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–ORIGINAL PAPERS –

Quantitative BCR::ABL1 Assay Using Digital Real-Time PCR System: Clinical Applications and Insights

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Abstract

Background: *Accurate quantification of BCR::ABL1 transcripts is paramount for the management of chronic myeloid leukemia (CML) and Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL). Despite advancements in real-time reverse transcription PCR (RT-qPCR), the need for more sensitive and precise assays remains. This study evaluated the diagnostic utility of digital reverse transcription PCR (dRT-PCR) for BCR::ABL1 quantification in assessing minimal residual disease (MRD).*

Material and methods. *Twelve patients with CML or Ph+ ALL were included. Peripheral blood or bone marrow samples, collected in PaxGene RNA preservation tubes, were extracted with the automated QIASymphony SP instrument. dRT-PCR conducted on the LOAA analyzer, utilized the Dr. PCR BCR-ABL1 Detection Kit CE_IVD (OPTOLANE Technologies, South Korea). By employing a dual-labeled hydrolysis probe and target-specific primers, this protocol enabled the simultaneous amplification and detection of BCR::ABL1 transcripts e13a2 and e14a2 and ABL1. The resulting copy numbers/reaction were used to calculate the International Scale percentage (%IS) and the molecular reduction (MR) value and analyzed for clinical correlations.*

Results*. Digital RT-PCR demonstrated high precision in quantifying BCR::ABL1 transcripts, the lowest %IS value being 0.02%. A consistent quality RNA sample totalizing 110 ng/reaction was crucial for successful reactions. This assay proved faster and easier than conventional RT-PCR and did not require standards or calibrators.*

Conclusion. *This study supports the feasibility of digital RT-PCR for quantitative analysis. Future objectives for our laboratory include expanding our diagnostic repertoire using this platform, for enhancing patient care.*

Keywords*: Digital PCR, BCR::ABL1, Optolane, Chronic Myeloid Leukemia, Acute Lymphoblastic Leukemia*

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Introduction

The BCR::ABL1 fusion gene arises from the reciprocal translocation between the BCR (breakpoint cluster region) gene on chromosome 22 and the ABL1 (Abelson tyrosine kinase 1) gene on chromosome 9, thus called the Philadelphia chromosome [1]. It results in a constitutively active tyrosine kinase, leading to uncontrolled cell proliferation and the development of several hematological malignancies, particularly chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and, less frequent, in acute myeloid leukemia (AML).

The presence of this fusion gene signals a poor prognosis in both AML [2] and in ALL [3], even with targeted tyrosine kinase inhibitors (TKIs), particularly imatinib, which have revolutionized treatment response rates over the decades [4]. Using both classical and advanced molecular biology techniques, it has been demonstrated that patients who do not respond to first-line TKI treatment exhibit resistance to this type of therapy [3,5,6]. This discovery has unveiled new resistance mechanisms, leading to the development of new generations of TKIs [7]. Among all responses to TKIs, achieving a major molecular response is the most important outcome for patients [8–10].

Following the latest guidelines, major molecular response (MMR) or molecular response (MR) 3.0 is defined as BCR::ABL1/ABL1 international scale (IS) ≤0.1% by real-time quantitative PCR (RT-qPCR), and deep molecular response (DMR) (≥MR4.0) is defined as BCR::ABL1/ABL1 IS ≤0.01% [8,9]. The MMR level of response is a crucial indicator of effective treatment in patients with chronic myeloid leukemia CML undergoing therapy with TKIs. Clinically, achieving MMR is associated with a reduced risk of disease progression and improved long-term survival rates [8,9]. Biologically, MMR reflects a substantial decrease in the leukemic cell burden, indicating effective suppression of the BCR::ABL1 oncogene. Accurate quantification of BCR::ABL1 transcripts is paramount for effective disease management [8].

The gold standard technique to measure BCR::ABL1 transcripts is real-time quantitative PCR (RT-qPCR). However, without following the minimum information for publication of quantitative real-time/digital PCR experiments (MIQE) guidelines, the validation and data analysis processes of the results can be unreliable [11–13]. Despite advancements in RT-qPCR technology, there remains a need to explore alternative laboratory workflows with enhanced sensitivity, precision and easier workflows [14,15].

In this paper, we aimed to evaluate the feasibility and potential clinical utility of introducing digital RT-PCR technology for BCR::ABL1 quantification in our laboratory, particularly for assessing minimal residual disease (MRD) status.

