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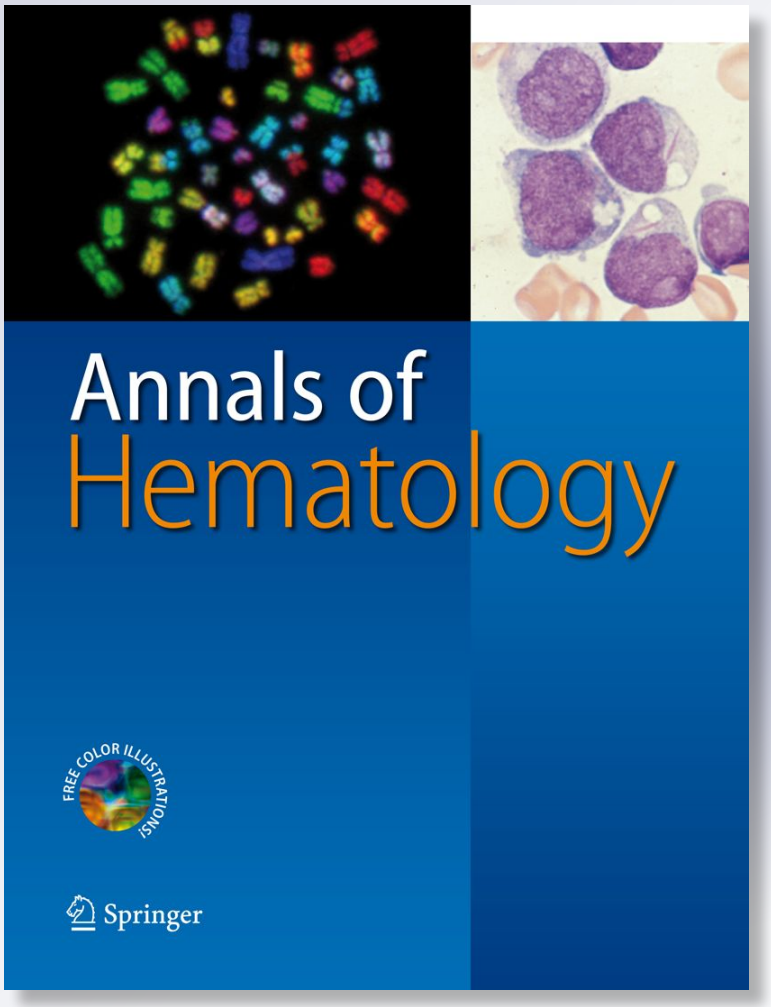

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ORIGINAL ARTICLE

Comparative study of *BCR-ABL1* quantification: Xpert assay, a feasible solution to standardization concerns

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Abstract The level of *BCR-ABL1* reached after treatment with tyrosine kinase inhibitors is an effective marker of the therapeutic response and a good survival predictor in chronic myeloid leukemia (CML) patients. However, no agreement has yet been achieved about either the standardization of the technique to determine *BCR-ABL1* or the interpretation of the results. The aim of this study was to compare the method currently recommended by the European Leukemia Net, which includes the application of a conversion factor to express the results in international scale, with an automated method (Xpert BCR-ABL™, Cepheid). *BCR-ABL1* transcript quantification was performed in 117 samples from CML patients in two different laboratories by both methods, and the results were compared by statistical procedures. A high linear correlation was obtained in the results between the two methods. The concordance at logarithmic intervals reached 62 %. When the major molecular response (MMR) was analyzed, 85 % agreement was achieved. The automated method provides reproducible results and does not show significant differences compared with the traditional method. As a clinical tool, Xpert correctly classified the patients in MMR and can be considered a useful alternative for the molecular follow-up of CML patients.

