

## Analysis and Identification of Mutation in Chronic Myelogenous Leukemia (CML) and BCR\_ABL in Leukemia Patients aged 40 to 50 Year

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(Received: 27 March 2012; accepted: 09 May 2012)

Chronic Myelogenous Leukemia (CML) is one of the few cancers that is known to be caused by a single, specific genetic mutation in more than 90% of cases. The transformation to CML is caused by a reciprocal translocation of the BCR gene on chromosome 22 (at q11) and the ABL gene on chromosome 9 (at q34), resulting in a fused BCR-ABL gene dubbed the "Philadelphia chromosome", approximately 44,000 people in the United States are diagnosed with some form of leukemia. The tyrosine kinase inhibitors (TKIs) Imatinib, Dasatinib, and Nilotinib inhibit activity of the BCR-ABL fusion protein, resulting in both hematologic responses. The aim of my study was to determine and confirm presence of Philadelphia chromosome and presence of BCR-ABL in patient with CML. Determination of the presence of the Ph chromosome by karyotyping at diagnosis is necessary as part of differential diagnosis and as prognosis. The standard methods of banding are the Q, G, R, and C banding techniques. In this study chromosomes have been treated to trypsin for taking different bands. karyotyping and FISH were equal, as shown in the present work, except for the fact that FISH was faster. In cases that did not present metaphases by karyotyping, FISH was extremely useful in providing a result. In daily practice, FISH can be very helpful since index of karyotyping, when the patient is receiving myelotoxic drugs, there may not be enough metaphases for analysis. RT-PCR is still more sensitive but its clinical meaning has to be clarified. Most of PCR include the detection of the translocation at the level of the RNA transcript. The method usually consists of two steps. The first reaction is that of obtaining cDNA from RNA by means of reverse transcription (RT). Subsequently, an aliquot of cDNA is transferred to another tube and subjected to the second reaction, that is, PCR amplification. Two different enzymes are used, namely, reverse transcriptase for the RT step and Taq polymerase for the PCR. Flow cytometry is a technique for analyzing large populations of single cells. For this method, the diagnostician has to analyze multiple bivariate dot plots from the cytometer. The flow cytometric immune bead assay is a fast and easy technique for specific detection of BCR-ABL proteins in leukemic cells. It is not dependent of the breakpoint position in the BCR gene, does not need special laboratory facilities other than a routine flow cytometer, provides results within several hours and it can be run in parallel to routine immunophenotyping.

**Key words:** Chronic Myelogenous Leukemia, Cancer, Mutation, Mid-age.

### Leukemia

Leukemia is the general term used to describe several different types of blood cancers. Each year, approximately 44,000 people in the United States are diagnosed with some form of leukemia. In this section, we discuss some promising results from clinical trials for people with the following types of leukemia.

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All of these forms of blood cancer affect various types of white blood cells, which are made by the bone marrow and help the body fight infections and other diseases. However, a major difference among these cancers is how they grow. The chronic forms of leukemia tend to grow slowly; the acute forms of leukemia grow more quickly (Margie Patlak, 1998).

#### **Clinical Recommendations**

Given the strong evidence demonstrating benefit with BCR-ABL TKIs in patients with CML, NCCN Clinical Practice Guidelines recommends their use in patients with CML with confirmed BCR-ABL transcripts in bone marrow or evidence of translocation on cytogenetics. Treatment-naïve patients with chronic phase CML should be started on imatinib, or dasatinib or nilotinib if resistant to or intolerant of imatinib. Patients in accelerated phase should be started on dasatinib or nilotinib, and patients in blast phase should be started on dasatinib. Importantly, if patients are BCR-ABL negative or Ph-negative, diagnosis of CML should be reconsidered (John Goldman, 2008). 4

#### **Mechanism of BCR-ABL in the Pathogenesis of Chronic Myelogenous**

If the ABL tyrosine-kinase activity is essential for BCR-ABL-mediated leukaemogenesis, then why are all the leukaemic cells in patients with CML not eradicated by imatinib treatment? Can studies using mouse models of BCR-ABL-mediated leukaemogenesis provide useful information about the mechanisms of this residual disease? (<http://www.ncbi.nlm.nih.gov/pubmed>).

In examining the role of the ABL kinase activity in mouse BMT models, bone-marrow cells that expressed an ABL-kinase-deficient form of BCR-ABL seemed to propagate normally in mice, although they did not become leukaemogenic. Similarly, human CD34<sup>+</sup> cells that express the ABL-kinase-deficient form of BCR-ABL respond to cytokines like normal CD34<sup>+</sup> cells. So how does the imatinib, which blocks the kinase activity of ABL, induce apoptosis in CML cells? (<http://www.ncbi.nlm.nih.gov/pubmed>).

Cytokines maintain the homeostasis of normal haematopoietic cells, by regulating their proliferation, survival and differentiation. As described above, BCR-ABL can promote the survival and proliferation of myeloid cells

by activating cytokine-receptor signalling. In addition, BCR-ABL induces overproduction of certain cytokines. These activities are likely to account for the massive expansion of haematopoietic cells that occurs during CML development. As the tyrosine-kinase activity of ABL is essential for most, if not all, of the activities of BCR-ABL in regulating cell survival and proliferation, inhibition of the ABL kinase could lead to inhibition of BCR-ABL signalling and reduced production of cytokines. The return to normal level of cytokine concentrations would not be able to support the excessive amount of haematopoietic cells, so the excess CML cells would die of growth-factor starvation (<http://www.ncbi.nlm.nih.gov/pubmed>).

