pediatric ALL was obtained. The Kaplan-Meier method was used to estimate overall survival (OS) and disease-free survival (DFS) rates. The log-rank test was used to compare the survival curves at the < 0.05 level of significance.

3. Results

3.1. Application of one-step RT-qPCR TaqMan for fusion gene transcript detection

The first step in this study consisted of the development of a combination of primers and TaqMan probes for the detection of the fusion gene transcripts *ETV6-RUNX1, KMT2A-AFF1, BCR-ABL1p190* and *TCF3-PBX1.* One-step RT-qPCR for detecting these alterations was used in the present study. It is considered an advantageous method because it is less expensive, reduces process time, and requires limited handling of samples, thus reducing pipetting errors, cross contamination between RT and PCR steps, and other sources of error. We tested the predesigned probe and oligonucleotides with different kits, and we observed differences in Ct values among different kits. With the QuantiTect Probe (Qiagen) kit, the best results were obtained with Ct between 16.7 and 27.5, while the other kits were greater than Ct 21.8 (Supplementary Fig. 2). Therefore, we proceeded to use the QuantiTect probe kit for the following tests to standardize the detection of fusion gene transcripts. Additionally, the Positive RNA controls showed satisfactory results using a QuantiTect probe kit (Table 1).

3.2 Optimization of one step RT-qPCR

To optimize the one-step RT-qPCR reaction, we performed a temperature gradient experiment using control positive RNA that was subjected to 6 different annealing temperatures (58°C – 64°C). We found that the optimal annealing temperature was 60°C for detection of all fusion transcripts, and no amplification product could be observed above 62°. Additionally, the optimal concentration of the different primer and TaqMan probe sets used was determined. We found significant differences among the various primers, but not among probe concentrations. Based on the results, a final concentration of TaqMan probes of 0.5 µmol/l was selected for detection of all fusion gene transcripts. The highest detection was obtained at a primer concentration of 0.4 µmol/l for all.

In addition, we were able to detect fusion genes using only 50–100 ng of total RNA with good analytical sensitivity for detecting ≥ 1000 copies/µl of transcripts using a different set of primers and probes for each fusion gene. Otherwise, the problems related to the presence of gDNA, degradation of RNA and contaminants that can inhibit RT or PCR were solved by isolation of RNA using column-based purification protocols and DNAse treatment (Direct-zol™RNAMiniprep, Zymo Research, California, USA) compared to other methods, such as RNAzol® RT (Sigma-Aldrich, Missouri, USA) or TRIzol® Reagent (Thermo Fisher Scientific, Massachusetts, USA) (data not shown).

3.3 PCR primers and probe design resulted in good amplification efficiency and specificity.

Construction of plasmids was used to obtain standard reference curves to infer the levels of transcripts of interest from clinical samples or cell lines (Fig. 2). GAPDH mRNA was used as an internal control for the evaluation of RNA integrity. In the GAPDH calibration curve, the Ct was linearly proportional to the logarithm of the number of input copies by at least five orders of magnitude (R2 = 0.9973, E%=107, Fig. 2E). To be considered adequate, the Ct values of the GAPDH signal must be less than 20, while Ct values above 20 may not be related to the quantification and quality of good RNA (Table 1). The amplification efficiencies obtained were 101% for *ETV6-RUNX1*, 97% for *KMT2A-AFF1*, 98% for *BCR-ABL1p190* and 106% for *TCF3-PBX1*. Efficiency was calculated using primer and probe sets considering a

slope (S) of the linear regression curve indicating an optimal PCR in which the number of copies was duplicated in each cycle. Typical calibration and amplification curves are shown in Fig. 2 (A-E).

Assay specificity was evaluated by testing RNA from the bone marrow of patients without leukemia but with various hematological diseases (e.g., pancytopenia, chronic myeloid leukemia, idiopathic thrombocytopenic purpura) and from plasmid mixtures. The results were negative for the detection of fusion gene transcripts (Ct value: 40), indicating the absence of nonspecific amplifications related to the detection of spurious sequences.