Material and Methods

Patients and samples

A total of twelve CML or Ph+ ALL patients, (6 males and 6 females) were included from the Hematology Department, University Emergency Hospital Bucharest. Samples were collected at diagnosis, after TKI treatment and at follow-ups. Samples were collected in PaxGene tubes (PAXgene® Blood RNA Tube, BD), with two patients providing bone marrow aspirates and the remaining 10 patients providing peripheral blood samples. All patients gave written informed consent, and the study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Automated RNA extraction was performed using the QIAsymphony PaxGene Blood RNA Kit on the QIAsymphony SP instrument (QIAGEN, Germany) and the RNA concentration and purity were checked through spectrometry using the NanoQuant Infinite 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland).

Digital PCR and data analyses

Digital RT-PCR assays were conducted on the LOAA analyzer (OPTOLANE Technologies, South Korea), utilizing the Dr. PCR BCR-ABL1 Major IS Detection Kit CE_IVD (OPTOLANE Technologies, South Korea). The kit employed a dual-labeled hydrolysis probe, TaqMan and target-specific primers, enabled the simultaneous amplification and detection of two BCR::ABL1 (Ch01) fusion transcripts (e13a2 and e14a2) and ABL1 (Ch02). The minimum threshold for a valid result, in the case of a negative sample for the BCR::ABL1 transcript, was 10000 ABL1 copies per reaction.

For each reaction, 10 µL of RNA template and 20 µL of PCR master mix were utilized, resulting in an approximate volume of 0.928 - 0.930 nL PCR-RNA reaction mix distributed to each of the 20000 wells of the dPCR cartridge. Valid wells (successfully filled with PCR-RNA mix) were recorded for each of the two channels: Ch01 (BCR::ABL1 transcript) and Ch02 (ABL1 reference gene).

Results consisted of the number of copies/reaction of BCR::ABL and ABL1. These were then used to calculate the percentage corresponding to the %IS and the log MR level for each individual sample. The kit's limit of detection and limit of quantification declared by the manufacturer, was 0.0025% IS (MR 4.6).

Results

Out of the 12 patients evaluated, three yielded inconclusive results due to failure to achieve the minimum threshold of 10000 ABL1 copies per reaction. Valid wells (successfully filled with RNA mixture out of the 20000 available) had counts which ranged from 12,891 to 18,686 for both channels. MR levels assessed varied across the cohort, ranging from 0.0376 to 3.6234 (Table 1). Additionally, IS% values determined for each patient ranged from 0.5485% to 91.697% (Table 1)

To assess the limit of detection of the LOAA system, we conducted a 1/200 dilution of the total RNA sample from patient #4. In the undiluted sample run, the BCR::ABL1 quantification yielded 1264.3 copies/reaction, with an associated IS% of 9.647% and an MR level of 1.0156. Subsequently, the diluted sample was analyzed, resulting in a BCR::ABL1 quantification of 5.2 copies/reaction, corresponding to an %IS of 0.023% and a MR of 3,62. The higher MR value was due to the higher ABL1 copy number. Notably, the ABL1 quantification value in the diluted sample was higher at 21212.3 copies/reaction.

Patient No.	Age (years)	Sex (M/F)	Sample Type (PaxGene BMA/PB)	Clinical Diagnosis	Test Result	Valid no. of wells	Ch01- Abs. Q. BCR-ABL1 (C/reaction)	Ch01- Abs. Q. BCR-ABL1 Thr.	Ch01- Positive Ratio (%)	Ch02- Abs. Q. ABL1 (C/reaction)	Ch02- Abs Q. ABL1 Thr.	Ch02- Positive Ratio (%)	% IS	MR Level	Well Image & Scatter Chart
1	52	F	BMA	B-ALL	Positive	18314	16520	494	40.07	55913,8	533	82.33	28.655	0.5428	
2	36	F	PB	CML suspicion	Positive	18036	9040	502	24.41	24992,7	511	53.84	35.115	0.4545	
3	73	M	PB	CML	Positive	14888	8105,7	528	22.22	27482	571	57.34	28.609	0.5435	
4	52	F	PB	B-ALL	Positive	18486	1264,3	497	3.84	12711,4	485	32.51	9.647	1.0156	
4*	52	F	PB	B-ALL	Positive	18661	5,2	498	0.02	21212,3	513	48.12	0,023	3.6234	
5	46	M	PB	CML	Inconclusive	18419	0	506	0.00	9467,2	490	25.39	0	N/A	
6	72	F	PB	CML	Inconclusive	18635	0	491	0.00	4479	461	12.94	0	N/A	
7	59	M	PB	CML blast crisis	Positive	18337	110822,7	495	96.76	117231,5	476	97.34	91.697	0.0376	
8	80	F	PB	CML	Inconclusive	18471	0	493	0	4058	469	11.80	0	N/A	
9	53	M	PB	CML suspicion	Positive	18072	26734,7	507	56.26	90310,8	495	93.88	28.714	0.5419	
10	27	M	BMA	ALL	Positive	17718	78387,3	467	91.15	179360,9	430	99.61	42.392	0.3727	
11	44	M	PB	CML under TKI	Positive	12891	30	569	0.09	5305,5	630	15.17	0.5485	2.2608	
12	67	F	PB	CML blast crisis	Positive	17831	333,5	555	1.03	16077,4	642	39.18	2.0121	1.6964	