Keywords BCR-ABL · QRT-PCR · CML · MDR

Introduction

BCR-ABL1 appears after a chromosomal translocation, t(9;22)(q34;q11), which is detected cytogenetically as the well-known Philadelphia chromosome. The molecular rearrangement of both genes (*BCR* and *ABL1*), located in chromosomes 9 and 22, results in the fusion transcript *BCR-ABL1*, which codes a non-regulated tyrosine kinase protein with a higher activity, leading to chronic myeloid leukemia (CML). Therefore, *BCR-ABL1* constitutes a specific leukemic marker that can be employed for minimal residual disease (MRD) follow-up [1], and its quantification after treatment with tyrosine kinase inhibitors may predict the outcome of CML patients, particularly in those who achieve a complete cytogenetic response [2].

However, despite the efforts made for standardization of real-time quantitative (RQ)-PCR to determine *BCR-ABL1* RNA levels, significant variability can still be found in the methodology employed by different laboratories throughout the world. Such differences range from the technique used for RNA extraction or cDNA synthesis, to the standard

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curves that have to be calculated and applied, or the reference gene used for the quantification process [3]. Therefore, in order to evaluate the molecular response and make the results obtained by different laboratories comparable, the CML meeting at the National Institutes of Health in Bethesda in October 2005 proposed several recommendations for the harmonization of MRD assessment. As a consequence, on behalf of the European Leukemia Net (ELN), an international scale (IS) was elaborated for *BCR-ABL1* RQ-PCR measurements [4].

Recently, Cepheid has developed the Xpert BCR-ABL Monitor™ assay for the performance, on a GeneXpert® automated analyzer, of each step needed for the PCR (RNA extraction, retrotranscription, amplification of target and reference genes), and also for the establishment of a ratio between *BCR-ABL1* and the reference gene *ABL1* [5]. The aim of this study was to compare, using the same samples, the results of *BCR-ABL1* quantification, employing the manual RQ-PCR method proposed by the ELN including the application of the conversion factor (CF), and the automated Xpert assay.

Methods

Fifty-one bone marrow and 66 peripheral blood samples from CML patients at different stages of the disease were analyzed, belonging both to MRD and diagnostic studies, in order to include a wider quantification range. Fifty-eight determinations were performed at the Dr Negrin Hospital in Gran Canaria, Spain (LAB1) and 59 at Carlos Haya University Hospital in Malaga, Spain (LAB2). All the patients had previously signed an informed consent to collect and handle their samples for scientific purposes.

Quantification of *BCR-ABL1* transcripts was performed in two different platforms based on the different methods: one method, recommended by the "European Against Cancer" (EAC) Program, was chosen as our standard method, employing a LightCycler equipment (Roche); the other method was based on the GeneXpert technology, and represents the automated procedure to be tested. In order to guarantee quantitative linearity, samples included in the analysis showed a *BCR-ABL1* expression below 10 %. All samples were positive for e13/e14-a2 (b2a2 or b3a2) *BCR-ABL1* transcripts.

At reception of the samples, different volumes were used to perform Xpert BCR-ABL assay, according to the manufacturer's recommendations (20 μl for diagnostic, 20 μl for follow-up bone marrow samples, and 200 μl for follow-up peripheral blood samples). At the same time, total RNA from the same samples was extracted and used to measure the transcript by the standard RQ-PCR method. In accordance with the European Treatment and Outcome Study (EUTOS) program, both laboratories had an accredited CF that allowed the expression of the results of *BCR-ABL1* using an IS when they were obtained by the RQ-PCR manual method.

BCR-ABL1 determination by TaqMan-based RQ-PCR, according to the standard assay

Automated RNA isolation was performed from total leukocytes using QIAcube device, after pretreatment of the samples with a monophasic solution of phenol and guanidine isothiocyanate. cDNA was synthesized from 1 μg of total RNA using MMLV reverse transcriptase (Promega kit or transcriptor cDNA synthesis from Roche) and random hexamer primers, according to the manufacturer's indications. The primers and TaqMan probes for *BCR-ABL1* and for the control gene *ABL1*, as well as the conditions for the assay, have been established by the EAC [6, 7]. The RQ-PCR LightCycler platform was used in both laboratories, and pattern curves were obtained from plasmids supplied by Ipsogen (France). Quantification was automatically obtained with the software provided for each platform, applying the CF (0.614 and 1.3677 for LAB1; 0.3048 and 1.1513 for LAB2), which had been previously obtained (from 30 duplicate aliquots, April 2010 and February 2012) in the accredited laboratory of the Clinic Hospital, Barcelona, Spain, according to the EUTOS recommendations [8].