Additionally, in parallel with our one-step RT-qPCR method, independent testing of 45 samples was conducted in an external laboratory (Hospital Juarez de Mexico1) using a QuanDX kit. The results showed 100% concordance between the two laboratories when the four fusion genes of interest were analyzed (Supplementary Table 1). Out of the 45 samples analyzed, only in 7 (15.5%) was a fusion gene transcript detected. Two were positive for ETV6-RUNX1 (4.4%), three were positive for *TCF3-PBX1* (6.6%) and two were positive for *BCR-ABL1p190* (4.4%). One sample tested with the QuanDX kit was positive for another MLL-ENL fusion gene (sample 20), and another sample showed RUNX1/CBFA2T3 fusion (sample 1) in addition to ETV6-RUNX1 (both fusion genes were not included in the one-step RT-qPCR) (Supplementary Table 1).

3.4 Reproducibility and repeatability of real-time RT-PCR.

To assess interlaboratory variation, positive RNA controls were tested by three independent analysts at different time periods. The Ct values for all the fusion genes are listed in Table 1. We obtained good precision, and the coefficients of variation (CV) of reproducibility and repeatability were 2.1-3.0% and 2.6–3.8%, respectively (Table 1).

Table 1
Precision of OneStep RT-PCR Taqman Assay

Assay		**Reproducibility**			**Repeatability**		
Cell line	**Target**	**Mean (range)**	**SD**	**CV (%)**	**Mean (range)**	**SD**	**CV (%)**
REH	*GAPDH*	17.1 (16.1–17.9)	0.4	2.6	16.9 (16.0 -17.9)	0.5	3.0
	TEL-RUNX1	26.4 (25.6–27.7)	0.6	2.3	26.3 (25.0–27.7)	0.7	2.6
P300	*GAPDH*	19 (18.4–19.6)	0.4	2.1	18.5 (17.4–19.4)	0.6	3.5
	E2A-PBX1	26.5 (25.6–27.9)	0.7	2.6	26.3 (25.0 -27.9)	0.8	2.9
RS4	*GAPDH*	17.4 (16.7–18.1)	0.5	2.7	17.3 (16.0-18.1)	0.5	3.1
	KMT2-AFF1	26.4 (25.4–27.5)	0.6	2.4	26.9 (26.0 -28.4)	0.7	2.7
SUP-B15	*GAPDH*	17.0 (16.0-17.9)	0.5	3.0	16.7 (15.3–17.8)	0.6	3.8
	BCR-ABL1 p190	26.1 (25.1–27.1)	0.6	2.4	26.0 (24.4–27.0)	0.7	2.9

Table 1. The results shown are the Ct means (ranges) of the independent determinations, with the standard deviation (SD) and coefficient of variation (CV) shown. (*) Independent assays were performed four times on five different days for repeatability and (**) four times on three days for reproducibility.

3.5 Prevalence and prognostic impact of fusion gene transcripts

Samples from 253 children with newly diagnosed ALL were analyzed. Samples with good quality for analysis were considered. A total of 136 boys (52.1%) were included with a mean age of 8.6 years (range, 0.2–17.7 years). The mean follow-up time was 398 days (95% CI: 378–419 days) for disease-free survival (DFS) and 404 days (CI: 383–425 days) for overall survival (OS). During the follow-up, 17 (6.7%) patients died, and 12 (4.7%) relapsed (Table 2). Seven patients had Down's syndrome, one of whom had the transcript *BCR-ABL1p*^{190}. Of the samples analyzed by one-step RT-qPCR, 75.9% were negative for the four fusion genes analyzed, while only 24.1% (61/253) were positive. The prevalence of fusion gene transcripts was as follows: *ETV6-RUNX1* (10.3%), *TCF3-PBX1* (7.5%), *KMT2A-AFF1* (2.8%) and *BCR-ABL1p*^{190} (3.6%) (Table 2).

