Molecular diagnosis of bcr-abl fusion gene in CML patients using Monoplex-Two Steps- Reveres Transcriptase–Polymerase Chain Reaction

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Summary:

Background: Chronic myeloid leukemia (CML) is a stem cell disorder associated with an acquired chromosomal abnormality, Philadelphia chromosome (Ph), which arises from the reciprocal translocation of part of long arm of chromosome 9, in which proto-oncogene ablson gene (*abl*) is located, to long arm of chromosome 22, in which break point cluster region gene (*bcr*) is located. The bcr-abl fusion gene can be detected using several molecular methods. For its simplicity, rapidity, and sensitivity, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is one of the most common techniques used for analyzing whether a target gene is being expressed or not.

Patients and methods: Venous blood (VB) sample from hematologically and clinically diagnosed 34 CML patients and10 acute lymphoid leukemia (ALL) were collected. Also, 10 healthy individuals were included as health negative control. RNA was extracted from these samples using commercial kit. Molecular screening for the presence of *bcr-abl* in these samples was done using Monoplex- Two Steps-Reverse Transcriptase-Polymerase Chain Reaction (M-TS-RT-PCR). Amplified products were electrophoresid in 1.5% agarose gel.

Results: The results showed that all CML patients were positive for *bcr-abl* while all the others were negative for this gene.

Conclusion: Monoplex - Two steps – RT-PCR has been successfully used to detect and subtype bcr-abl fusion gene. It is a fast and effective technique that should be done upfront at diagnosis in patients with CML, as its molecular type is crucial in the treatment follow-ups.

Key words: CML- *bcr-abl* detection- monoplex- two steps – RT-PCR

Introduction:

Chronic myeloid leukemia (CML) is a pluripotent stem cell disorder characterized by the presence of the Philadelphia chromosome (Ph). Ph is the result of a translocation of the c-abl oncogene from chromosome 9 to the breakpoint cluster region (bcr), within the bcr gene on chromosome 22, forming a chimeric bcr-abl gene(1,2) .The fused genes encode an 8.5-killo base(kb) chimeric mRNA that is translated to a 210-killo dalton(kd) protein(BCR-ABL) (3) .This p210 bcr-abl protein shows tyrosine kinas activity, is present in the leukemia cells of patients with CML, and is necessary and sufficient for transformation (4). In 95% of patients, the breakpoint in the bcr gene occurs either between bcr exon 2 (b2) and 3 (b3) or between bcr exon 3 (b3) and 4 (b4). Hence, 2 alternative chimeric p210 bcr-abl proteins, comprising either a b3a2 or a b2a2 junction, can result from this fusion gene (5, 6).BCR-ABL has become a target for the development of therapeutics to treat leukemia. Most recently, Gleevec® (STI571) or Imatinib, a small molecule inhibitor of the ABL kinase, has been approved for the treatment of CML (7). Although conventional cytogenetic studies remain the cornerstone of genetic testing, molecular-based technologies have emerged as a most useful tool for the detection of disease-defining genetic lesions (8, 9). Most translocations, when evaluated at the Molecular level, are detected by RT-PCR in the routine diagnostic setting. RT-PCR detection of the major leukemia translocations has numerous advantages over conventional cytogenetic, including shorter turn-around time, no requirement for dividing cells, detection of translocations that may be missed by conventional cytogenetics ("cryptic" translocations) and providing a sensitive marker for subsequent minimal residual disease testing (10,11).

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Patients, Materials and Methods:

Patients

This is a prospective study enrolled 44 leukemic patients at The National Center of Hematology (NCH) /Al-Mustensseria University from February 2006 to August 2008. They were diagnosed clinically and hematologically as CML (34patients) and ALL (10 patients). Also, they were diagnosed molecularly and cytogenetically as CML *bcr-abl* positive-Ph positive and ALL *bcr-abl* negative-Ph negative using Multiplex Single Step —Reverse Transcriptase–Polymerase Chain Reaction and conventional cytogenetic karyotyping, respectively. CML patients were treated with imatinib for different durations. Patients were randomly selected concerning to age, gender, pre-treatment and disease phase (12). Also, 10 healthy individuals were included as healthy negative control. They were evaluated hematologic ally and molecularly in the

same manner as the patients. Commercially available extracted RNA from BCR-ABL cell line K562 (Ambion,USA) was used as a positive controls in amplification reactions. One ml of venous blood (VB) sample was obtained from each subject, placed in tube containing EDTA (as anti-coagulant).Then, 250µl of VB was mixed with 750µl of lyses reagent contain Guanidinium Thiocyanate (GTC) and the suspension was passed several times through a pipette for about 5min at 25°C.The lysate was used in RNA extraction or kept at –80ºC until used (13).