BCR-ABL1 determination by the Xpert BCR-ABL method

As specified above, different sample volumes were used, depending on the source of the sample (bone marrow or peripheral blood) and the moment of its collection (at the time of diagnosis or at MRD follow-up). Red blood cell lysis was performed according to the manufacturer's instructions, after which the sample was introduced in the reagents cartridge, which was placed into the GeneXpert equipment. Inside the device, sample processing, RNA extraction, retrotranscription, and real-time nested PCR for *BCR-ABL1* and the control gene *ABL1* were performed. The system generates a calibration curve for each reagent batch with a determined slope and efficiency. Transcript quantification takes place automatically with the software provided, applying the formula: % of $BCR-ABL1/ABL1 = (E_{\Delta Ct})^{\Delta Ct} \times 100$, where Ct is the threshold cycle, $E_{\Delta Ct}$ is the efficiency of the PCR which is specific for each batch and is calculated from the calibration curve slope ($E_{\Delta Ct}=10^{(1/slope)}$). The ΔCt=*ABL1* Ct−*BCR-ABL1* Ct. In order to guarantee the optimal sample conditions, *ABL1* Ct values must range from 12 to 18, and the *BCR-ABL1* Ct value should not be higher than 32 [9, 10].

Statistical analysis of the results

All data were transformed to logarithmic scales in order to fit linearity. Regression analysis and correlation coefficients