Table 2
Clinical features of patients with ALL by molecular subtype

	ETV6-RUNX1	*TCF3-PBX1*	*BCR-ABL1 p^{190}*	*KMT2A-AFF1*	*Nondetected*
	n = 26	**n = 19**	**n = 9**	**n = 7**	**n = 192**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Sex					
Male	18 (69.2)	9 (47.4)	5 (55.6)	4 (57.1)	100 (52.1)
Female	8 (30.8)	10 (52.6)	4 (44.4)	3 (42.9)	92 (47.9)
Age (years)					
< 1	-----	-----	-----	-----	3 (1.6)
1-4.9	10 (38.5)	7 (36.8)	1 (11.1)	1 (14.3)	54 (28.1)
5-9.9	11 (42.3)	4 (21.1)	2 (22.2)	2 (28.6)	58 (30.2)
10-14.9	2 (7.7)	7 (36.8)	5 (55.6)	2 (28.6)	56 (29.2)
≥ 15	3 (11.5)	1 (5.3)	1 (11.1)	2 (28.6)	21 (10.9)
Down's syndrome					
Yes	-----	-----	1 (11.1)	-----	6 (2.8)
No	26 (100)	19 (100)	8 (88.9)	7 (100)	186 (97.4)
WBC count (cells/ul)					
< 50,000	22 (84.6)	18 (94.7)	8 (88.9)	4 (57.1)	185 (96.4)
≥ 50,000	4 (15.4)	1 (5.3)	1 (11.1)	3 (42.9)	7 (3.6)
NCI-risk classification					
Standard	16 (61.5)	8 (42.1)	1 (11.1)	1 (14.3)	107 (55.7)
High	10 (38.5)	11(57.9)	8 (88.9)	6 (85.7)	85 (44.3)
Relapse					
Yes	-----	1 (5.3)	1 (11.1)	3 (42.9)	7 (3.6)
No	26 (100)	18 (94.7)	8 (88.9)	4 (57.1)	185 (96.4)
Death					
Yes	1 (3.8)	1 (5.3)	3 (33.3)	1 (14.3)	11 (5.8)

	ETV6-RUNX1	*TCF3-PBX1*	*BCR-ABL1 p^{190}*	*KMT2A-AFF1*	*Nondetected*
	n = 26	**n = 19**	**n = 9**	**n = 7**	**n = 192**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
No	25 (96.2)	18 (94.7)	6 (66.7)	6 (85.7)	181 (94.2)

Kaplan-Meier estimations for OS and DFS by considering each fusion gene transcript are shown in Fig. 3. As indicated, there were no significant differences in OS; in contrast, the differences in DFS were statistically significant among the fusion genes (p = 0.02).

ETV6-RUNX1 was the most common fusion gene transcript (10.3%), present in all age groups, mainly between 1.0 and 10 years. Approximately 84.6% of these patients had a white blood cell count below 50,000, and 61.5% were classified as standard risk. In this study, almost all patients with this fusion gene transcript remained in first complete remission at the time of conclusion of this study; only one exhibited a poor response and died (Table 2, Fig. 3). It therefore had a favorable impact on DFS and OS (100% and 96%, respectively). The least prevalent fusion gene transcript was *KMT2A-AFF1*, observed in 2.8% of patients aged 1–16 years at diagnosis. These patients showed a higher overall white blood cell count (> 50,000, 42.9%) at the time of diagnosis. Approximately 85.7% of these were classified as high risk, three relapsed, and one died. *KMT2A-AFF1* patients displayed low DFS and OS rates (56% and 80%, respectively). On the other hand, the t(1;19) translocation (q23;p13) causing *TCF3-PBX1* was present in nineteen patients (7.5%) aged 1–16 years. Of these, 94.7 had a white blood cell count below 50,000 at the time of diagnosis. Eleven (57.9%) were classified as high risk, one relapsed and one patient died. In these patients, this translocation had a favorable impact on DFS and OS (89% and 88%, respectively). Finally, transcribed *BCR-ABL1p^{190}* showed a positivity rate of approximately 3.6%, and most patients achieved complete remission. One relapse and three deaths occurred within this molecular subgroup. OS was 63% while the DFS was 86%.

4. Discussion

In children with ALL attended at public hospitals in Mexico City, the identification of common fusion gene translocations associated with the prognosis of the disease is not routinely performed, but is available through research projects such as the present study. The purpose of this study was to identify the prevalence of *ETV6-RUNX1, KMT2A-AFF1, TCF3-PBX1*, and *BCR-ABL1p190* fusion genes using a one-step TaqMan RT-qPCR assay and present the technical details of this assay. In Mexico, as in other countries, several groups have published different protocols for the detection of leukemia-specific fusion transcripts using conventional RT-PCR, and valuable prognostic information on human leukemias has been provided [16, 19–21]. To improve the specificity and timeliness of the detection of common fusion gene transcripts, the abovementioned assay was developed and validated in the present study. All one-step RT-qPCR assays for each fusion gene showed good sensitivities and specificities. The sensitivity could be

explained, at least in part, by the elimination of the cDNA synthesis step using reverse transcription prior to PCR. Currently, a different enzyme with a higher efficiency is available in one-step kits. In our work, we compared different one-step kits and found that QuantiTect Probe RT-PCR (QIAGEN) showed high sensitivity for detecting the four fusion genes assessed.

