Highly Sensitive Multiplex RT-PCR System for the Detection of all Common BCR-ABL Transcripts Associated with Different Leukemias

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Abstract

Objective: After the recent characterization of leukemia-specific DNA rearrangements, molecular methods have become primary tools for the diagnosis and monitoring of many hematological malignancies, due to their superior sensitivity and accuracy over other conventional methods. The BCR-ABL fusion transcripts resulting from the t(9;22) translocation are distinct hallmarks of Philadelphia chromosome positive (Ph^+) leukemias. There are clear associations between different isoforms of the BCR-ABL fusion protein and specific phenotypes of these leukemias. Each isoform also has a significant prognostic value and can be a critical indicator of the clinical outcome. In this study we have adopted, with modifications, a highly sensitive and specific method to screen simultaneously for the most frequent BCR-ABL fusion transcripts, namely, p210 (b3a2/b2a2), p190 (e1a2) and p230 (e19a2).

Methods: A multiplex reverse-transcriptase polymerase chain reaction (RT-PCR) protocol with nested primer strategy for each of the above fusion transcripts was carefully optimized. RNA integrity, cDNA synthesis and PCR amplification were checked using internal control primers for the normal untranslocated BCR gene. Over 100 clinical samples were collected from hospitals in Amman between 2003 and 2005.

Results: This system was applied successfully on 100 clinical samples previously diagnosed as chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL). RNA extracts from established leukemic cell lines carrying the translocations of interest were used as external positive controls. Representative PCR products were sequenced to verify the specificity of the amplification system.

Conclusion: Our multiplexed nested RT-PCR assay provides a sensitive, accurate, time-saving and cost-effective diagnostic tool for the diagnosis and monitoring of patients with Ph^+ leukemias.

Keywords: Chronic Myeloid Leukemia, CML, BCR-ABL, Philadelphia Chromosome, Polymerase Chain Reaction (PCR).

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Introduction

The BCR-ABL fusion protein is the molecular consequence of the Philadelphia (Ph) chromosome translocation and is an active cytoplasmic tyrosine kinase. [1] This fusion protein can vary in size from 190 kd to 230 kd, depending on the site of the breakpoint within the BCR gene. [2] Almost all patients with Chronic Myeloid Leukemia (CML) express a 210-kd BCR-ABL protein, while patients with Ph^+ Acute Lymphoblastic Leukemia (ALL) most commonly express a 190-kd BCR-ABL protein. [3] A larger, 230-kd BCR-ABL fusion protein is rarely found in a subgroup of patients with chronic neutrophilic leukemia (CNL). [4]

Reverse transcription-polymerase chain reaction (RT-PCR) is becoming the method of choice for the diagnosis of Ph^+ leukemias. The advantages offered by this method, when compared with other conventional diagnostic methods, include its high sensitivity and specificity, as well as being time-saving and cost-effective. For example, cytogenetic analysis is time-consuming and has a relatively low sensitivity. Furthermore, it is not practical with peripheral blood samples and yields sufficient metaphases only in 60% to 80% of the bone marrow samples. [5] Likewise, Fluorescence In Situ Hybridization (FISH) and Immunophenotypic analysis by flow cytometry (FACS) fail to achieve the sensitivity of PCR and are relatively expensive. [5] PCR allows the identification of a single Ph-bearing cell among 10^5 to 10^6 normal cells, a sensitivity unparalleled by any other available method. [6] Thus, efforts to design PCR and RT-PCR for Ph-chromosome translocation have been ongoing for many years. [7]

The BCR-ABL gene is the molecular marker of CML, [8] which is present in more than 95% of patients. [9] In ALL, the BCR-ABL transcripts are found at lower frequencies (20-30%) but it was shown that their presence has a diagnostic value as a poor prognostic indicator. [10]