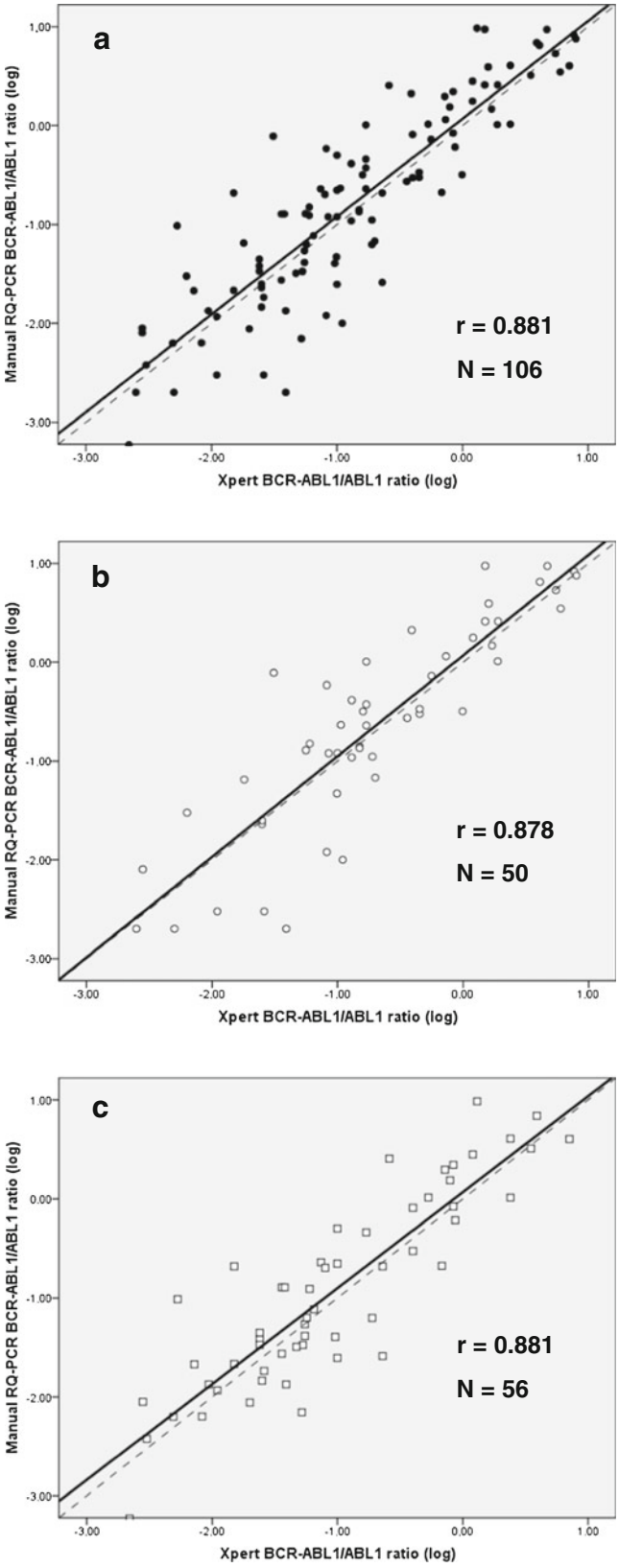

Fig. 1 Regression analysis of the whole series (**a**), and the samples assayed separately in LAB1 (**b**) or LAB2 (**c**). *Dashed lines* represent the equality between the two techniques. *Continuous lines* show an estimation of the relation between the *BCR-ABL1* values obtained by the two methods. Correlation coefficients are included in each chart

were calculated to relate the results obtained by both methods. The Bland and Altman estimations, and the analysis of the concordance between both methods, were also employed in the comparisons. Proportions were compared using the Chi-square test. SPSS for Windows Statistical Package (v 15.0.1) was used for calculations.

Results

Comparison between both methods (standard RQ-PCR and Xpert)

Each laboratory CF was applied to the values obtained by the standard RQ-PCR method in order to express all the results according to the IS. When these data were compared to those obtained by Xpert in the same samples, 106 of 117 (90.6 %) were *BCR-ABL1* positive with both methods. In the remaining 11 cases, the transcript could not be detected with the manual RQ-PCR method (although it was positive when Xpert was used). In all these undetectable *BCR-ABL1* samples, *ABL1* copies were always above the limit of 1×10^4 recommended by the ELN to consider patients in complete molecular response.

The regression analysis of the positive cases showed a good linear fit with a high correlation (r=0.881), as depicted in Fig. 1a. The slope of the regression line (0.989; 95 % confidence interval (CI), 0.89–1.09) was not significantly different from 1, and values with both methods remain very similar with the *Y*-axis intersection of the regression line close to zero (0.07; 95 % CI, −0.06 to 0.20). The Bland and Altman analysis of differences against averages of the values obtained by both assays (Fig. 2a) confirmed this similarity (average difference of −0.08; 95 % CI, −0.08 to 0.17). However, a slight correlation (r=0.236) with a small positive slope (0.12; 95 % CI, 0.02 to 0.22) was found, indicating a continuous bias over the whole range of determined values. Furthermore, when the results of the two laboratories were independently analyzed (Figs. 1b, c and 2b, c), no differences could be found between them. Similarly, the source of the sample, either bone marrow or peripheral blood, did not influence the correlation between the two methods (r=0.913 for bone marrow vs r=0.893 for peripheral blood).

The concordance analysis showed that 62 % of samples agreed at the same logarithmic interval (Table 1), with a similar distribution of Xpert disagreement in values above and below those of the standard RQ-PCR method (20 vs 18 %, respectively). In this case,