Another important aspect of the one-step RT-qPCR assay was the precision obtained. It was estimated by the coefficient of variation, which reflects the day-to-day differences and variations of operators with different levels of experience. This coefficient was acceptably low to propose the use of this assay. Our results also showed high repeatability and reproducibility, and all cross-reactivity assays demonstrated that the primers and probes were specific for each fusion gene. The results of the interlaboratory comparison showed an acceptable concordance of results.

While commercially available multiple fusion gene transcript detection kits are relatively expensive, when considering the importance of detecting the four most common fusion gene transcripts associated with prognosis, the developed one-step real-time RT-PCR assay may be more cost-effective than QuanDx. This is supported by the fact that major problems for the determination of common fusion genes in Mexican health institutions are the lack of resources and specialized personnel.

Prevalence and prognostic impact of common fusion genes

It is well recognized that the B-lineage ALL (B-ALL) subtype has large genetic heterogeneity. Of importance, the fusion gene transcripts evaluated here are relevant for stratifying the risk of children with this disease (Table 2, Supplementary Fig. 3).

The frequencies of *ETV6-RUNX1, TCF3-PBX1, KMT2A-AFF1* and *BCR-ABL1p190* were 10.3%, 7.5%, 2.8% and 3.6%, respectively, similar to a previous study conducted by our research group where a comparable number of clinical samples were analyzed [22]. Of note, the prevalence of these four fusion gene transcripts was 24.2%, which is similar to the 20% detection reported in a recent study carried out in southern Mexico using an in vitro diagnostic test (HemaVision-Q28, DNA Diagnostic) [23]. Nonetheless, it has a low prevalence compared with studies conducted in other countries, particularly developed nations [24, 25]. This highlights the importance of discovering new molecular determinants possibly contributing to the high incidence and mortality of ALL in the Mexican population.

ETV6-RUNX1

ETV6-RUNX1 is the most prevalent fusion gene transcript reported in children with ALL from developed countries (~ 25%), and it is associated with a favorable prognosis of the disease, currently reaching survival rates higher than 90% at 5 years of follow-up [26]. However, in the present study, the analysis revealed that 26 of 253 cases (10.3%) presented the *ETV6-RUNX1* fusion; most were male (69.2%), with a < 50,000 WBC count (84.6%) in peripheral blood at diagnosis. It was present at all ages but most commonly observed in children under 10 years of age (Table 2). The low prevalence of *ETV6-RUNX1* is consistent with previous Mexican reports using different detection methodologies: 8.7% [27], 8.5% [28],

7.4% [22], 6.9% [23], 13.5% [19], and 14.9% [29]. These results are similar to those reported in Hispanics living in other countries 4.5% in Guatemalan and 14% in Hispanic residing in the Northern California [30, 31]. This finding is probably due to the genetic background of these populations [32].

However, it also raises concerns about whether this finding is due to a misdiagnosis. In this regard, we used RT-qPCR, which is considered a highly sensitive method to detect fusion gene transcripts. In addition, it is well known that fusion genes may have different breakpoints, making them undetectable using standard methods; however, this phenomenon occurs infrequently. Notably, the full identification of fusion gene transcripts is possible using next-generation sequencing (NGS) technology [33]. In this context, in a previous study by our research group using RNA-Seq analysis of 24 bone marrow samples of children with ALL, only one patient was *ETV6-RUNX1* positive, which was consistent with the prevalence found in the present study[34]. Conversely, we found a novel fusion gene, *ETV6-NUFIP1* and *ETV6-SNUPN*, that interrupts the *ETV6* gene with break sites in the first and second exons, respectively; therefore, it is possible that these novel fusions play a causal role in tumorigenesis [34]. Nevertheless, although the prevalence of genetic fusion in the Mexican population is low, several groups have reported other genetic alterations related to *ETV6* and *AML1* genes, such as deletions or duplications. Perez-Vera, 2005 reported 35% alterations in *AML1* copy numbers in LLA, and structural changes in *ETV6* increased the number of *ETV6* and *AML1* consequences of polysomy of chromosomes 12 and 21, which play significant roles in disease progression [28].