Several methods have been used to detect low numbers of malignant cells during and after treatment. [11] This is referred to as monitoring of Minimal Residual Disease (MRD), in which the number of remaining leukemic cells, or the leukemic burden, is precisely measured. Most of the conventional techniques are not suitable for clinical MRD detection because of their limited sensitivity, specificity, or applicability. [11] However, current RT-PCR approaches for MRD monitoring can reach sensitivities of 10^{-5} to 10^{-6} and is sufficiently specific, and broadly applicable. [12] The chimeric BCR-ABL mRNA is an attractive target for monitoring MRD by RT-PCR assays because it is a persistent leukemia specific marker. [13] Detection of MRD in acute leukemia is useful for evaluating early response to treatment and consequently for improving stratification. [12] This includes treatment intensification in patients at high risk of relapse and reduction in patients in low-risk of relapse. [12] The most relevant clinical application of the diagnosis of MRD in CML is the assessment of treatment response after bone marrow transplantation (BMT). [13]

In this study, we present a multiplexed nested RT-PCR system for the detection of all common variants of the BCR-ABL fusion gene. The nested strategy offers superior sensitivity and enhanced specificity making this system ideal for early diagnosis of Ph+ leukemias as well as monitoring of MRD cases.

Materials and Methods

Patients and Samples: Peripheral blood samples from one hundred patients were obtained for molecular studies. Eighty patients were diagnosed as CML, and twenty patients as ALL. The patients were attending Jordan University Hospital, King Hussein Medical Centre, and Al-Bashir Hospital, all in Amman city, between January 2003 and March 2005. The median age was 40 years (range: 16-80 years), and the male to female ratio was 1:1.

RNA Extraction: Total RNA was extracted from peripheral blood samples in EDTA tubes according to the RNA isolation kit protocol (Gentra, USA). RNA was extracted within few hours of blood collection. The quality of RNA was evaluated on electrophoresis gels, and the concentration was determined spectrophotometrically. RNA was stored at - 70°C.

cDNA Synthesis: RNA (1 µg) was heated to 70°C and placed on ice. A reagent blank was included at this stage. Synthesis of cDNA was achieved by adding 10 µl cDNA mix (Promega, USA) made of 10 mM Tris-HCl pH 9.0, 50 mM KCl, 5mM MgCl2, 0.1 % Triton, 1mM of dATP, dCTP, dTTP and dGTP, 1u/µL RNasin, 15u/µg AMV reverse transcriptase, and 0.5 µg random hexamers to samples and then incubating at 40°C for 1 hour . The reaction was terminated by heating to 95°C for 5 minutes and cDNA was stored at –20°C.

Primer selection: The primer sequences are listed in Table 1, and their locations on the BCR and ABL genes are shown in Figure 1.

Multiplex PCR amplification: cDNA (10µl) was mixed with 40µL of multiplex mix made of 12 mM Tris pH 8.3, 2.0 mM MgCl2, 50 mM KCl, 0.2 mM dNTPs (Promega, USA), 0.6 µM primers (210-F, 190-F, ABL-R & BCR-R), and 30 u/ml Taq polymerase (Promega, USA). PCR was performed using an MJ Research Inc. programmable thermal cycler for 35 cycles of 96°C/1 min, 61°C/1 min, and 72°C/1 min followed by a 10 min extension step at 72°C.

Uniplex PCR amplification: cDNA (10µl) was mixed with 40µL of PCR mix made of 12 mM Tris pH 8.3, 2.0 mM MgCl2, 50 mM KCl, 0.2 mM dNTPs (Promega, USA), 0.6 µM of the indicated primer pair (210-F/ABL-R, 190-F/ABL-R or 230-F/ABL-R), and 30 u/ml Taq polymerase (Promega, USA). PCR was performed for 35 cycles of 96°C/1 min, 61°C/1 min, and 72°C/1 min followed by a 10 min extension step at 72°C.