Materials and Methods:

Total RNA was extracted from 750µl VB lysate using TRIZOL LS Reagent (invetrogen,USA) according to the manufacturer's specifications. RNA concentration and purity were estimated using UV-spectrophotometer according to Sambrook *et al.* (13). The integrity of extracted RNA was checked by size fractionation on a formaldehyde agarose gel electrophoresis according to Sambrook *et al.* (13).

First strand complementary DNA (cDNA) was synthesized. The first master mix (MM-1) was prepared by mixing the following components (per one reaction): 1µl of 500µg/ml Random primer (promega,USA), 1µl of dNTPs(200µM each) (promega, USA), RNA (final concentration 1 µg) and DEPC-H2O to final volume 13 µl. The mixture was heated to 65ºC for 5 min and then incubated on ice for at least 1 min. The second master mix (MM-2) was prepared by mixing the following components (per one reaction): 4µl of 5X first strand buffer (invitrogen,USA), 1µl of 0.1M DTT (invitrogen, USA), 1µl of 40U/µl Rnase Inhibiter (invitrogen, USA)and 1µl of Superscript lll reverse transcriptase enzyme at final concentration 200U/reaction (invitrogen, USA).Then, MM-2 was added to MM-1 and the components were mixed by pipetting gently. Reaction tubes were incubated at 50ºC for 50min in order to reverse transcription of RNA and then heated at 70 ºC for 15 min.

After reverse transcription, the cDNA was amplified using primer set specific for housekeeping gene, Glycerol aldehyde phosphate dehydrogenes (GAPDH) , in order to estimate the integrity of cDNA according to Barbany *et al.* (14). The master mix was prepared by mixing the following reagents(per one reaction) : 2.5 µl of 5X green PCR buffer(promega, USA), 0.5µl of 200µM dNTPs (promega, USA), 1µl of each Forward primer(WMG): (CTG GGG TCT TCA CTA CCA) and Reverse primer: (TTG AGA GGG CCC TCT GA) (final concentration 0.4µM), 0.2µl of 5U/ µl Tag polymerase(Promega), 0.5µl of 25mM $MgCl_2$(promega) and DEPC-H2O to final volume 23 µl. The components were mixed well by pipetting and spin briefly. Then, 2 µl (equivalent to 100 ng) of cDNA was added and mixed by pipetting. Reaction tubes were transferred into the thermal cycler(XP-thermal cycler, Bioer) and the following program profile was used for amplification:94ºC for 10 min(X1), 94ºC for 30 sec(X39), 59ºC for 30 sec(X39),72ºC for 1min(X39), 72ºC for 10 min(X1).

PCR products were electrophoresd on agarose gel (1.2%) according to Sambrook *et al*. (13), or stored at -20 C until used. To detect the presence of *bcr-abl,* Monoplex-Two Steps-RT-PCR was used according to Cross (15). *bcr-abl* and *bcr* (internal control) were amplified in two separated test tubes for each sample. Master mix was prepared according to the number of patient`s samples and controls plus one additional reaction to ensure adequate volume. It was planned to perform positive control reaction tube (using RNA extracted from *bcr-abl* positive cell line K562), and negative controls (one no-template control (NTC) reaction tube and one no amplified control reaction tube (NAC) (using DEPC-H2O and RNA extracted from healthy individual, respectively). Two master mixes were prepared, first master mix was prepared for amplifying fusion gene as following(per one reaction):10 µl of 5X green PCR buffer(promega, USA), 1.5µl of 10 mM dNTPs (promega,USA),1µlof Forward primer, (5'-, ACAGAATTCCGCTGACCATCAATAAG-3')l µl of Reverse primer, (5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3'),(final concentration 1.25 µM for each), 0.2µl of 5U/ µl Go Tag DNA polymerase, (promega,USA) and DEPC-H2O to final volume 45 µl/reaction.