Inhibition of the tyrosine-kinase activity of BCR-ABL by imatinib does not simply cause cells to revert to cytokine-dependent growth, however. Imatinib induces apoptosis of patients' leukaemic progenitor cells that are cultured in the presence of cytokines, with little effect on normal haematopoietic cells. These results indicate that the cytokine signalling is disrupted in CML cells, and that the survival of CML cells has become BCR-ABL dependent. It is possible that 5

BCR-ABL, in activating intracellular signalling pathways, might suppress the normal cytokine response through an unknown mechanism (<http://www.ncbi.nlm.nih.gov/pubmed>).

Although *in vitro* colony formation by CML progenitor cells is significantly inhibited by imatinib, not all CML cells are sensitive to the drug a subset of CML cells can grow in response to external cytokines independently of the tyrosine kinase activity of BCR-ABL. Furthermore, CD34<sup>+</sup> progenitor cells isolated from nucleated bone-marrow cells of patients with a complete cytogenetic response (assayed on unsorted nucleated bone-marrow cells, in which CD34<sup>+</sup> cells are rare) to imatinib therapy tested positive for the presence of *BCR - ABL* when cells were analysed by fluorescence *in situ* hybridization. Quantitative PCR assays have also demonstrated the level of the *BCR - ABL* transcript was higher in the CD34<sup>+</sup> cell population, indicating an enrichment of BCR-ABL<sup>+</sup> cells in the progenitor fraction. Colony-formation assays showed that BCR-ABL<sup>+</sup>CD34<sup>+</sup>

cells isolated from imatinib-treated patients retain functional committed and primitive progenitor capacity. So CML progenitor cells seem to be refractory to imatinib therapy, compared with more mature cells (*M.W. Drummond, T.L. Holyoake, 2001*).

The mechanism of resistance of CML progenitor cells to imatinib treatment is not known. It is possible that the intracellular concentration of the drug in these cells is low because of their unique efflux-pump activity. More potent BCR-ABL tyrosine-kinase inhibitors, or combinations of BCR-ABL kinase inhibitors, might overcome this problem. It is also possible that residual leukaemic cells express mutant forms of BCR-ABL that are resistant to imatinib. It has been shown that imatinib-resistant mutant forms of BCR-ABL exist in some patients even before imatinib treatment. Furthermore, genetic defects other than t(9; 22)(q34; q11) might cause resistance of CML cells to imatinib. However, rapid expansion of such mutant cells and relapse of the disease are expected. Therefore, this mechanism might not apply to most imatinib-treated patients with a durable and complete cytogenetic response (*M.W. Drummond, T.L. Holyoake, 2001*).

The most worrisome possibility, with regard to minimal residual disease in patients undergoing treatment with imatinib, is that the survival and even proliferation of some CML progenitors is not fully dependent on BCR-ABL. This would mean that such kinase inhibitors might not be able to kill the cancer stem cells, even when they can fully inhibit the kinase activity of BCR-ABL. 6

There is some evidence to support this idea. BCR-ABL expression promotes cytokine-independent growth of haematopoietic cell lines, such as the murine myeloid cell line 32D and the human megakaryocytic cell line MO7e. Imatinib treatment did not inhibit the proliferation of these cells in the presence of IL-3 (for 32D cells) or GM-CSF (for MO7e cells), although it did induce apoptosis of BCR-ABL-expressing 32D cells and MO7e cells in the absence of cytokines. The proliferative capacity of BCR-ABL expressing 32D, but not MO7e cells, in the presence of imatinib, however, could be rescued by addition of exogenous cytokines. So, imatinib just causes some cells to regain cytokine-dependent proliferation

(*Ruibao Ren, 2005*).

What does this mean for treatment of patients? Residual disease in CML patients on imatinib might be maintained, at least partially, by the normal haematopoietic environment. A study of human CML cells showed that imatinib functioned by inhibiting the abnormally increased proliferation rates of progenitor cells, rather than by selective induction of apoptosis. Stem-cell quiescence could contribute to such imatinib resistance. So, haematopoietic cells might differ in terms of developing BCR-ABL dependency. In addition, the tyrosine-kinase-defective form of BCR-ABL retains the ability to dysregulate cell adhesion and migration. The effects of these altered cell activities in the maintenance of residual disease in CML patients on imatinib therapy will require further study (*Ruibao Ren, 2005*).

There are other ways in which CML cells might survive and proliferate even in the presence of a BCR-ABL kinase inhibitor such as imatinib. Recent studies have shown that expression of the AML-associated oncogene *MOZ - TIF2*, formed by a fusion of the gene encoding the acetyltransferase *MOZ* with the gene encoding the nuclear receptor coactivator *TIF2*, in committed myeloid progenitors such as common myeloid progenitors and GMPs (which have no self-renewing capacity) can induce leukaemia in a BMT mouse model. However, expression of BCR-ABL in these same cell types, using the same model system, fails to induce disease. These results indicate that *MOZ-TIF2*, but not BCR-ABL, can confer properties of leukaemic stem cells to some haematopoietic progenitors. So it is possible that BCR-ABL-mediated leukaemogenesis relies on the self-renewal capacity of the original haematopoietic stem cell, and that the activity of BCR-ABL might be dispensable for the self-renewal capacity of CML stem cells, (*Ruibao Ren, 2005*).

#### **Maps for BCR-ABL and BCR-ABL mRNA Transcripts**

In these map three major kinds of breakpoint is applicable, major, minor and micro. In major point important area b2a2 and b3a2 was investigated with size of P210 KDa. In the case of minor break point e2a2 and for micro break point e9a3 has been investigated, which micro point had size of P230 KDa (figure 1), (*A Hochhaus, et al, 1996*).

