Clin. Lab. 2018;64:XXX-XXX

SHORT COMMUNICATION

Use of the Polymerase Chain Reaction as a Complementary Method for the Detection of Central Nervous System Involvement in Children and Adolescents with Acute Lymphoblastic Leukemia

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SUMMARY

Background: **Cytological analysis of the cerebrospinal fluid (CSF) remains the most widely used method for diagnosing central nervous system (CNS) involvement in acute lymphoblastic leukemia (ALL). This study aimed at evaluating the use of polymerase chain reaction (PCR), in comparison to other methods, for the assessment of the presence of blast cells in the CSF at the time of diagnosis of ALL.**
Methods: **This was a prospective, single-centre, study enrolling all patients up to the age of 18 years who were admitted to a university hospital between November 2011 and November 2014 with a diagnosis of ALL and from whom it was possible to draw a sufficient amount of CSF for analysis by conventional cytology (CT), immunophenotyping (IMP), and PCR.**
Results: **A total of 46 CSF samples from 44 ALL pediatric patients were included. CT was performed in all samples, IMP in 44, and PCR in 34. Thirteen (28.2%) samples showed positive results: two by CT, four by IMP, four by PCR, and three by both IMP and PCR.**
Conclusions: **The results of this study showed that PCR should be considered a complementary method for the evaluation of the CSF in ALL patients at diagnosis.**
(Clin. Lab. 2018;64:xx-xx. DOI: 10.7754/Clin.Lab.2017.170622)

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Short Communication accepted August 22, 2017

KEY WORDS

acute lymphoblastic leukemia, central nervous system, cerebrospinal fluid, polymerase chain reaction, diagnosis

INTRODUCTION

Cytological analysis of the cerebrospinal fluid (CSF) remains the most widely used method for diagnosing central nervous system (CNS) infiltration in acute lymphoblastic leukemia (ALL) [1,2]. However, cytology (CT) is not the ideal method, since CSF lymphocytes may present with atypical morphology, and this may hamper the distinction between a mature lymphocyte and a leukemic cell [2].
A number of diagnostic methods may be used, along

with the morphological assessment, in order to distinguish blasts from other cells. Immunophenotyping (IMP) and molecular biology, particularly polymerase chain reaction (PCR), comprise the main methods for this purpose [3,4].
In the present study, PCR was used for detecting CSF blast cell clonality at diagnosis or at relapse of ALL in children and adolescents. PCR results were compared to those of the other two methods used for evaluating the CSF, namely: CT and IMP.

MATERIALS AND METHODS

This study accrued 44 patients with a diagnosis of ALL who were admitted to Hospital das Clinicas of Federal University of Minas Gerais (HC-UFMG) between November 2011 and November 2014, and for whom it was possible to draw a sufficient amount of CSF to perform three diagnostic assays: conventional CT, IMP, and PCR. Two patients who were included at diagnosis also had CSF samples drawn at the time of relapse of their disease. All patients included at first diagnosis were treated using the Brazilian Group for the Treatment of ALL (GBTLI-LLA) protocol [5].
This study was approved by the Institutional Research Ethics Committee, and, for accrual, all patients had their informed consent forms signed by their parents or legal guardians.
In accordance with the GBTLI-LLA protocol, a cytological examination of the CSF was performed in all patients at diagnosis of ALL. For every accrued patient, whenever possible, a total of 5 mL of CSF was drawn and subdivided as follows: 1 mL for CT; 3 mL for IMP, and 1 mL for future PCR, since these samples were to be frozen at -80°C before the test was performed. CSF classification, according to the cytological analysis, was as follows: CNS 1- atraumatic lumbar puncture, absence of blasts; CNS 2 - atraumatic lumbar puncture, leukocyte count < 5/μL, presence of blasts; CNS 3 - atraumatic lumbar puncture, leukocyte count ≥ 5/μL, presence of blasts; TLP *negative* - traumatic lumbar puncture (TLP): ≥ 10 red blood cells/μL, absence of blasts; TLP *positive* - TLP and presence of blasts. CSF analysis using IMP by flow cytometry has previously been described by Cancela et al. [6]. By means of this method, the detection of an equal to or more than 10 cell cluster harboring the same IMP profile as that of the BM was considered as being positive for CNS leukemia.
For performing PCR, DNA extraction was used based on the technique of Biojone et al., whereas the method defined by the GBTLI Molecular Biology group and adapted from the BIOMED team was used for detecting clonal BM rearrangements at the time of diagnosis (Yunes et al, personal communication, Centro Infantil Boldrini, Campinas/SP, Brazil) [7-9]. DNA samples from the BM were amplified using 19 consensus primer mixes that are adjunct to the immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangement regions and that vary depending on the multiple subtypes of ALL. For the children with B-ALL subtypes, pairs of primers were used for screening for complete and incomplete gene rearrangements of *IgH* (VH-(DH)-JH, DH-JH), *IgK* (Vk-Kde, Intron-Kde), *TCRG* (Vg-Jg1.3/2.3 + Jg1.1/2.1), and *TCRD* (Vd2-Dd3, Dd2-Dd3). Those with T-ALL, in turn, were tested for complete and incomplete gene rearrangements of *IgH* (DH-JH), *TCRG* (Vg-Jg1.3/2.3 + Jg1.1/2.1), and *TCRD* (Vd-(Dd)-Jd1, Dd2-Jd1, Vd2-Dd3, Dd2-Dd3), as well as for microdeletions of the *SIL-TAL* genes (SIL-TAL1, SIL-TAL2). BM rearrangements identified for each patient at diagnosis were chosen for assessing neoplastic clonal cells in the corresponding CSF.
For each CSF specimen in which PCR was performed, 6 μL of extracted DNA was used. In samples where amplification was not possible, DNA integrity was assessed by testing for amplification of the FLT3 gene. PCR tests were performed using the *Veriti 96 Well Thermal Cycler* (*Applied Biosystems*) thermocycler. The negative control consisted of material without DNA, and a non-specific amplification control consisting of healthy DNA donors (PBL) was also used. After performing PCR, a homo/heteroduplex analysis was used for characterization of clonality [10].
Considering the fact that, in accordance with the GBTLI-LLA-2009 protocol, a parallel monitoring of minimal residual disease (MRD) was to be performed, specific primers for certain V-D-J junction regions of leukemic cells identified in the BM at diagnosis were accessible for some of the studied patients. For these patients, CSF was tested using real-time quantitative (RQ)-PCR. The methodology used for amplification, normalization, and data analysis of gene rearrangements through RQ-PCR was previously described by Paula et al. [11].
The *kappa* coefficient was used for the analysis of concordance between the methods used for CSF evaluation. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