Nested PCR amplifications: cDNA (2.5µl) was mixed with 47.5 µL of nested mix made of 12 mM Tris pH 8.3, 2.0 mM MgCl2, 50 mM KCl, 0.2 mM dNTP (Promega, USA), 0.5µM primers (Table 1) and 30 u/ml of Taq polymerase (Promega, USA). PCR was performed for each BCR-ABL fusion transcripts separately. p210 BCR-ABL amplification consisted of initial dentauration at 94°C for 4 min, followed by 35 cycles of 94°C/55 sec, 57°C/55 sec, and 72°C/1 min with a final extension step at 72°C for 10 min. p190 BCR-ABL amplification consisted of initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 45 sec, 57°C for 45 sec, and 72°C for 1 min with a final extension at 72°C for 10 min. p230 BCR-ABL amplification consisted of initial denatuaration at 94°C for 4 min, followed by 35 cycles of 94°C/50 sec, 59°C/50 sec, and 72°C/1 min with a final extension step at 72°C 10 min.

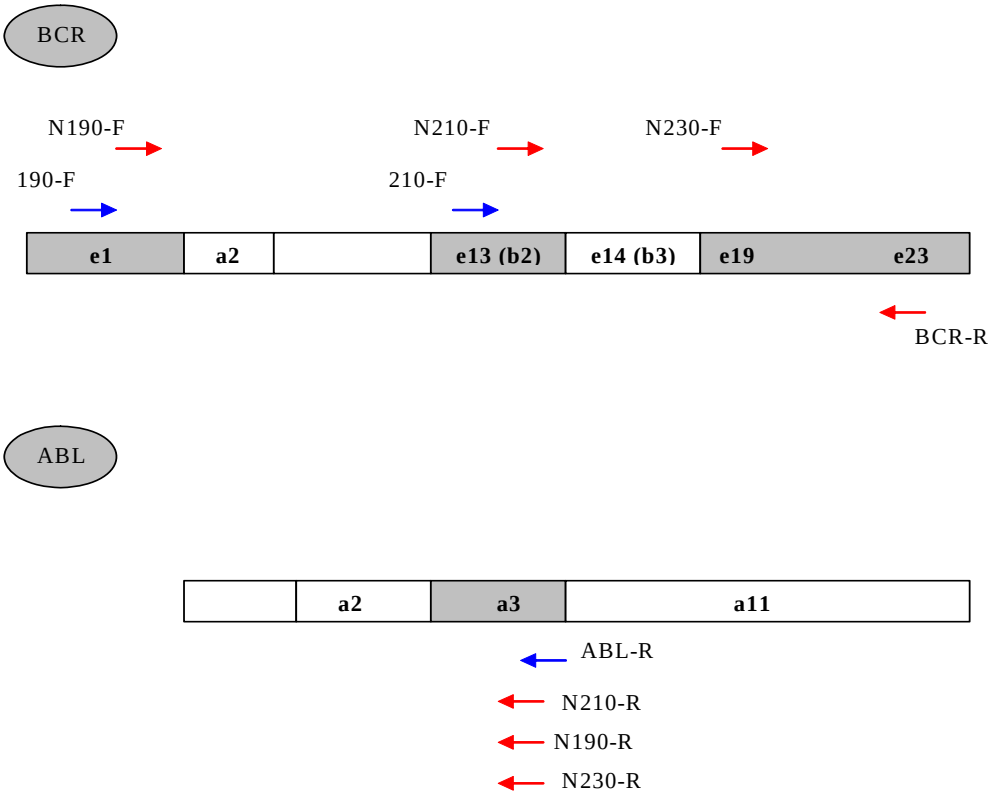

Figure1. Schematic representation of BCR and ABL mRNAs indicating locations of the first and nested PCR primers. Primers 190-F, 210-F, BCR-R and ABL-R are used for the first multiplex PCR, while primers N190-F, N210-F, N230-F, N190-R, N210-R and N230-R are used for the nested PCR.

Table 1: Oligonucleotide primer sequences.