## MATERIAL AND METHODS

### Karyotyping

#### Spread Chromosome

5ml of patient's blood (for 12 patients) has taken and centrifuged at 2000 rpm for 5 minutes, at that time the erythrocytes settled by gravity, approximately 4 ml of leukocyte-rich plasma on the top has been left, and a white Buffy coat of leukocytes was formed in the middle. Very carefully this buffy coat has been removed by using of a needle without any mixing. Slowly expel the leukocyte-rich plasma and the buffy coat into a sterile tissue culture flask containing 8 ml of Eagle's spinner modified media supplemented with 0.1 ml of phytohemagglutinin (PHA). Cultures have been incubated for 66-72 hours at 37° C and gently agitate the culture once or twice daily during the incubation period.

0.1ml of colcemid (10 micrograms/ml) was added to the all culture flasks and incubated for an additional 2 hours. The colcemid-treated cells were transferred to a 15 ml centrifuge tube and centrifuged at 225 xg for 10 minutes. Then aspirated and discarded all but 0.5 ml of the supernatant. The bottom of the centrifuge tube has been gently taped to resuspend the cells in the remaining 0.5 ml of culture media. 10 ml of 0.075 M KCl, was added dropwise at first, and then with gentle agitation to the centrifuge tube, gently mixed with each drop. The cells has been stand exactly 6 minutes in the hypotonic KCl. The cells were centrifuged at 225 xg for 6 minutes. The KCl was discarded but 0.5 ml of the supernatant, gently resuspended the cells in this small volume of fluid. 10 ml freshly prepared fixative was added dropwise at first and then with gentle agitation. If the cells were not properly resuspended in previous step, the cells will clump beyond any further use. The cells ha been allowed to stand in fixative at room temperature for 30 minutes. Centrifuged at 200 xg for 5 minutes and removed all but 0.5 ml of supernatant. Resuspend the cells in fresh fixative. The cells were washed twice more in 10 ml volumes of fixative. The fixative was added slowly, recentrifuge, and aspirate the fixative as previously directed (table2). 9

A piece of dry ice against the side of a styrofoam container was kept and lace a clean slide onto the dry ice to chill the slide, then the slide

was removed and air dried, after dried the slide, drop few drops of DNA sample with optimum distance (2 to 3 feet) on the slide, and perform suitable stain (Repeat for all samples).

#### Staining and banding

Slides before staining have been dried (air dried only); the banding which used in this study was G-banding. Treat fixed and flamed slides in alkaline solution, kept at room temperature for 30 seconds. They have been rinse in saline-citrate solution, 3changes for 5-10 minutes, and then they were incubated in saline-citrate solution, 65° C for 60-72 hours. All the slides treated with 3 changes of 70% ethanol and 3 changes of 95% ethanol (3 minutes). They treated with trypsin for 45 seconds. Air dried and Stained in buffered Giemsa for 5 minutes, briefly was rinsed in distilled water, finally air dried and mounted. Trypsine has been used for detection of bands (Banding) in chromosome.

Photograph from the chromosomes was taken by which have been detected under camera, and then printed them out, arranged the chromosomes and identified the Philadelphia chromosome (figure 3).

#### Fluorescence in Situ Hybridization (FISH)

Fluorescent in situ hybridization (FISH) is a molecular cytogenetic method used to investigate chromosomal abnormalities associated with leukemias, solid tumors, bone marrow transplants and genetic disorders. The chief advantage of FISH is that it can be applied to non-dividing, uncultured cells (interphase nuclei) when applicable to help establish or confirm diagnoses; whereas, classical cytogenetics require dividing cells. FISH is performed on a variety of specimen types such as blood, bone marrow and paraffin-embedded tissue sections. Because of the higher sensitivity, FISH testing can detect mosaicism for clonal chromosome changes, and is therefore useful in the detection of minimal residual disease following treatment (e.g., interferon for chronic myelogenous leukemia [CML]. Because the majority of specific deletions is sub-microscopic and can only be detected by molecular tests, FISH plays a critical role in the confirmation of microdeletion syndromes (*Gordon W, et al, 1998*).

The blood sample of the patients has been taken (12 patients). The slides were Incubated for 30 min in 2xSSC (pH 7.0) prewarmed to 37°C in a coplin jar (4 slides at a time in the coplin jar), and

dehydrated sequentially in 70%, 85% and 100 % ethanol series, 2 min each, all has been air dried. Probe was prepared in following procedure, 7  $\mu$ l buffer was added to 1  $\mu$ l of distilled water, then 1  $\mu$ l of each probe (in this paper CEP6 Spectrum Green probe and CEP17 Spectrum Orange probe) has been Centrifuged 1-3 seconds (short spin), and they have been Vortex, Recentrifuged and were Heated for 5 min. at 73 °C in a water bath to denaturation process. Denaturant solution (70% Formamide/ 2xSSC) has been placed in 73 °C water bath inside coplin jar; slides were denatured for 5 min, dehydrated in 70%, 85% and 100% ethanol for 2 min. each, and air dried. 10  $\mu$ l denatured probe has been applied and cover with a cover glass. Hybridizing area was marked on the all slides used a diamond scribe, they were sealed carefully with rubber cement. the slides has been placed in a prewarmed humidified box (wrapped in metal foil to protect against light) and were incubated overnight at 42°C. 0.4 X SSC/ 0.3% NP-40 has been placed in a 73°C water bath, cover glasses were removed and immediately placed into wash tank with 0.4xSSC/ 0.3% NP-40. All slides were left in coplin jar for 2 min. The slides were placed in 2xSSC/ 0.1% NP-40 at room temperature for 1 min and air dried in darkness. 20  $\mu$ l of probe (DAPI) solution has been applied to the target area and putted on cover glass (it has covered hybridized area). Examined slides on a fluorescence microscope. Slides showing more than 50% cells with fluorescent dots were selected for analysis. From each slide, at least 100 overlap signals. Red dots (Rhodamine) corresponded to the ABL (9q34.1) gene and green dots (Fluorescein) to the BCR (22q11) gene, so when one cell with two isolated red and green dots was seen, it was counted as normal, without rearrangement. When a cell with one isolated red dot, one isolated green dot and one fused red and green signal was seen, this was considered as presenting rearrangement (Figure 4). All slides were analyzed by at least two observers. One samples of normal blood donors served as a control. The control provided an estimation of overall hybridization quality of the test material

