Technical challenges for standardization of molecular monitoring in chronic myeloid leukaemia in developing countries

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Abstract Quantification of *BCR-ABL* after treatment with tyrosine kinase inhibitors is an important therapeutic indicator for patients with chronic myeloid leukaemia (CML). An international scale (IS) for *BCR-ABL* was established in 2012 to improve the comparability of results between different laboratories. *BCR-ABL* measurement is technically challenging, especially in developing countries where the process of standardization isn't easily available. The aim of this study was to assess the technical advantages of reporting results on the IS using the GeneXpert *BCR-ABL* assay, compared to the RQ-PCR standard method currently recommended by the European Leukaemia Network but not standardised in our laboratory and to compare the results obtained by both methods. *BCR-ABL* transcript quantification was performed in 51 samples from CML patients by both methods. The operator hands-on time was reduced using the automated method. A good linear correlation was observed in the results between the two methods. The concordance at logarithmic intervals reached 64%. When the major molecular response (MMR) and the early molecular response (EMR) were analysed, 90% and 80% agreement, respectively, were achieved. However, the discordances in the remaining cases could have misled the therapeutic approach if only a standard method was used. The complexity of the process to establish a laboratory-specific conversion factor in developing countries can be overcome by the use of the GeneXpert automated system, which represents a rapid, accurate and reliable clinical tool to correctly define molecular responses on the IS and to allow clinicians to achieve better patient management.

Keywords: BCR-ABL, Chronic myeloid leukaemia, qRT-PCR, Standardization

Introduction

Molecular testing for the *BCR-ABL* fusion gene provides important prognostic information for individual chronic myeloid leukaemia (CML) patients treated with tyrosine kinase inhibitors. International treatment recommendations include specific time-dependent molecular milestones to assess whether optimal molecular response is achieved (Huet et al., 2014; Recchia et al., 2015). To date, real-time quantitative polymerase chain reaction (RQ-PCR) is considered the gold standard method to measure the level of *BCR-ABL* mRNA relative to an internal reference gene (Cross et al., 2015a). An international scale (IS) was elaborated to express results, with the value of 100% *BCR-ABL* corresponding to the International Randomized Study of Interferon and STI571 (IRIS) study standardised baseline and the value of 0.1% *BCR-ABL* being defined as a major molecular response (MMR) (Hughes et al., 2006). However, despite the efforts made for standardisation, significant variability remains across laboratories worldwide, especially in developing countries where establishing a laboratory-specific conversion factor (CF) is a complex and expensive procedure.

Cepheid has developed The GeneXpert platform, which can be used for *BCR-ABL* quantification by a real-time nested PCR in an automated and closed system, reporting results on the IS (López-Jorge, 2012).

Based on the clinical needs of alignment of *BCR-ABL* values to the IS, we have recently acquired a GeneXpert automated system in our laboratory in Tunisia. We assessed the technical advantages of reporting results on the IS using the GeneXpert *BCR-ABL* assay, compared to the RQ-PCR standard method currently recommended by the European Leukaemia Network and routinely used in our laboratory and we compared the results obtained by both methods.

Materials and methods

At different follow-up stages, we tested 51 peripheral blood samples from CML patients treated with tyrosine kinase inhibitors. Analyses were performed at the Farhat Hached Hospital in Tunisia. All samples were positive at diagnosis for e13a2 and/or e14a2 (b2a2/b3a2) *BCR-ABL* transcripts.

Quantification of *BCR-ABL* transcripts was performed by two different methods: The first method, which was chosen as our standard method, is recommended by the "European Against Cancer" (EAC program) and employing a LightCycler 480 equipment (Roche). This method has not been standardised yet in our laboratory. The second method was based on the GeneXpert technology, and represents the automated procedure to be tested. Blood samples were processed within 24 hours of sampling. Samples were conditioned for RNA extraction using Trizol (Invitrogen) to measure the transcript by the standard RQ-PCR method. Xpert *BCR-ABL* assay was performed according to the manufacturer's recommendations.

BCR-ABL measurement by TaqMan-based RQ-PCR using LightCycler 480 platform

RNA isolation was performed from total leukocytes according to the method using acid guanidinium thiocyanate-phenol-chloroform as described (Chomczynski et al., 1987). cDNA was synthesised from 1µg of total RNA using MMLV reverse transcriptase and. random hexamers primers (Invitrogen). The primers and TaqMan probes for *BCR-ABL* and for the control gene *ABL* have been established by the EAC (Beillard et al., 2003; Gabert et al., 2003). Pattern curves were obtained from plasmids (Qiagen).