Code	Primer sequence
BCR-R *	5` ATA GGA TCC TTT GCA ACC GGG TCT GAA 3`
ABL-R *	5` TGT TGA CTG GCG TGA TGT AGT TGC TTG G 3`
210-F *	5` ACA GAA TTC GCT GAC CAT CAA TAA G 3`
190-F *	5` ACC GCA TGT TCC GGG ACA AAA AG 3`
N210-R †	5` TTC ACA CCA TTC CCC 3`
N210-F †	5` CTG ACC ATC AAT AAG GAA G 3`
N190-R †	5` GGC CAC AAA ATC ATA C 3`
N190-F †	5` ATG GAG ACG CAG AAG 3`
N230-R ‡	5` CAG ACT GTT GAC TGG CGT GA 3`
N230-F ‡	5` GAG AGA GAG GTC CAA GGT GC 3`

**** Cross et al 1994***
† Nogva et al 1997
‡ Bertorelle et al. 2000

Results

Multiplex PCR amplification: The performance of the developed multiplex PCR system was first optimized by detecting the different fusion transcripts in established leukemic cell lines, namely K562 (p210 BCR-ABL), SD-1 (p190 BCR-ABL) and AR230 (p230 BCR-ABL). RNA from these cell lines also served as positive controls for our assay. All four translocations, b3a2, b2a2, e1a2 and e19a2, along with the un-translocated BCR gene as the internal control, were successfully amplified and the PCR products were discriminated by their different fragment sizes upon agarose gel electrophoresis (Figure 2).

Once the PCR conditions were established and optimized, eigthy CML and twenty ALL samples were successfully assayed using this system. Our results were in accordance with previous diagnosis by other methods such as FISH. The percentages of BCR-ABL positive samples were slightly lower than internationally reported figures, most likely due to the fact that some of the patients were in the remission phase.

It is worth noting that in patients who were positive for any of the BCR-ABL transcripts, no BCR band was visible. However, this band was effectively amplified when the primers p210-F and BCR-R were used in a uniplex reaction (Figure 3). This can be explained by assuming that the BCR-ABL amplification product out-competes or suppresses the BCR amplification effectively under the reaction conditions described.

Nested PCR amplification: With the sensitivity of BCR-ABL transcript detection being a major technical advantage in the diagnosis and monitoring of Ph+ leukemias, we have adopted a second round of PCR amplification using nested primers for each of the fusion transcripts targeted by the first round primers. These individual split-out reactions also improve the specificity of PCR detection of such transcripts.

Uniplex PCR amplification: In parallel to the multiplex system, single uniplex reactions with nested strategies for each of the main BCR-ABL fusion transcripts were also optimized (Figures 4-6). Such reactions are known to be more sensitive than multiplex systems, and can be used to monitor patients with specified BCR-ABL translocations.

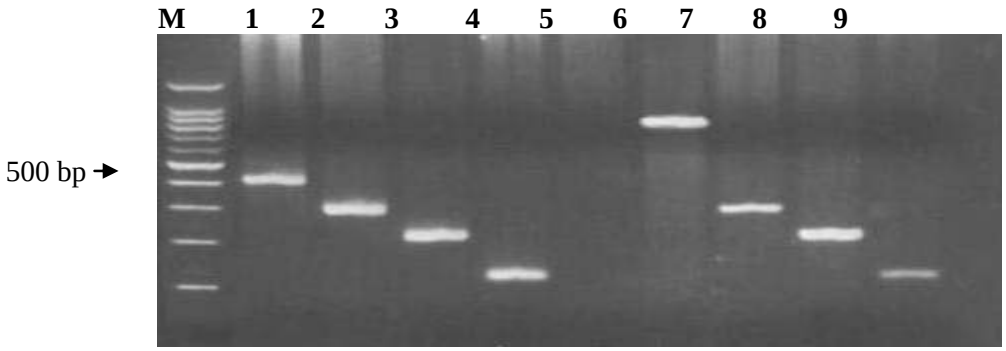

Figure 2. Multiplex RT-PCR results. Lanes 1-4 represent bands from positive control cell lines for e19a2 (p230), b3a2 (p210), b2a2 (p210) and e1a2 (p190) respectively, while lane 5 is an H_2O negative control, and lane 6 is Ph negative blood sample showing a BCR internal control band. Lane M is a 100 bp ladder (500 bp band indicated). Lanes 7-9 represent Ph positive clinical samples with b3a2 (CML), b2a2 (CML) and e1a2 (ALL) respectively.