#### PCR

#### Extraction of RNA from the blood sample of the patient

Sample has taken from 12 patients 40 to 50 years and RNA isolation from leukocytes from

fresh peripheral blood samples was performed by TRI method. In this method I have used of TRI solution, which is composed of the following solutions:

- TRI reagent 750  $\mu$ l
- Patient blood 200  $\mu$ l
- Acetic acid 20  $\mu$ l

Tri-RNA reagent is a reagent from the improved phenol and guanidine isothiocyanate (GSN) method for the single-step RNA isolation. During sample homogenization or lysis, Tri-RNA reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. The composition of Tri-RNA reagent includes phenol and GSN in a mono-phase solution. A biological sample is homogenized or lysed in Tri-RNA reagent and homogenate is separated into the aqueous and organic phases by chloroform addition, vortexing and centrifugation. RNA remains exclusively in the aqueous phase (clear upper phase), DNA in the interphase, and proteins remain in the organic phase (red color). RNA is precipitated from aqueous phase by addition of isopropanol and solubilized/concentrated. If needed, DNA and proteins can be sequentially precipitated from the interphase and organic phase with ethanol and isopropanol respectively, and solubilized/concentrated.

In the first step of my project, 200  $\mu$ l of patient sample was taken and 750  $\mu$ l of TRI reagent has been added followed by 20  $\mu$ l of acetic acid, vortexing till gain a uniform solution. Incubate at room temperature for five minutes. 200  $\mu$ l of chloroform was added and shake it vigorously for 30 seconds, whenever a uniform solution has been formed incubate at room temperature for another five minutes.

Centrifuged the sample at 12000 rcf for 8 minutes at 4°C, in this stage we have observed three layers which, upper layer was RNA middle was DNA and precipitated layer was include protein and other organic matter, I have taken the upper layer which was involved RNA in the separate tube. 500  $\mu$ l of isopropanol was added to that and incubate at room temperature for 5 to 10 minutes. Centrifuged at 12000 rcf for 8 minutes at 4°C, discard supernatant and pellet was taken, 1  $\mu$ l ethanol (70%) was added for washing of the pellet, vortexing the solution properly.

Centrifuged sample at 7600 rcf for

5 minutes at 4°C, supernatant was discarded and pellet has been dried which was RNA sample (between 5 to 10 min at room temperature), 20 µl nuclease free water has been added to dried pellet and kept in thermo mixer at 55°C for 10 minutes. It has kept in nano drop spectrophotometer now for quantification of RNA sample (steps have been repeated for all samples).

#### **RT-PCR for bcr-abl**

For some years, this chromosomal damage has been detected cytogenetically. However, recently, cytogenetic procedures have been extensively replaced by polymerase chain reaction (PCR) based protocols. The advantages of PCR detection of BCR-ABL translocation lie in its high specificity and unique sensitivity<sup>5-7</sup>: PCR is able to detect the mutated gene even in some cytogenetically normal (Ph negative) leukaemic clones and can even detect it in the presence of thousands of normal genomes. Thus, PCR is much more informative than cytogenetics alone as a means of t(9, 22) detection (*S. Menif, et al, 2007*).

RT-PCR was used to amplify the chimeric BCR-ABL mRNA. The nucleotide sequences of the PCR primers used in *BCR-ABL* amplification are listed in Table 3. For all PCR reactions a 1025 and 1026 dilution of an Amplichek BCR/ABL positive CML control RNA (BioRad, Hercules, CA) into the Amplichek BCR/ABL negative RNA served as the positive controls. Negative controls included a no-RNA PCR mix ("blank") and the Amplichek negative control RNA. A separate amplification of β<sub>2</sub>m was used as a control of RNA integrity and was performed as previously described. Precautions to eliminate PCR carryover contamination included separate rooms for pre-PCR and amplification procedures, aerosol-resistant pipette tips, and no-nucleic acid PCR reactions as negative controls in all PCR amplification reactions.

First step was creation of the complimentary DNA (cDNA) from RNA with the help of RTPCR which was as follow:

2.2µl of magnesium chloride (MgCl<sub>2</sub>), 2.5 mMolar was added to the sample, and then 2µl of dNTPs (A, G, C and T) followed by random hexamer or primer 0.5 µl was added to the tube. After that 0.25 µl of enzyme (Reverse transcriptase or RTase) has been added, in the last stage for prevention of degradation of RNA by enzymes (RNase), the

RNase inhibitor has been add 0.2 µl to the tube, this used for all vessels (table 4).

In this stage the samples have transferred to the PCR set and regulate that to convert RNA to cDNA in the following stage which is involve three major stages as follow:

In the first stage samples in the set have regulated to keep at 25°C for 10 minutes, in the second stage set was at 95 °C for 5 minutes and in the last stage it was set at 48 °C for 30 minutes.