Table 1: *Patients clinical data and dPCR data analysis*

ALL – acute lymphoblastic leukemia, B-ALL – B-cell acute lymphoblastic leukemia, BMA – bone marrow aspirate, CML – chronic myeloid leukemia, %IS – International standard, MR – molecular response, PB – peripheral blood, TKI – tyrosine kinase inhibitor, Thr – flourescence threshold value
4= repeated test with dilution factor of 1/200*
Within the scatter charts – blue positive wells represent the detection of BCR::ABL1 transcripts; where as red positive wells represent the detection of ABL1 transcripts.

Discussions

This study provides strong preliminary data supporting the feasibility and diagnostic utility of digital RT-PCR technology in our molecular pathology laboratory for quantitative BCR::ABL1 analysis. Digital PCR demonstrated good sensitivity and good detection limits, with %IS values reaching as low as 0.02% which is an equivalent MR 3.6. Apart from total RNA extraction, total sample preparation time was around 10-20 minutes, with the amplification and detection completed in 90 minutes. Also, this assay performed well in the dilution test for sample #4, in which the undiluted sample detected 1264.3

BCR::ABL1 per reaction, and the 1/200 dilution yielded a total of 5.2 BCR::ABL1 copies per reaction. This result is within the theoretical dilution of 6.3 copies (1264.3/200 BCR::ABL1 copies). Thus, confirming the method's capability to detect BCR::ABL1 transcripts at very low quantities.

We also observed inconclusive results from patients #5, #6, and #8, due to ABL1 copies falling below the threshold of 10000 copies per reaction, underscoring the importance of sample quality and RNA concentration in achieving reliable results. Although sample #9 also presented <10000 ABL1 copies per reaction (5305.5 ABL1 copies per reaction), we were able to validate the result due to the detection of the BCR::ABL1 transcript.

Although the initial application of the digital PCR method in our laboratory was limited to a small cohort of patients, composed mostly of patients at diagnosis and only one under treatment with TKI, the promising results obtained underscore the potential of this technique for routine in MRD monitoring.

However, the method is constrained by the available sample size and the number of partitions, which can affect the accuracy and reproducibility of the results. Regarding this aspect we did observe a change in the total number of ABL1 copies detected between sample #4 and the 1/200 dilution – where in the undiluted sample a total of 12711 ABL1 copies/reaction were detected, and in the diluted sample the copy number rose to 21212 ABL1 copies/reaction. This difference is indicative of an inhibition of the PCR reaction due to high copy number of ABL1 transcripts in the undiluted sample. To address these limitations, further refinement of the current protocol and optimization of workflows are necessary to maximize the method's sensitivity and reproducibility.

Compared to NGS, dPCR is less expensive and more specific for the absolute quantification of target genes but has a lower throughput capacity [16]. Whereas RT-qPCR remains an accessible and rapid method, its major disadvantage is the need of a standard curve and/or normalization using reference genes [17]. The choice of the appropriate technology depends on the specific needs of the laboratory, available resources, and type of results needed for the management of patients.

Our study highlights the clinical applicability of dPCR in improving patient outcomes through a readily available and reliable quantification of BCR::ABL1 transcripts. Furthermore, the method's independence from standards or calibrators enhances its diagnostic potential. Thus, we also consider that the digital RT-PCR method offers significant advantages in terms of sensitivity and accuracy, making it ideal for monitoring minimal residual disease in BCR::ABL1 positive hematological malignancies, as previously stated [18].

In the near future, our laboratory aims to expand its diagnostic capabilities by incorporating MRD testing for AML with recurrent mutations, focusing at first on the quantitative detection of NPM1 insertions [2]. By leveraging advanced molecular techniques, we seek to enhance our abilities to monitor disease progression, predict treatment response, and guide personalized therapeutic interventions for patients.

Conclusion

Overall, the integration of dPCR into routine clinical practice for the management of CML and Ph+ ALL represents a promising step forward in the pursuit of personalized medicine. Future studies with larger cohorts and long-term follow-up are warranted to further validate the clinical benefits of dPCR and to explore its potential in other settings of molecular diagnostics.

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Conflicts of interest

I undersign, certificate that I do not have any financial or personal relationships that might bias the content of this work. The authors declare no conflict of interest.

The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study.

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