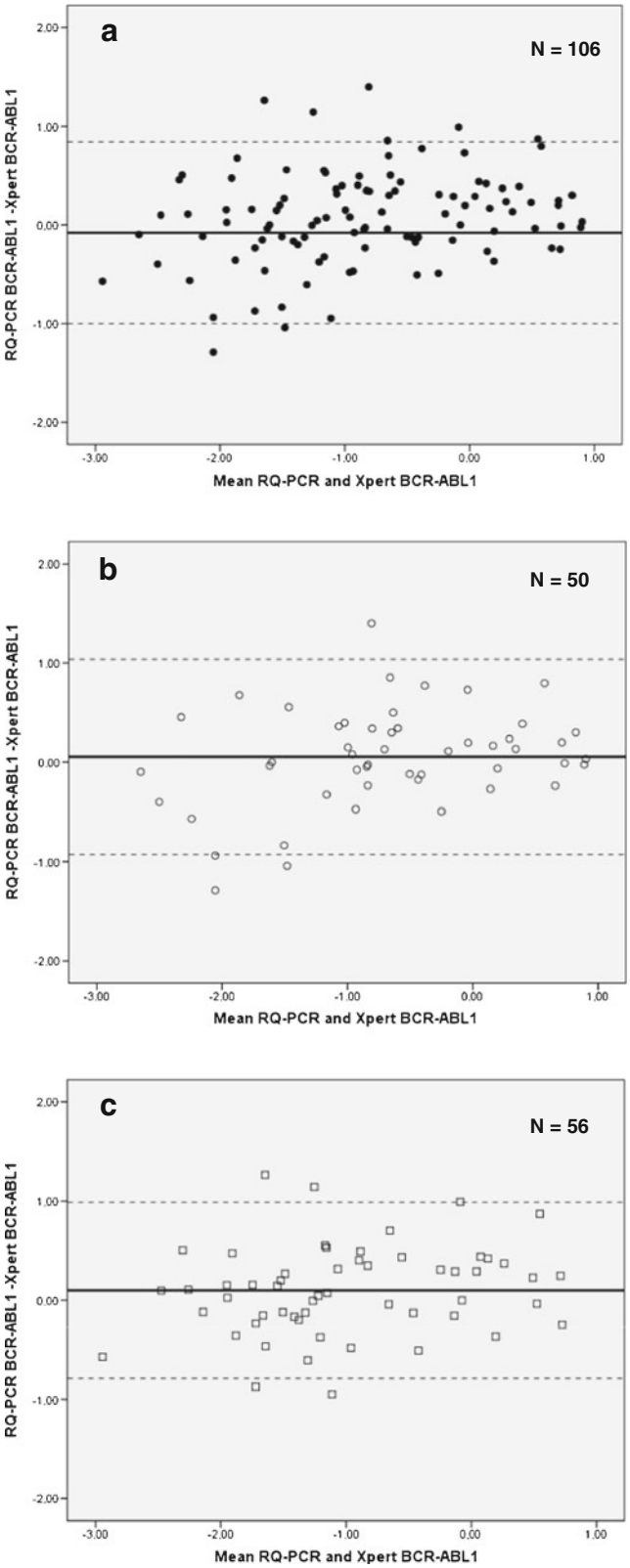

Fig. 2 Bland and Altman analysis of the whole series (**a**), and the samples assayed separately in LAB1 (**b**) or LAB2 (**c**). *Continuous lines* show the mean difference between *BCR-ABL1* values determined by manual RQ-PCR and Xpert. *Dashed lines* represent 95 % confidence limits for the difference

the analysis by intervals did not show any significant difference in concordance (p=0.934, Chi-square test), and the same occurred when the samples from the two laboratories were separated (Table 1).

Analysis of the molecular response

According to the ELN recommendations, the major molecular response (MMR) can be defined as a *BCR-ABL1* % (IS) ≤0.1 %. This term has great prognostic value in CML patients, and it has been used to predict relapse and survival [2]. In our series, the standard RQ-PCR showed 54 samples in MMR (including those not detected), whereas Xpert detected 64 samples in this situation. Globally, 85 % of cases (99 of 117) would have been concordant, and the patient would have been classified similarly with both methods. Eighteen samples (15 %) showed different results in the two assays: 14 of them were considered in MMR by Xpert and not by the RQ-PCR standard method, and the remaining 4 samples were found in MMR only by the RQ-PCR manual method. In all cases, discordant values were placed very close to the range limit (median of 0.20 in the RQ-PCR group and 0.19 in the Xpert samples), corresponding therefore to samples that were difficult to classify.