#### **Qualitative PCR**

After preparation of the cDNA the qualitative PCR has been done by below protocol: First 8µl of positive control (PC) was added to a vessel, after that 2µl of the sample has been added to that, in this stage I have added 1µl of NTC (major β<sub>2</sub>M), which was reagent of negative control for this experiment, it was followed by adding of 9µl of NT (β<sub>2</sub>M). The samples were ready for PCR now and samples have kept that in following pattern:

Stage i) kept at 94 °C for 2 minutes which is called as predenaturation.

Stage ii) puts at 94 °C for 30 seconds (denaturation satage), followed by 58 °C for 30 seconds (Annealing stage) and 72 °C for 1 minutes (extension stage).

Stage iii) again kept at 72 °C for 7 minutes (final extension stage), in case of any forgetting or later requiring of sample PCR set are able to preserve that at 4 °C at least for 24 hours.

Note: β<sub>2</sub>M negative control has to be house kept for taking proper result.

After PCR amplified cDNA patient sample was loaded in 1.2% agaros electrophoresis gel, the 1X, TE buffer and electric field was applied. The running of sample was takes place and observed under UV radiation.

#### **Quantitative RT-PC**

The 59 exonuclease-based fluorogenic real-time RT-PCR of BCR-ABL and β<sub>2</sub>m was performed in single tubes in an ABI Prism 7700 sequence. Quantification was performed on samples that were qualitatively positive. The initial qualitative assay allowed for the determination of the b2a2 or b3a2 BCR-ABL breakpoints. In the quantitative PCR (Q-PCR) assay, different BCR primers were used, depending on the BCR breakpoint, in order to generate PCR products in the optimal size range. Thus, in this PCR assay yielded an 85–base pair (bp) product for the b2a2

BCR-ABL breakpoint and an 84-bp product for the b3a2 breakpoint. The amplification product for the  $\beta$ 2m gene was 70 bp.

In the Q-PCR assay, 100 nmol/L of a template-specific (either BCR-ABL or  $\beta$ 2m) oligonucleotide probe was used. The probes were labeled at the 59 end with 6-carboxy fluorescein and at the 39 end with 6-carboxy-tetramethyl rhodamine. Labeled probes were synthesized by Synthesgen (Houston, TX). RT-PCR was carried out in 50-mL reactions containing 1 X PCR buffer (PerkinElmer, Foster City, CA), 1.5 mmol/L  $MgCl_2$ , 100  $\mu$ M each deoxynucleoside triphosphate, template-specific primers, 7.5 U Prime RNase Inhibitor (5 Prime-3 Prime, Boulder, CO), 50 U Superscript II RT (Gibco BRL), 1.25 U Amplitaq DNA Polymerase (PerkinElmer), and 1.25 U Platinum Taq Antibody (Gibco BRL). In addition, each reaction contained a 6-mer attached to a fluorescent dye, 6-carboxy-X-rhodamine to serve as a passive internal reference. The sequences of the primers and probes used for the BCR-ABL and  $\beta$ 2m Q-PCR assays are listed in Table 3. The specific primers and amounts for the Q-PCR reaction were as follows: for b2a2, ABLTM (37.5 pmol) and BCRTM2 (37.5 pmol); for b3a2, ABLTM (37.5 pmol) and BCRTM3 (25pmol); and for  $\beta$ 2m,  $\beta$ 2TMF (12.5 pmol) and  $\beta$ 2TMR (12.5 pmol). In the Q-PCR assay,

the initial RT step was carried out at 42°C for 30 minutes followed by an inactivation step at 95°C for 5 minutes. The complementary DNA was immediately subjected to 40 cycles of a 2-step PCR. Each cycle consisted of a 15-second denaturation step at 95°C and a 1-minute combined annealing/extension step at 60°C.

## RESULTS

The aim of my study was to determine and confirm presence of Philadelphia chromosome and presence of BCR-ABL in patient with CML. Following data acquisition, plots such as those shown in Figure 6 can be generated using software such as BD FACSDiva™ software. After the capture beads have been identified, the presence of sandwich complexes is confirmed by the bead population's median fluorescence intensity (MFI) as generated by the detector reagent. A sample positive for BCR-ABL fusion proteins is identified by a PE MFI higher than a predetermined analytical detection limit.

In this study for this test I have used of two different types of blood sample, one from Ph negative (a person with BCR-ABL negative) and another one from a Ph positive (a person with BCR-ABL positive), which the difference is detectable

**Table 1.** Classification of Leukemia (Margie Patlak, 1998)

Cell type	Acute	Chronic
Lymphocytic Leukemia (or "lymphoblastic")	Acute Lymphoblastic Leukemia (ALL)	Chronic Lymphocytic Leukemia (CLL)
Myelogenous Leukemia (also "Meyloid" or "Nonlymphocytic")	Acute Myelogenous Leukemia (AML)	Chronic Myelogenous Leukemia (CML)

**Table 2.** Material requirement for karyotyping (for each slide)

S. No.	Compound Name	Amount of Compound
Spread DNA		
1.	Eagle's spinner	8 ml
2.	phytohemagglutin (PHA)	0.1 ml
3.	colcemid (10 1/4g/ml)	0.1ml
4.	KCl (0.075 M)	10 ml
Sataining & Banding		
1.	saline-citrate solution	Enough amount
2.	Ethanol (70% & 95%)	Enough amount
3.	Giemsa	2-3 drops
4.	Trpsin	Enough amount

in the following figure (figure 6). However it is preferred to use of karyotyping and PCR method due to highly specificity for BCR-ABL.