BCR-ABL measurement by the Xpert BCR-ABL method

Red blood lysis was performed at reception of the sample according to the manufacturer's instructions. Then, the sample was introduced in the reagents cartridge, which was placed into the GeneXpert equipment. During the test process, the GeneXpert system isolates the RNA from whole blood and performs retro transcription followed by

nested real-time PCR for *BCR-ABL* and the control gene *ABL*. The system generates a calibration curve for each reagent lot with a determined slope and efficiency. Transcript quantification is performed automatically with the software provided, according to the equation: % of *BCR-ABL/ABL* = $(E\Delta Ct)^{\Delta Ct}$ x 100, where Ct is the threshold cycle, $E\Delta Ct$ is the efficiency of the PCR which is specific for each lot and is calculated applying a formula: $E\Delta Ct = 10^{(1/slope)}$ ($\Delta Ct = ABL$ Ct − *BCR-ABL* Ct). In order to optimise sample conditions, *the BCR-ABL Ct value should not be higher than 32, and the ABL Ct value should* range from 12 to 18 (Dufresne et al., 2007; Jobbagy et al., 2007; López-Jorge et al., 2012).

Statistical analysis

The data were transformed to logarithmic scales to fit linearity. Regression analysis and the coefficient of determination were determined to compare the results obtained by both methods. Analysis was performed using Microsoft Excel and SPSS for Windows Statistical Package v17.0.
Concordance analysis at logarithmic intervals were defined as follows: the number of samples attributed to a given logarithmic interval by both methods divided by the number of samples attributed to the same logarithmic interval by the RQ-PCR standard method.

Results

The values obtained by the standard RQ-PCR method were compared to those obtained by Xpert BCR-ABL in the same samples; 35 of 51 (69%) were *BCR-ABL* positive and 10 of 51 (20%) were undetectable using both methods. In the remaining 6 cases, the transcript could not be detected with the Xpert assay, but it was positive with the manual RQ-PCR. In all undetectable *BCR-ABL* samples with the two methods, *ABL* copies were always above a value of 1 x 10^4 which represents the limit recommended by the ELN to consider patients in deep molecular response.

The regression analysis of the positive cases showed a good linear fit with a high correlation (R^2 = 0,82), as depicted in Figure 1.
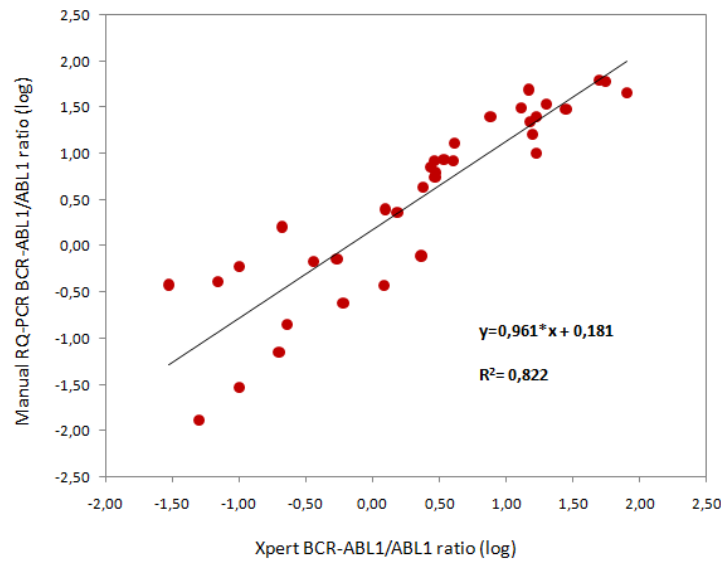
Figure 1. Regression analysis. - Continuous line shows the relation between the *BCR-ABL* values obtained by both methods.

Concordance analysis showed that 33/51 (64 %) samples agreed at the same logarithmic interval (Table 1) with a higher distribution of Xpert disagreement in values below than values above those of standard RQ-PCR method (24% vs 18% respectively).

Table 1: Concordance between results of standard RQ-PCR and those of Xpert method at different logarithmic intervals

BCR-ABL/ABL ratio (%)	Number of samples at the logarithmic interval		Concordance between methods
	RQ-PCR standard	GeneXpert	
>10	11	12	10/11 (91%)
1-10	13	11	9/13 (69%)
0,1-1	6	9	4/6 (67%)
0,01-0,1	9	3	2/9 (22%)
0,001-0,01	2	0	0/2 (0%)
Not detected	10	16	8/10 (80%)
Total	51	51	33/51 (64%)

A key marker of molecular response is the major molecular response (MMR) which is defined as a *BCR-ABL* transcript level (IS) $\leqslant$ 0,1%. Results were considered concordant when both automated and non-automated assays generated *BCR-ABL* transcript values of 0,1% or less for a particular sample. In our series, the standard RQ-PCR detected 21 samples in MMR (including those not detected), whereas Xpert showed 19 samples in this situation. Globally, 90% of cases (46/51) were concordant and the patient would have been classified similarly with both methods. Five samples (10%) showed different results in the two assays: 3 were considered in MMR by the RQ-PCR standard method and not by the Xpert, and 2 were found in MMR only by the Xpert. Another milestone is represented by the early molecular response (EMR), which is defined as a BCR-ABL transcript level $\leqslant$ of 10% at 3 months after starting therapy. In our series, we tested 5 samples 3 months after starting treatment. Four samples were concordant (80%): two had a *BCR-ABL* > 10% and 2 others < 10% using both methods. The fifth case has a ratio $\leqslant$ of 10 % with Xpert and > 10% with the standard method.