TCF3-PBX1

The *TCF3-PBX1* t(1;19)(q23;pl3.3) translocation results in the expression of a protein that combines the transactivation domains of *TCF3* with the DNA binding homeodomain region of *PBX1*[35]. *TCF3-PBX1* fusion can promote cell transformation both in vitro and in vivo. Interestingly, the existence of a loss-of-function allele of TCF3 as a consequence of translocations in this gene has also been proposed. It reduces the levels of wild-type TCF3, which contributes to the development of leukemia [36]. Because TCF3 plays an important role as a mediator of B cell differentiation, TCF3 functions as a negative regulator of tumorigenesis [37]. The prevalence reported for *TCF3-PBX1* fusion gene transcripts varies between 3–5% in childhood ALL; however, in this study, the prevalence was 7.5% (19 of 253 patients). *TCF3-PBX1* patients were distributed across all age groups, particularly in children with < 50,000 WBCs at diagnosis. One child relapsed, and another died early during treatment (16.2 and 17.7 months after diagnosis, respectively). *TCF3-PBX1*-positive ALL has been related to an intermediate disease prognosis [29, 38]. Nonetheless, currently, with the increase in the intensity of the subgroup of *E2A-PBX1*-positive patients, they have a similar survival as those patients classified as standard risk [39]. In the present study, we observed a DFS of 89% in patients with *TCF3-PBX1*, which correlates with previous reports in children with ALL and *TCF3-PBX1* positivity. Importantly, in public hospitals in Mexico City, the *TCF3-PBX1* group showed significant improvements in survival rates over time (data in preparation to publish) after the detection of this rearrangement through research project funding. In particular, the adverse prognostic value of these rearrangements has been overcome by using contemporary treatments such as Berlin-Frankfurt-Münster (BFM) [40, 41] and CCLG-ALL2008, a risk-based intensified treatment trial [42].

BCR-ABL1 p ^{190}

The t(9;22)(q34;q11) reciprocal translocation encodes a tyrosine kinase and is considered a pathogenetic driver that can be therapeutically targeted. There are several breakpoints in BCR that produce distinct BCR-ABL1 isoforms in children with ALL. In patients with chronic myeloid leukemia, the most common is the BCR-ABL1 p^{220} isoform, while in the ALL subtype, it is the p^{190} isoform associated

with a dismal prognosis [43]. However, treatment with tyrosine kinase (TK) inhibitors has improved the overall survival of patients with this rearrangement [18]. In the present study, the BCR-ABL1p^{190} translocation was identified in 3.6% of patients, with the majority being over 10 years of age at the time of diagnosis confirmation, which is similar to that reported in other populations. The survival analysis showed low survival rates (DFS: 86% and OS: 63%) in patients with this translocation, but they are similar to those reported in other studies [44]. Several mechanisms have been related to relapse and death among patients with *BCR-ABL1*. Among these mechanisms is resistance to imatinib due to mutations within the ABL1 (tyrosine kinase) domain of BCR-ABL1 (T315I). Another important mechanism is secondary chemotherapy resistance for different reasons. Therefore, it is necessary to study all the possible causes in our population, such as long-term adverse effects, resistance to TKIs, effects of other chemotherapy drugs and treatment noncompliance. In addition, a periodic follow-up of patients would be important to know the proportion of children with resistance to imatinib and to propose alternative treatments.

KMT2A-AFF1

The *KMT2A-AFF1* t(4;11) fusion was observed in this study in children between 4–16 years of age. This frequency highlights that this rearrangement is not exclusive to infants with ALL, as has been previously reported [13]. Patients with ALL and the t(4;11) translocation have a poor prognosis [12]. In the present investigation, *KMT2A-AFF1* patients had a poor prognosis with a high frequency of relapse, which was reflected in a low DFS (56%) (Fig. 3).

Mexico is a country with limited financial resources where the detection of fusion rearrangements is not routinely performed in all hospitals due to high cost and lack of trained personnel in molecular biology. This assay could be used routinely to detect common fusion genes in children with ALL and may contribute significantly to clinical outcomes.