#### BCR-ABL

The karyotyping of erythrocytes from the patient's samples has been done by G-banding staining, and Philadelphia chromosome was observed as it is illuminated in the figure 7, so presence of Ph chromosome is showing that sample is positive for BCR-ABL t(9;22)(q34;q11.1) by conventional karyotyping

The chromosomes were observed under fluorescence microscope, by which three different signals have been detected, green, orange and yellow. Green signals were BCR, orange signal were ABL and yellow signals has detected the BCR-ABL gene present in the chromosome. The green signal is seen in the entire nucleus possessing t(9;22).

#### BCR

One orange, one green and one fused

**Table 3.** Rt-pcr Primers and Probes

Qualitative RT-PCR	
First-round RT-PCR	
CMLNA	59TGGAGCTGCAGATGCTGACCAACTCG39
CMLNB	59ATCTCCACTGGCCACAAAATCATACA39
Second-round nested PCR	
CMLNH	59GATCTCCTCTGACTATGAGCGTCCA39
CMLNI	59-TTCACCTTTAGTTATGCTTAGAGTG39
Quantitative RT-PCR (Q-PCR)	
bcr-abl b2a2 primers	
BCRTM2	59CAT TCC GCT GAC CAT CAA TAA39
ABLTM	59AAC GAG CGG CTT CAC TCA GA39
bcr-abl b3a2 primers	
BCRTM3	59CCA CTG GAT TTA AGC AGA GTT CA39
ABLTM	59AAC GAG CGG CTT CAC TCA GA3
bcr-abl probe	
ABLTMPROBE	59AGCGGCCAGTAGCATCTGACTTTGAGC39
$\beta$ 2-microglobulin primers	
$\beta$ 2TMF	59CAT TCG GGC CGA GAT GTC39
$\beta$ 2TMR	59CTC CAG GCC AGA AAG AGA GAG TAG39
$\beta$ 2-microglobulin probe	

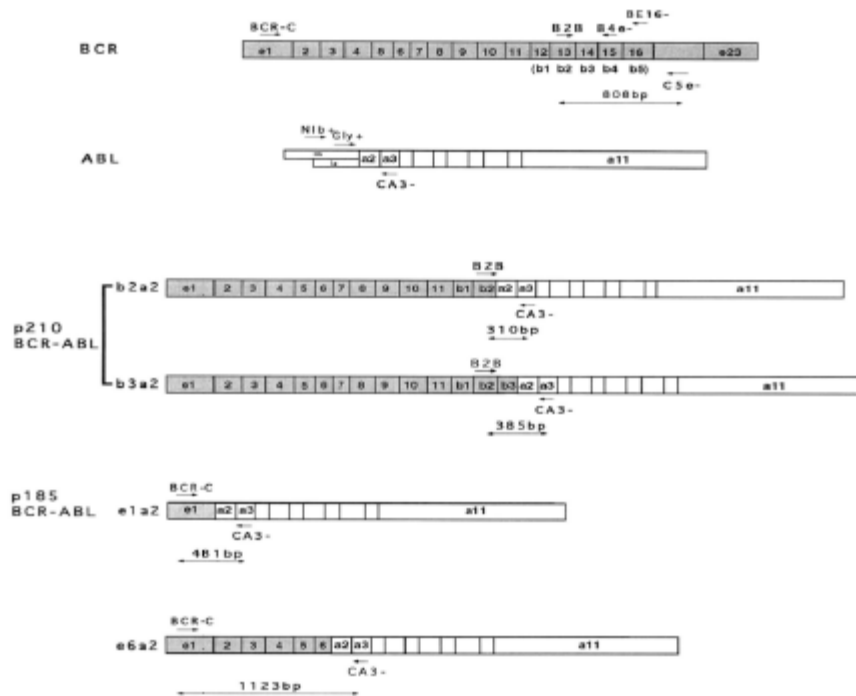
**Table 4.** Qualitative RTPCR material requirement

S. No	Compound name	Amount of compound ( $\mu$ l)
1.	Mgcl2	2.2 $\mu$ l
2.	dNTPs	2 $\mu$ l
3.	primer	0.5 $\mu$ l
4.	RTase	0.25 $\mu$ l
5.	RNase inhibitor	0.2 $\mu$ l
6.	DNA sample	4 $\mu$ l
7.	H2O	0.85
Total	10 $\mu$ l	

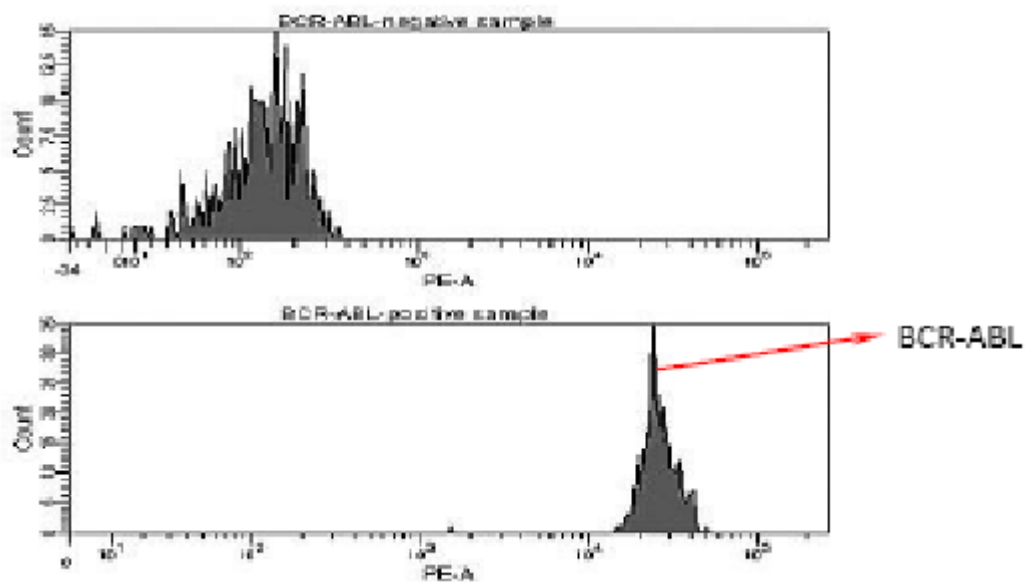
**Table 5.** DNA Extraction Result

Purity in 260/280	Purity in 260/230	Concentration of RNA (ng/ $\mu$ l)
1.91	1.13	244.5