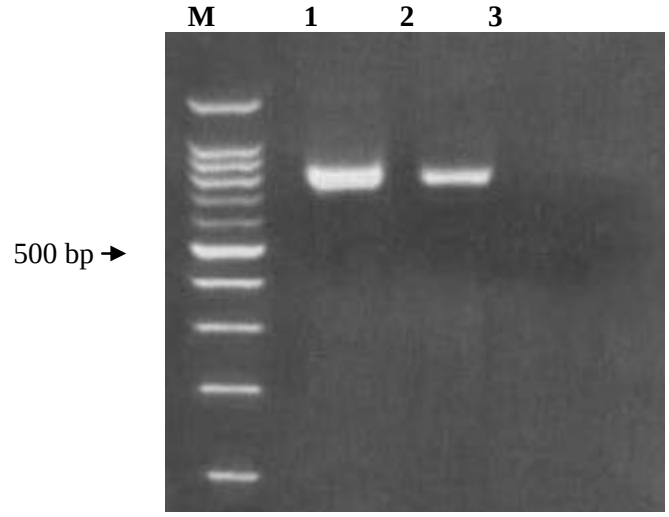

Figure 3. RT-PCR results for BCR internal control. Lane M: 100 bp marker (500 bp band indicated), lane 1: CML patient sample positive for normal BCR, lane 2: ALL patient sample positive for normal Bcr, lane 3: H_2O negative reaction.

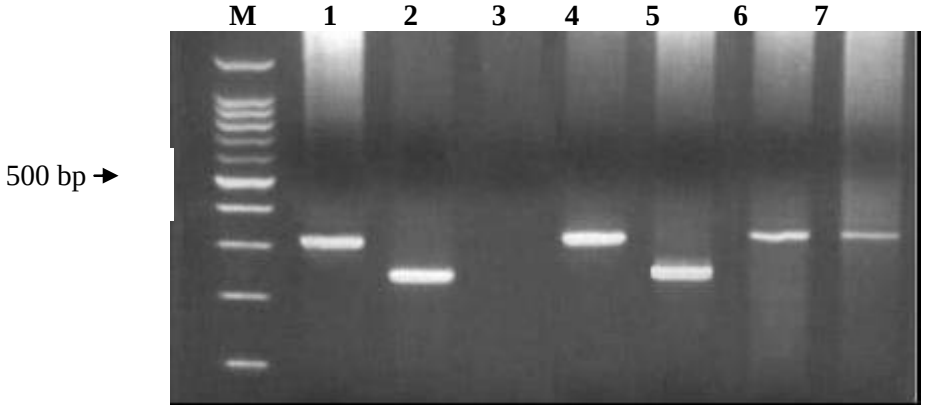

Figure 4. p210 Nested RT-PCR. Lanes 1 and 2 represent b3a2 and b2a2 bands from positive control cells respectively, while lanes 4-7 represent four CML clinical samples that are Ph positive for p210 variants. Lane M is the 100 bp ladder (500 bp band indicated), and lane 3 is the H_2O negative reaction.