5. Conclusions

In conclusion, we developed a one-step RT-qPCR method that provides a sensitive and reliable assay for the rapid detection of four common fusion genes in pediatric patients with ALL. It could also provide less time to detect these alterations with cost savings. Moreover, the present study highlights the importance of the detection of common genetic fusions in Mexican children with ALL, taking into account that some have molecular subtypes associated with a poor prognosis. Mexico is a country with limited financial resources where the detection of fusion rearrangements is not routinely performed in all hospitals due to

its high cost and the lack of trained personnel in molecular biology. This assay could be used routinely to detect common fusion genes in children with ALL and may contribute significantly to clinical outcomes.

Declarations

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Conflicts of Interest: The authors declare no conflicts of interest.

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Figures

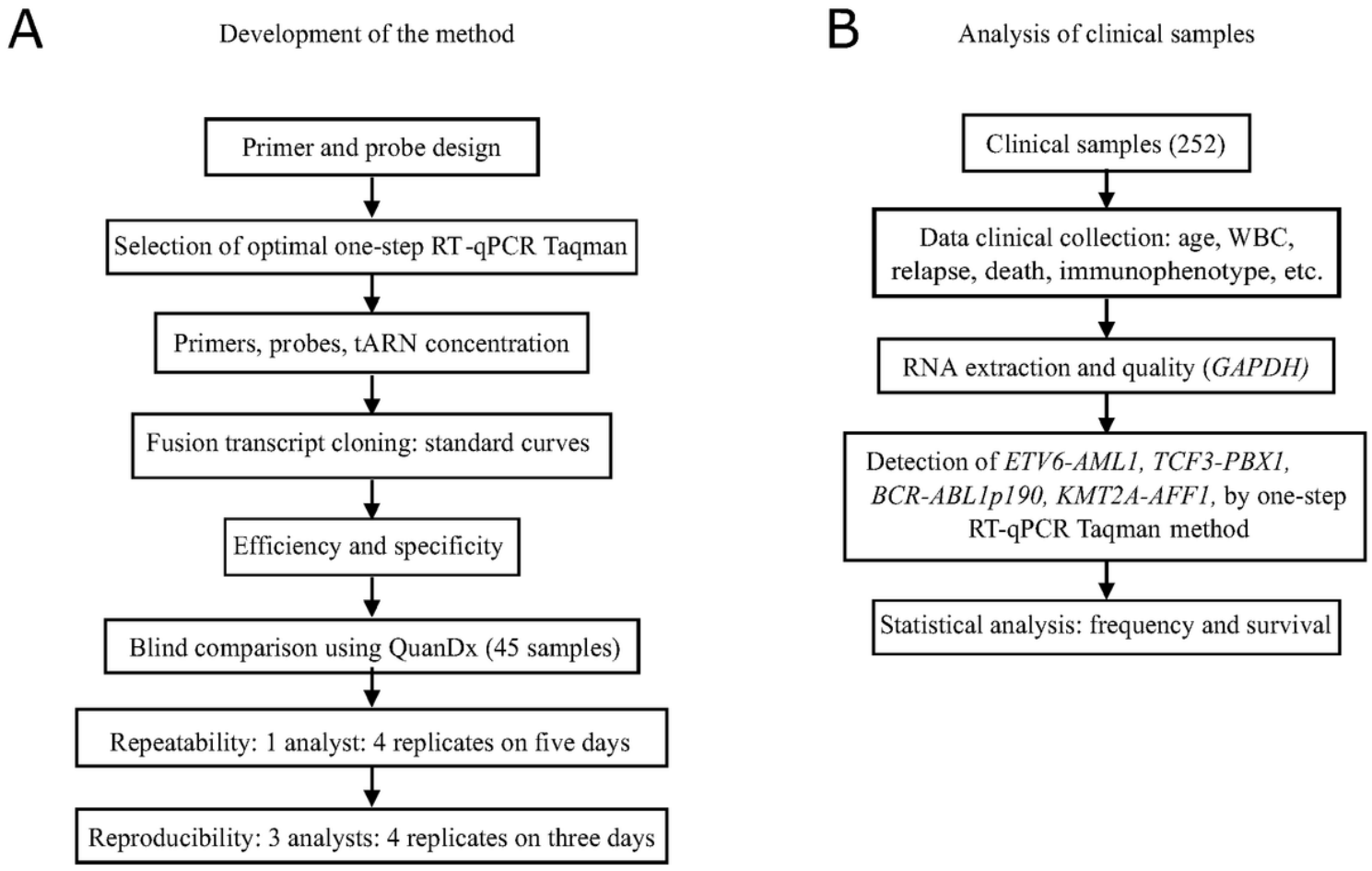

Figure 1

Schematic diagram showing the research workflow. The two main stages of research were A) development of the method (design, concentration optimization, efficiency, repeatability, reproducibility) and B) analysis of clinical samples (sample validation, RT-qPCR assay, data collection and data analysis).