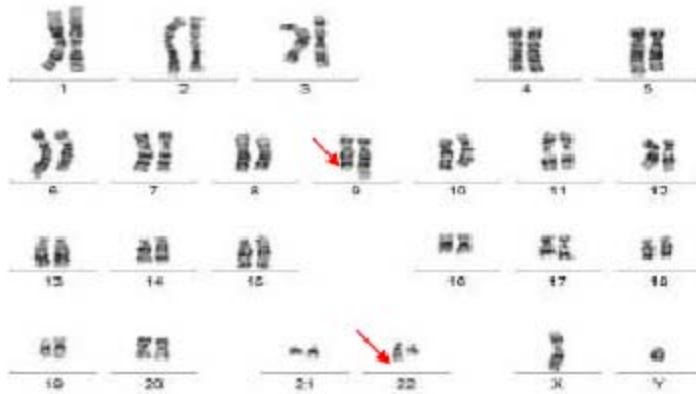




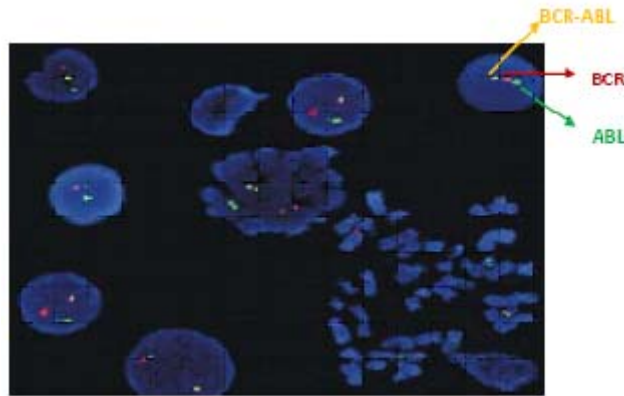
**Fig. 1.** Maps of BCR, ABL, and BCR-ABL mRNA transcripts. Primers BCR-C, 828, CA3-, and C5e- were used for multiplex PCR for BCR and BCR-ABL; primers BCR-C and CA3- were used for single-step PCR for BCR-ABL; and primers Nbl, Gly, Me-, and BE16- were used for nested PCR for ABL-BCR (adapted from: A. Hochhaus, *et al.*, 1996)



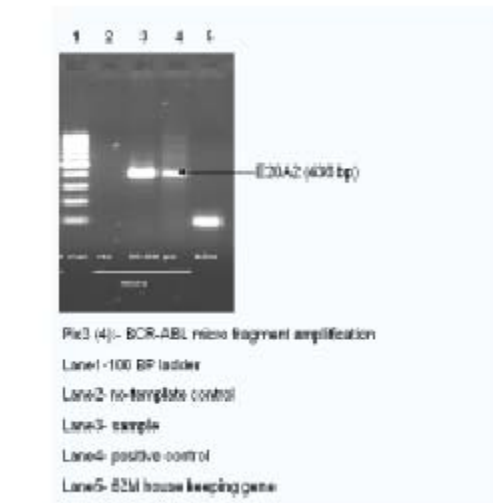
**Fig. 2.** PE-A histograms from a BCR-ABL-negative sample (top) and a BCR-ABL-positive sample (bottom)



**Fig. 3.** Presence of Ph (Philadelphia) chromosome t(9;22)(q34;q11.1) by conventional karyotyping



**Fig. 4.** In Interphase FISH showing presence of BCR/ABL gene fusion. One orange, one green and one fused orange and green signal is seen in all the nucleus possessing t(9;22)



**Fig. 5.** The electrophoresis of the samples showed e20a2 band with 436bp size, when analyzed by qualitative and quantitative PCR methods

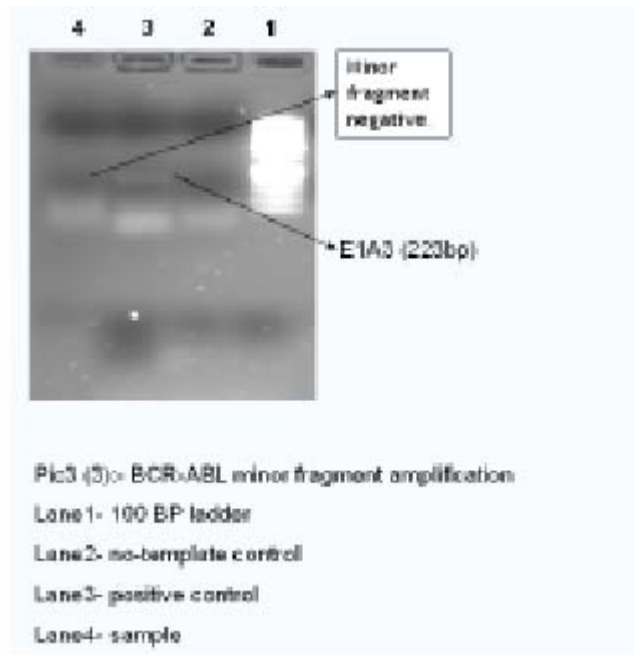
orange and green signal is seen in all the nucleus possessing t(9;22).