A total of 46 CSF samples were obtained from the 44 patients accrued in this study (two of the patients included at diagnosis also had CSF samples included at relapse).
CT was performed in all of the 46 CSF samples, only two of which (4.3%) were positive for leukemic cells (one was classified as TLP *positive* and the other as CNS 2).
Likewise, IMP was performed in all 46 available samples. In two, there was no cell yield for analysis. Of the 44 remaining specimens, seven (15.9%) were considered positive for the presence of blasts.
All of the CSF samples were also tested using conventional PCR. In 12 (26%) of these, the FLT3 control

Table 1. Cerebrospinal fluid samples with blast cell positivity in at least one of the four studied methods in patients with acute lymphoblastic leukemia.

Patient	Cytology	IMP	PCR	RQ-PCR
1	NEG	POS	NEG	NP
2	NEG	POS	POS	NP
3	NEG	POS	NEG	NEG
4	NEG	POS	POS	POS
5	NEG	NEG	POS [a]	NEG
6	NEG	POS	NEG	NEG
7	NEG	POS	NA	NEG
8	POS [c]	NEG	NA	NEG
9	NEG	NEG	POS [d]	NP
10	NEG	NEG	POS [f]	NP
11	NEG	POS	POS	NP
12	POS [e]	NEG	NEG	NP
13	NEG	NEG	POS [b]	NP

IMP - immunophenotyping, PCR - conventional polymerase chain reaction, RQ-PCR - real-time polymerase chain reaction, NEG - negative, POS - positive, NA - control gene not amplified, NP - not performed, [a] - Weak gene amplification (doubt in interpretation of the results), [b] - Positive for the VH3 rearrangement and negative for V2D3, [c] TLP pos - positive traumatic lumbar puncture, [d] - Positive for the VGI rearrangement (weak amplification) and negative for V2D3, [e]- CNS 2, [f] - Positive for the VH3 rearrangement (weak amplification) and negative for V2D3.

Table 2. Concordance analysis of three techniques for evaluating the cerebrospinal fluid at diagnosis of acute lymphoblastic leukemia.

Concordance	Kappa	p-value	Classification
IMP x CT	-0.050	0.679	No agreement
PCR x CT	-0.054	0.605	No agreement
RQ-PCR x CT	-0.077	0.773	No agreement
IMP x PCR	0.325	0.064	No agreement
IMP x RQ-PCR	0.323	0.101	No agreement
PCR x RQ-PCR	0.600	0.064	Trend to agreement

IMP - immunophenotyping, CT - cytology, PCR - conventional polymerase chain reaction, RQ-PCR - real-time polymerase chain reaction.