The second master mix was prepared as the first one, but using forward primer (5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3') and a reverse primer, (5'-ATAGGATCCTTTGCAACCGGGYCYGAA-3').

Then, 5 µl (equivalent to100 ng) of cDNA prepared from patient samples was added to each test tube. Also, 5µl of DEPC-H2O was added to NTC reaction tube, 5µl of healthy RNA was added to NAC reaction tube and 5µl of K562 extracted RNA was added to CML p210 positive control reaction tube.

Reaction tubes were transferred into the thermal cycler(XP-thermal cycler, Bioer) and the following program profile was used for amplification : 94°C for 5 min(X1), 92°C for 1 sec (X35),62°C for 1 min(X35),72°C for 1 min(X35) and 72 °C for 10 min(X1).PCR products were electrophoresd on agarose gel (1.2%) according to Sambrook *et al.* (13), or stored at – 20 C until used .

Results:

A total of 54 VB samples related to 34 CML patients, 10ALL patients and 10 healthy individuals were monitored for the presence of *bcr-abl* using Monoplex –Two Steps -RT-PCR assay. Samples that give a sufficient RNA purity (≥1.7) and quality (as estimated by denatured agarose gel electrophoresis) were selected for this assay.

CML patient's characteristics involved in Monoplex –Two steps -RT-PCR assay, including gender, age, prior treatment and CML phase were shown in table (1). All these factors studied at time point of molecular analysis.

Table 1: Characteristics of CML patients involved in Monoplex-Two Steps-Reverse Transcriptase-PCR assay.

Factors	Patient no. (%)
Gender :	
- Male	11(32.35)
- Female	23(67.64)
Age :	
- ≤ 40 ys.	25(73.52)
- > 40 ys.	9(26.47)
Prior treatment :	
- Previously treated	26(76.47)
- First line IM	8(23.52)
CML phase :	
- CP	30(88.23)
- AP	4(11.77)
-CML= Chronic myeloid leukemia, CP=Chronic phase, AP=Accelerated phase, IM=imatinib	

The efficiency of reverse transcription step was demonstrated by PCR reaction using synthesized cDNA as a template and specific primer set to amplified GAPDH gene. This reaction was considered as a screening step that will allow for the selection of cDNA samples with sufficient quality in order to apply in RT-PCR reactions. The results of GAPDH amplified reaction was shown in figure (1). Only single bands with molecular size about 600bp were detected in amplified products using cDNA from CML patients. That indicated a sufficient synthesis of cDNA.