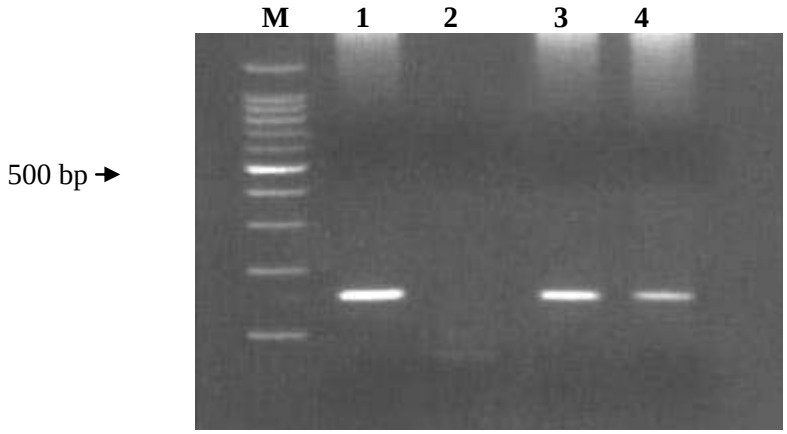

Figure 5. p190 nested RT-PCR. Lane 1 represents an e1a2 positive control (SD-1) while lanes 3 & 4 represent two ALL clinical samples that are Ph positive for p190. Lane M is the 100 bp ladder (500 bp band indicated) and lane 2 is an H_2O negative reaction.

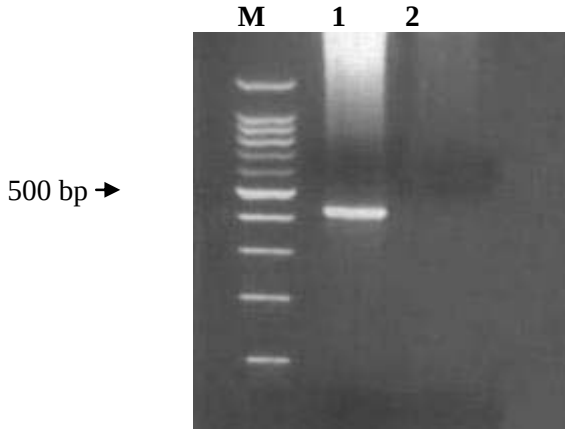

Figure 6. p230 nested RT-PCR. Lane 1 represents e19a2 positive control (AR230). Lane M is the 100 bp ladder (500 bp band indicated) and lane 2 is an H_2O negative reaction.

Discussion

Multiplex RT-PCR has been used previously for the characterization of individual or small groups of translocations found in different leukemic cells. [14- 16] These reports concluded that, compared to single RT-PCR reactions, multiplex RT-PCR systems allow rapid, specific, less expensive, less laborious and less time consuming detection of groups of the most frequent fusion transcripts in leukemic patients. Our aim in this study was to optimize such a multiplex RT-PCR system to screen simultaneously for the most frequent BCR-ABL fusion transcripts associated with CML and ALL cases.

Among the various reported combinations of PCR primers, we have adopted the multiplex PCR primers described by Cross et al. [18] In this system, sense primers, e1 and b2 (e13) specific, and antisense primers, a3 and c5 (e21) specific, were used (Figure 1). This set of primers allows the simultaneous amplification of all described molecular variants of the BCR-ABL gene along with the un-translocated BCR gene as an internal control. The sensitivity of this test was greater than 0.1%, i.e., can detect one BCR-ABL positive cell in 1000 or more normal cells. [18] However, in our study, nested primer strategies were added to increase the sensitivity of the analysis even further as described by Nogva et al. [19] The primers in this reaction were designed to bind inside the primers of the first-round amplification, and thus result in slightly smaller products. These nested primers are specific for the p210, p190 fusion transcripts as well as the rare p230 variant, which is not detected by many of the previously described systems and commercially available kits. [20- 23]

In recent years, real-time PCR technology has been heavily introduced into diagnostic medical applications. In molecular oncology, this technology made it possible to quantify the tumor burden and monitor the response to therapy. However, the parallel use of other molecular techniques such as qualitative nested PCR, FISH, and cytogenetics is still recommended. Each of these techniques has its own specific advantage at specific stages or conditions of the disease.

When compared with real-time PCR and other techniques, the main advantages of a qualitative, nested and multiplexed system such as the one described here, is its superior sensitivity and low cost. This makes our system most suitable for early diagnosis of Ph+ leukemia as well as the sensitive detection of MRD, and it is advisable to be acquired by diagnostic laboratories that cannot afford the expensive equipment needed for FISH and real-time PCR.

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