Discussion

Since the finding of tyrosine kinase inhibitors, *BCR-ABL1* quantification has taken on great clinical relevance, and it has highlighted the necessity for standardizing the methodology [11, 12]. Great efforts have been made in this sense, which have led to the publication of methodological recommendations (including three valid control genes) and which attempted to make the different measurements comparable and reproducible between different laboratories [3, 4]. However, standardization attempts have faced several difficulties related to the process itself since these determinations are

Table 1 Concordance between standard RQ-PCR and Xpert results at different logarithmic intervals, overall and according to the individual laboratory

BCR-ABL1/*ABL1* ratio (%)	Number of samples at the interval		Concordance between methods		
	Standard	Xpert	All samples	LAB1	LAB2
1–10	28	20	20/28 (71 %)	13/16 (81 %)	7/12 (58 %)
0.1–1	35	37	24/35 (69 %)	15/20 (75 %)	9/15 (60 %)
0.01–0.1	30	41	21/30 (70 %)	5/8 (63 %)	16/22 (73 %)
0.001–0.01	13	19	8/13 (62 %)	3/6 (50 %)	5/7 (72 %)
Not detected	11	0	0/11 (0 %)	0/8 (0 %)	0/3 (0 %)
Total	117	117	73/117 (62 %)	36/58 (62 %)	37/59 (63 %)

done by different procedures, each with its own standardization requirements (preanalytical stage, RNA extraction and instability during storage and transport, cDNA obtention, the use of probes and primers, and the analysis in different platforms), all of which might add errors to the whole assay [13].

GeneXpert is one of the platforms that can be used for *BCR-ABL1* quantification. It has been designed to detect and quantify the e13/e14-a2 transcripts of *BCR-ABL1* by a real-time nested PCR, in an automated and closed system, taking approximately 2 h and 20 min. The Xpert BCR-ABL uses *ABL1* as its control gene, which is one of the three genes accepted for this purpose by the EAC, and is recommended for MRD follow-up [7]. The GeneXpert platform automates both the preanalytical and analytical phases, reducing many of the previously mentioned variability concerns, which led to differences in the quantification efficiency. In addition, the amount of sample needed for the assay is minimal (200 μl in peripheral blood or 20 μl in bone marrow), contributing to a reduction in the variability, and the risk of contamination with the equipment or with other samples becomes insignificant since the assay is performed inside a closed cartridge. These reasons support the choice of Xpert for comparison with the current standard method, both in *BCR-ABL1* quantification and CML monitoring.

The Xpert method has been reported to overestimate the high levels of *BCR-ABL1* more than the low values [9], suggesting that it could be less useful for diagnosis than for MRD. In fact, ELN also establishes that quantification of *BCR-ABL1* is not actually required at the moment of diagnosis [11]. For this reason, samples with more than 10 % *BCR-ABL1* expression have not been included in our study.

However, our results show that the concordance between both measurements is quite good (correlation coefficient of 0.881), and that standard values, obtained by the ELN method, could be predicted reliably by Xpert. Furthermore, in our series, MMR was shown to be properly determined by Xpert when compared to the standard method. Finally, it is interesting to highlight that Xpert was able to detect a low number of copies in cases that turned out to be negative when determined by the standard method, indicating a higher sensitivity. This might be related to the fact that Xpert performs a more sensitive assay, a nested PCR, which is actually able to detect up to 5 log reduction in *BCR-ABL1* transcript concentrations. Furthermore, the reproducibility of the results in two independent laboratories, even with different samples, also contributes to showing the great reliability of this method.

On the other hand, although the financial cost of *BCR-ABL1* determination by the Xpert method compared to the standard method could be higher, we should take into account the cost of the whole process, including the reduction in personnel achieved by the automated processing of the samples and the reduction in the number of replications needed to rule out contamination and to avoid mistakes in sample handling. Some recent publications have confirmed this fact, calculating most of these variables and concluding that Xpert could be even less expensive than the manual RQ-PCR method [14]. Indeed, the reproducibility study performed by the group of Winn-Deen [5] suggests it would not be necessary to perform replications of the samples. Since Xpert uses a batch-specific calibration curve, the results come already as a ratio, and it has also been demonstrated that such calibration allows a great reproducibility between different batches [5].

Therefore, we believe that determination of *BCR-ABL1* by the Xpert method may contribute to correcting the present standardization deficits and may be considered a good alternative method, especially if we take into account that Xpert has developed *BCR-ABL1* standard reagents to adjust measurements to the IS when reagent batches are changed.

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