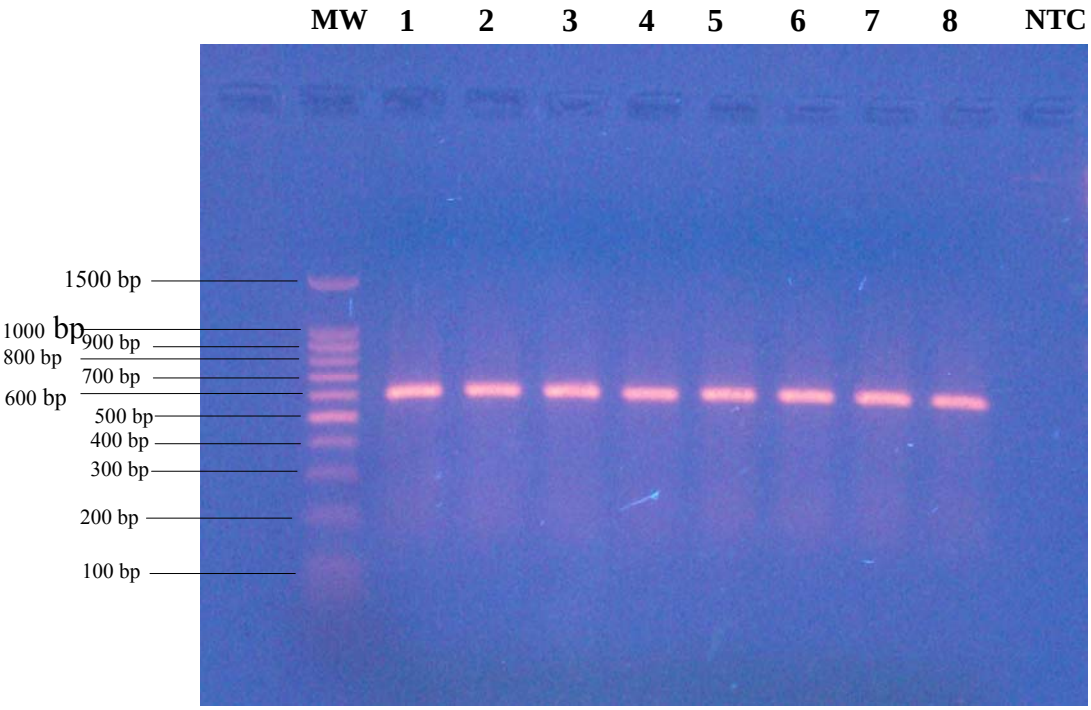

Figure (1): PCR amplified products of GAPDH gene in cDNA samples.
Lane (1-8) indicated amplified products of GAPDH gene in cDNA samples related to CML patients. (MW): DNA ladder. (NTC): No template control. Electrophoresis was carried out in 1.2% agarose gel at 5V/cm for 60 minuets.

The results of Monoplex –Two Steps -RT-PCR assay for all CML patient`s samples included in this study indicated that they were *bcr-abl* positive when detected in different intervals from starting imatinib (IM) treatment. The result of agarose gel electrophoresis of amplified products from CML using Monoplex –Two Steps -RT-PCR assay was shown in figure (2). The assay considered

satisfactory for interpretation when the internal control (normal *bcr* allele) was present, PCR product of the fusion gene positive control (K652) was positive and the NTC shown no amplified product.