Table 3. Results of the polymerase chain reaction (PCR) and cytology (CT) analyses of the cerebrospinal fluid (CSF) in different studies in patients with acute lymphoblastic leukemia (ALL).

	Positive PCR samples (%)	Positive CT samples (%)
Present study	20.6% *	4.3%
BIOJONE, 20127	49.2% *	4.6%
SCRIDELI, 200413	45.9% *	5.4%
PINE, 200514	20.0% **	16.6%
SAYED, 200912	60.0% **	22.2%

*** - Conventional PCR, ** - Real-time quantitative RQ-PCR.**

gene did not amplify. Amongst the remaining 34 specimens, seven (20.6%) showed a positive result for the presence of the same gene rearrangements seen in the BM at diagnosis of ALL. All of these seven patients had a B-immunophenotype.

RQ-PCR was performed in 16 of the available CSF samples. In two of these samples, no amplification of the control gene was observed. In the remainder, only one (7.1%) was positive for blasts. The sensitivity of the primers for RQ-PCR varied from 10^{-3} to 10^{-4}.

A total of 13 samples (28.3%) revealed a positive result for the presence of blasts based on at least one of the four methods (Table 1).

Comparison between the three methods (CT, IMP, and PCR) did not show any agreement (Table 2).

DISCUSSION

The present study was intended to evaluate the use of the PCR method as an additional diagnostic tool to conventional CT in the analysis of CSF in patients with ALL. As has been previously reported in the literature, there was a greater proportion of patients having leukemic cells detected in the CSF using PCR compared to that observed with conventional CT (Table 3).

A total of 28.3% of the analysed samples were positive for the presence of leukemic cells when combining the four methods (CT, IMP, PCR, and RQ-PCR). Sayed et al. conducted a similar study in children with ALL and noted that 26 (57.8%) samples yielded positive results: 10 by CT; 21 by IMP, and 12 by RQ-PCR, wherein only four of the samples were simultaneously positive in all three assays [12]. It is noteworthy that, in this same study, 12 patients presented with symptoms suggesting CNS involvement, which may account for the greater number of positive cases observed by these authors [12].

Despite the fact that it may offer a greater chance of identifying leukemic cells, PCR analysis of the CSF has some technical challenges that may impact the success of the exam and should be considered when interpreting its results [2,13]. In the current study, there was no control gene amplification in 26% of the samples evaluated through conventional PCR. Similarly, Sayed et al. reported that in 33% of their samples, RQ-PCR was not performed due to inadequate DNA material [12]. On the other hand, Biojone et al. noted only 9% of their samples to be inadequate [7]. A possible explanation for these unconformities may be the interference of inhibitors in this reaction. Another would be the absence of a sufficient number of cells or of DNA for performing the assay. Notably, the sensitivity of a test has a significant influence on the observed results. In the present study, when a reaction with a greater sensitivity, such as RQ-PCR, was used, six out of 12 samples that had not had the control gene amplified using conventional PCR were found to be positive for this gene.

When comparing the four methods used for the detection of blast cells in the CSF no agreement was shown. The two samples that had positive results by CT were negative using the other methods (IMP and PCR), which may suggest a false-positive result of the first technique. Amongst the samples showing isolated PCR positivity, three displayed a weak amplification. Importantly, in all three samples that were positive in both IMP and PCR assays, there was no doubt regarding the interpretation of amplification in the PCR gel. Comparison between conventional PCR and RQ-PCR showed some concordance, but the reduced number of amplified cases using RQ-PCR affected the analysis. RQ-PCR is a sensitive technique, but it harbors a greater cost and time for preparing the specific primer, which, in turn, reflects on a longer period of time to perform the test. In the case of CSF analysis, this may not be appropriate for a timely therapeutic decision-making process.

CONCLUSION

Based on the results presented herein, PCR was able to show a greater CSF blast cell yield compared to that of the other studied methods. Nonetheless, the impact of the presence of blasts in samples with fewer than 5 cells/μL in the CSF and the possibility that complementary methods, such as PCR, be included in the selection process of patients toward a more intensified CNS-targeted therapy need to be better refined using a larger study population and a longer follow up period.

Acknowledgement:
We are indebted to Antonio Vaz de Macedo, MD, MSc, from the Hematopoietic Stem Cell Transplant Unit of Hospital das Clinicas da Universidade Federal de Minas Gerais, for his valuable contributions to the elaboration of this manuscript.

Source of Support:
This work was supported by grants of Fundação do Amparo à Pesquisa de Minas Gerais (FAPEMIG).

Declaration of Interest:
The authors have nothing to declare.

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