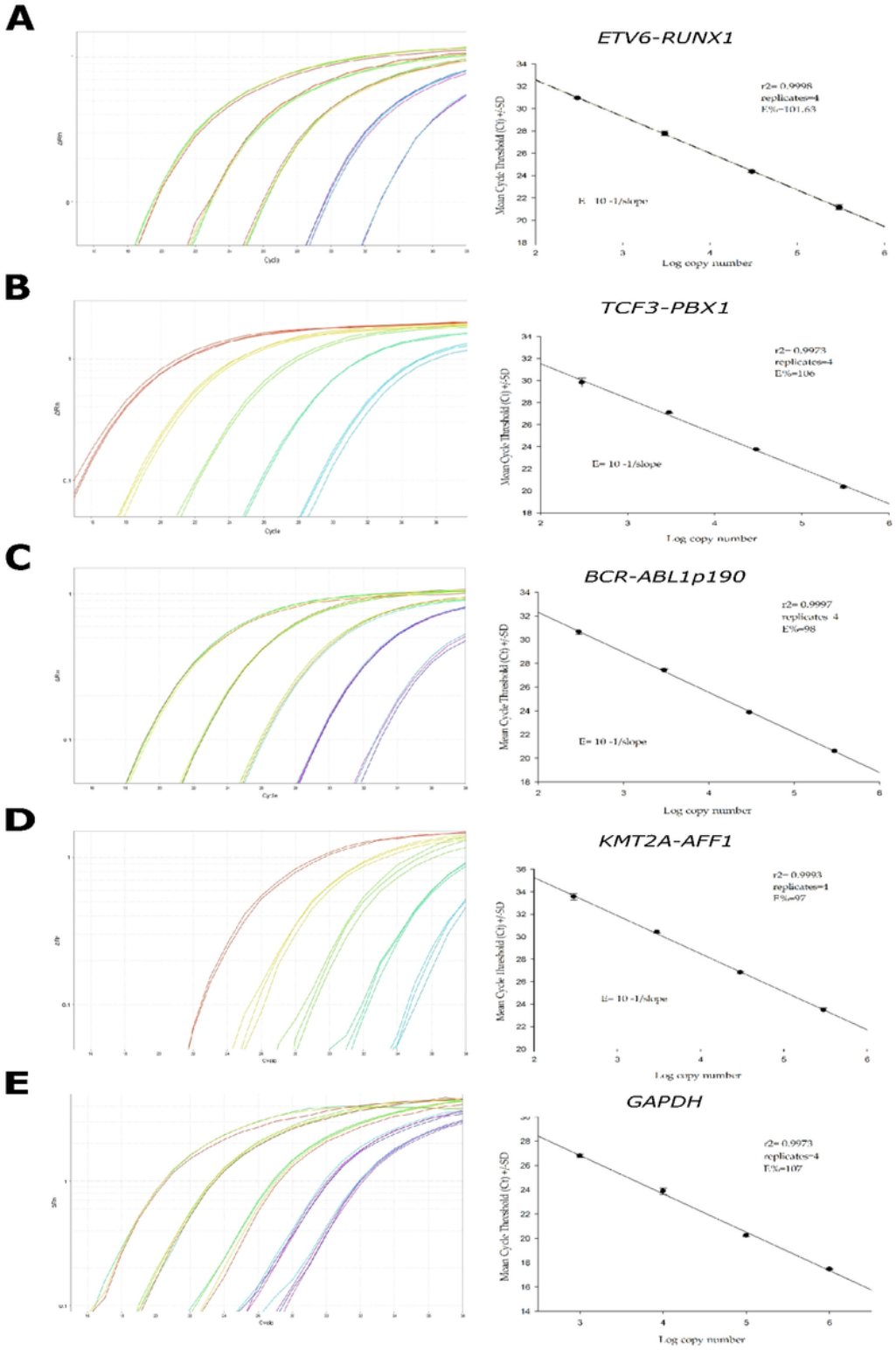

Figure 2

Linear dynamic range of one-step RT-qPCR using plasmid DNA. The standards used in this study were well-characterized plasmid DNA dilutions that had been directly quantitated using a Qubit fluorometer. The standard curve for real-time RT-PCR was generated from the Ct values plotted against 10-fold serial dilutions (4×106 to 4×102 copies). Triplicate samples were used for the assay. Amplification plot and standard curve of ETV6-RUNX1 (A), TCF3-PBX1 (B), BCR-ABL1p190 (C), KMT2A-AFF1 (D) and GAPDH (E).