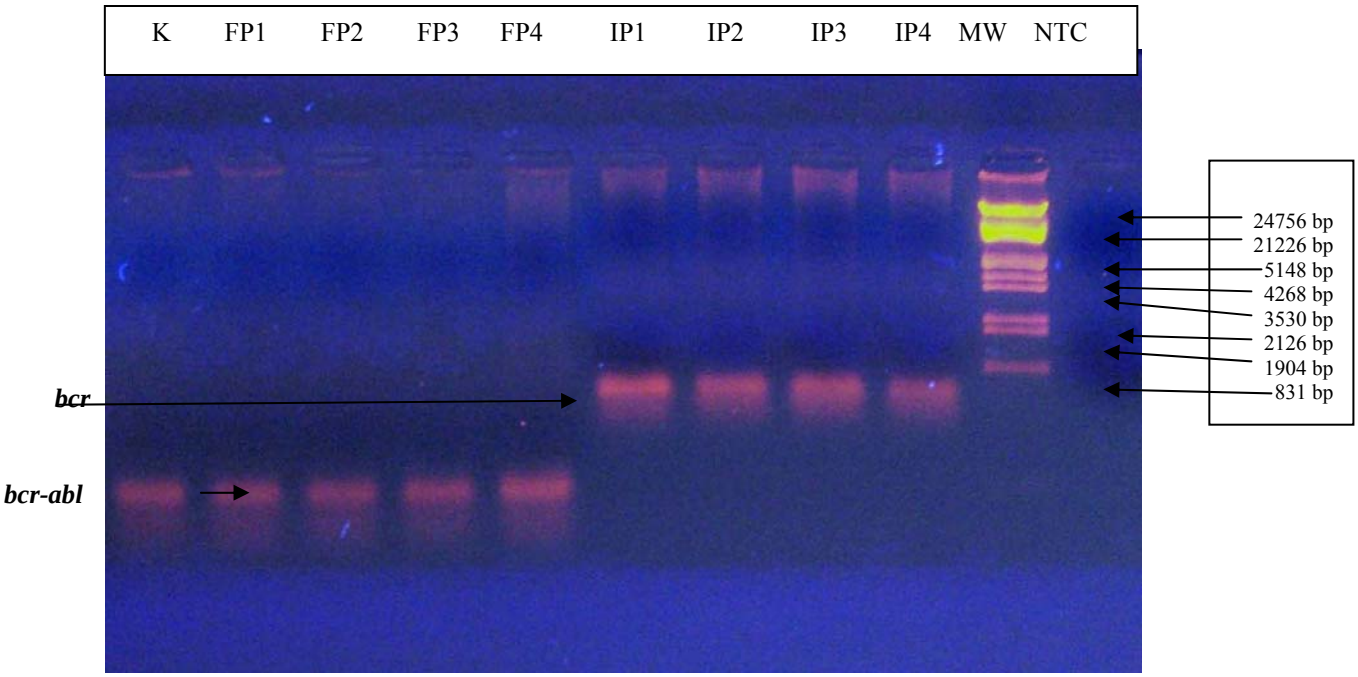

Figure (2): Monoplex-Two Steps–Reverse transcriptase–PCR amplified products.
Lane FP1-FP4 indicated amplified products of *bcr-abl* while Lane IP1-IP4 indicated amplified products of internal control (*bcr*) in CML patient's samples. Lane (K): Amplified product of standard RNA extracted from cell line K652.Lane (NTC): No template control. MW: Molecular Wight marker of lambda DNA restricted with *Eco RI+Hind III.* Electrophoresis was carried out in 1.5% agarose gel supplied with ethidiume bromide at (5V/cm) for 60 minuets

As shown, there was one band with a molecular size approximately of 350 bp (fusion transcript) detected in lane (FP1-FP4) referred to amplification products from 4 CML patients samples. The other band with a molecular size approximately of 800 bp (referred to *bcr* allele, as internal control) detected in lane (IP1-IP4), in compare with molecular weight marker and amplified product of K652. The type of transcript was identified in CML patients as b3a2 (according to molecular size of k652). Interestingly, two of those patients were identified as *bcr-abl*, b3a2 in other lab (Molecular Biology Lab/College of Medicine/Jordanian University). None of the *bcr-abl* fusion variants were detected in amplified products of ALL or healthy individuals.

Discussion:

The purpose of this study was to validate the clinical value of monoplex- reverse transcriptase –PCR reaction as laboratory routine molecular screening for the presence of bcr-abl fusion gene.Quality of extracted RNA is a critical point that limited the successful of further steps in RT-PCR analysis. It is affected since sample collection. The best way to store sample is as lysate in Guanidinium Thiocyanate (GTC) and at -20ºC, in which the RNA is stable for months or stored at -80ºC in which the RNA is stable for years. Some samples shown degraded extracted RNA or RNA with insufficient purity repeatedly, even with new sample obtained from the same patient. That may be due to either contamination with RNase or an individual physiological factors effecting RNA extraction (16). All CML patients were *bcr-ab*l positive after different IM treatment periods. The observation of that the vast majority of patients on IM treatment whom achieved complete hematological response and /or major cytogenetic responce but still have measurable disease after at least 12 to ≥18 months of IM therapy is consistent with experimental observations of Hughes *et al.* (17).Reason behind this status is that IM acts mainly by inhibiting proliferation rather than by inducing apoptosis in *bcr-abl* positive leukemic cells (18). In this study, *bcr-abl* was detected in blood samples. In other study, they have conducted a quantitative analysis and serial comparison between peripheral blood and bone marrow sample. They demonstrate that blood samples provide information of similar quality to that obtained by the assay performed on bone marrow samples (18). An amplified product from the bcr gene is the only band detected in those healthy individuals who were considered as negative control. The sole presence of this band indicates that the quality of RNA was good, thus the individual confidently can be considered to be negative for *bcr-abl* . The notion that monoplex-two steps-RT-PCR methods and conventional cytogenetics are

complementary tests is supported by our results and that conserned with what mentioned by (19, 20). The assay detected the translocation and, despite the use of multiple primers in a multiplexed PCR scenario. The specificity of this assay was improved by that there were no bands produced in the split-out reactions for the *bcr-abl* that could have been misinterpreted as positive. All of the former cases, with a submitted clinical diagnosis of "rule out CML" that were *bcr-abl* negative with the Monoplex-Two steps-RT-PCR assay, were also negative (for all fusion transcripts) with the multiplex-one step-RT PCR system. Accordingly, this facet of the study validated the diagnostic accuracy and diagnostic sensitivity of the monoplex assay.

Conclusion:

Importantly, however, this study illustrates how the availability of a broad spectrum RT-PCR assay may improve clinical diagnostic accuracy. Also, molecular-based screening via RT-PCR system is still extremely valuable in clinical practice, given the quite high false-negative rate of conventional cytogenetic.

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