Using Xpert automated assay reduced operator hands-on time from several hours to approximately 20 minutes and also reduced the time of results delivery from about six days to a few hours.

Discussion

Molecular monitoring of CML patients by qRT-PCR has taken on great clinical relevance since the finding of tyrosine kinase inhibitors. An international scale (IS) was established to express *BCR-ABL* mRNA levels to make the different results reproducible and comparable between different laboratories (Hughes et al., 2006; Branford et., 2008). However, assessing an IS conversion factor is a complex and expensive procedure, especially in developing countries. In addition, it has been showed that standardisation itself has faced several difficulties related to the different procedures, each with its own standardisation requirements (RNA extraction and its instability during storage, retro transcription protocol, the use of primers and probes and the analysis in different platforms), all of which might add bias to the measurement (White et al., 2010). Efforts to standardise molecular monitoring to the IS have led to the development of a cartridge Xpert *BCR-ABL* for *BCR-ABL* quantification by Cepheid. The GeneXpert system integrates sample processing, nucleic acids extraction and amplification and target sequence detection in peripheral blood specimens collected in PAXgene or EDTA tubes (Jobbagy et al., 2007). The cartridge includes reagents to detect *BCR-ABL* fusion genes resulting from two major breakpoints, e13a2 (b2a2) and e14a2 (b3a2), and the *ABL* transcript as an endogenous control. Alignment to the IS, is based upon the quality control standards derived from the World Health Organization (WHO) *BCR-ABL* Standards (White HE et al., 2010; WHO, 2010). is performed lot to lot automatically by the software of the GeneXpert Instrument. In several studies, Xpert *BCR-ABL* has been demonstrated to be a reliable and accurate test for monitoring BCR-ABL1 transcript levels (Jobbagy et al., 2007; Cayuela et al., 2011; López-Jorge et al., 2012).

Based on the clinical needs of standardisation, we have recently acquired the GeneXpert system in our laboratory in Tunisia. To our knowledge, only a rare series of standardised results of CML patients have been reported in Africa. Otherwise, we compared patient test results obtained with Xpert BCR-ABL1 and with the RQ-PCR standard method to assess the advantages of the automated method. Our prospective study showed that the concordance between both measurements is quite good ($R^2 =$ 0.82). These results are consistent with Jobbagy et al., 2007 and López-Jorge et., 2012. However, we observed some discordances in classifying patients within logarithmic intervals. These situations are especially critical when we need to define specific molecular response levels associated with therapeutic milestones such as EMR at 3 months after starting treatment or MMR. Therefore, harmonising the various laboratory procedures is essential to standardise optimal treatment response criteria (Griffiths et al., 2015). In this sense, the automated assay allowed us to classify patients correctly and hence to aid clinicians in correctly addressing therapeutic decisions and achieving better CML patient management for optimising outcomes. Both methods use *ABL* as a control gene, which is recommended for the MRD follow-up by the EAC (Beillard et al., 2003). The GeneXpert system has the obvious advantage of automation in both the pre-analytical and analytical phases, reducing many variability parameters which led to differences in the quantification efficiency. In addition, the hands-on assay setup time is less than 30 minutes, and the total test time is about 2 hours. Some studies concluded that automated processing of the samples could be even less expensive than the manual RQ-PCR method (Cayuela et al., 2011). Another benefit of the automated test is the sample volume needed for the assay, which contributes to a reduction in the variability, and the risk of contamination with other samples or with the equipment is reduced as the assay is performed inside a closed cartridge.The Xpert *BCR-ABL* can detect a 6 log reduction in *BCR-ABL* transcript levels. This high sensitivity allows identifying patients who might be considered for a trial of therapy discontinuation (Cross et al., 2015 (b); Alikian et al., 2016).

In conclusion, Xpert BCR-ABL automated assay is a rapid and reliable test for monitoring BCR-ABL mRNA transcript levels. It is interesting to highlight that this assay offered us the possibility of standardisation which is not easy in developing countries. Reporting our results to the IS allows clinicians to achieve better patient management. With Xpert BCR-ABL's implementation, we could accurately detect deeper molecular responses. From a technical point of view, the cartridge-based automated systems significantly reduce operator hands-on time and the risk of contamination. Finally, the availability of a rapid response using a

GeneXpert assay may contribute to reduce anxiety linked to delay in results delivery and hence to improve patient quality of life.

Conflict of Interest

The author has no financial interest and no competing interest.

Author contribution

Ines Ouahchi designed this study, analysed the data and wrote the manuscript.

Patient consent

All patients consented for molecular analyzes to be carried out.

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