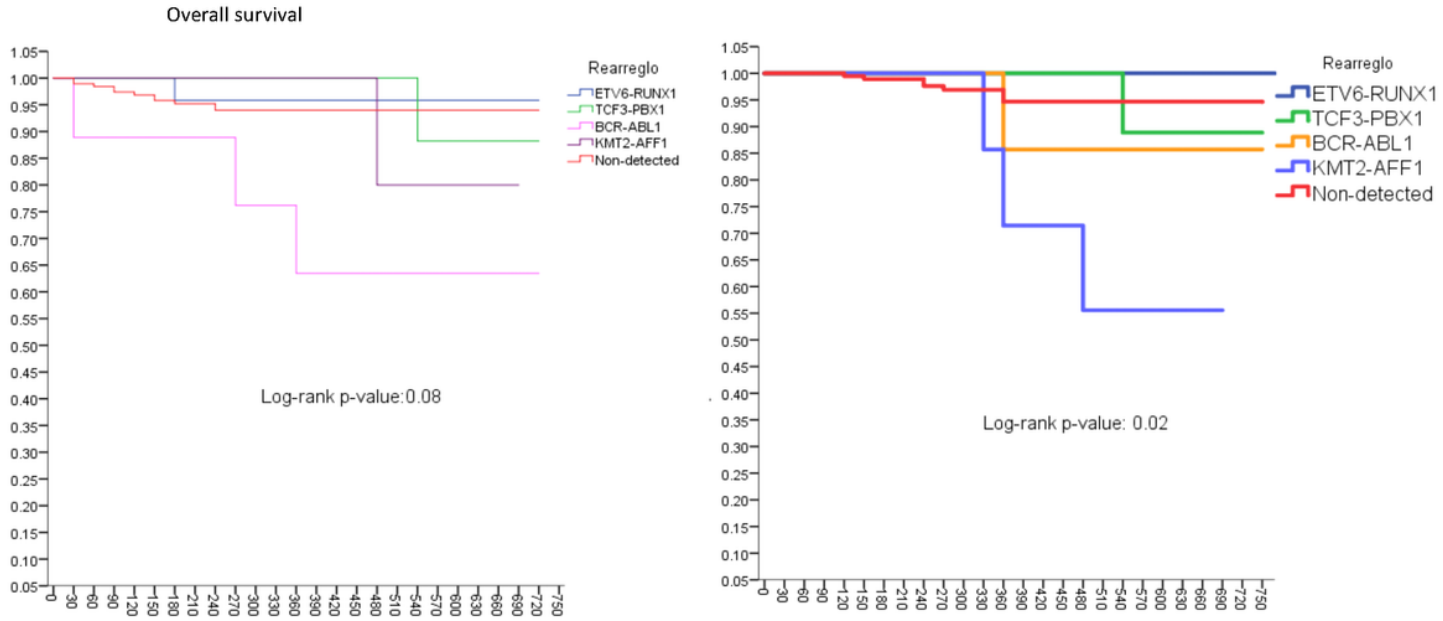

Figure 3

Overall survival (OS) and disease-free survival (DFS) rates for childhood with ALL by each fusion gene. Outcome was significantly better in patients with ETV6-RUNX1, TCF3-PBX1 and nondetected fusion gene transcripts than in those with rearranged KMT2A-AFF1. DFS was 100, 97, 89, 86, and 56% and overall survival (OS) was 96, 94, 88, 63, and 80% for ETV6-RUNX1, no fusion gene transcripts detected, E2A-PBX1, BCR-ABL1p190, and KMT2A-AFF1, respectively.

Supplementary Files

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