**ABL**

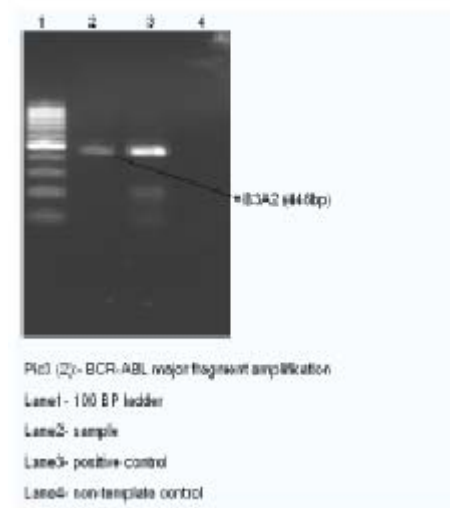
In this sample amount of the purification of RNA has been calculated according to the Nanodrop Spectroscopy in the wave length of 260 nm, 280 nm and 230 nm. In this purity in 260/280 was 1.91 and purity in 260/230 was 1.13 by which

the concentration has estimated 244.5 ng/μl. This has shown that the RNA sample is present in good and reliable concentration; we can start other procedure of determination of BCR-ABL.

The samples showed BCR-ABL translocation (for major and minor break point) when stained by cytochemical methods, and has been analyzed by qualitative and quantitative PCR



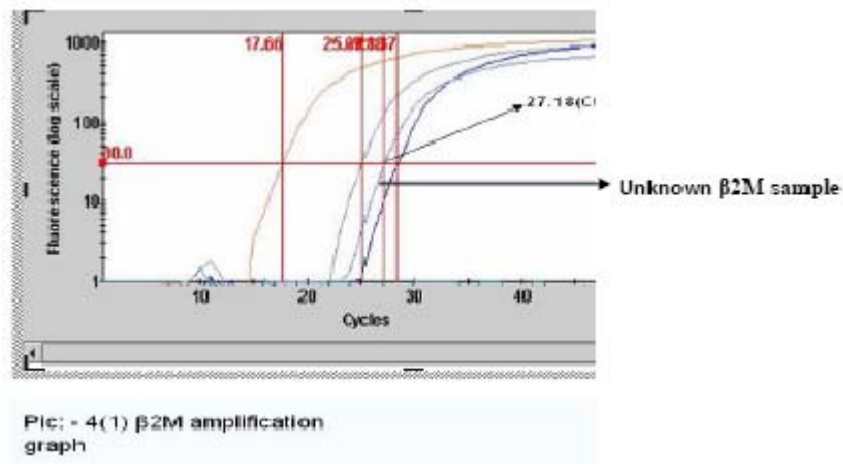
**Fig. 6.** Shows the implication for micro fragment. Positive for both micro and minor breakage point a clear product size corresponding to this translocation has been detected, which detected e1a3 with 223 bp sizes



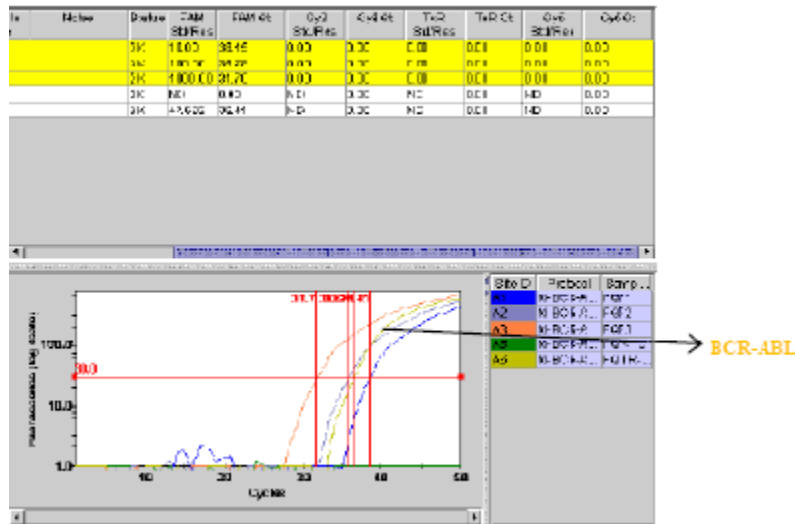
**Fig. 7.** Implication for BCR-ABL major fragment amplification with RQ-PCR and b3a2 brake point (446 bp)

methods. Figure 4 shows PCR product for minor fragment run along with electrophoresis gel with no template control and positive control. In this e20a2 band is clear with 436 base pair (bp) size. In the figure 10 the implication for micro fragment has been shown, since the sample was positive for both micro and minor breakage point a clear product size corresponding to this translocation has been detected, which detected e1a3 with 223 bp size. Figure 12 has shown implication for BCR-ABL major fragment amplification with RQ-PCR and b3a2

brake point has been detected with the size of 446 bp. Graph 1 shows implication for house keeping gene  $\beta 2M$  by RQ-PCR method. Clear implication has been noted here which qualified the sample for further analysis of fusion quant BCR-ABL. The unknown  $\beta 2M$  has been detected for 27.18(ct) which is light blue. The implication of BCR-ABL major fragment by RQ-PCR method has been shown in the graph (2) and it has been quantified by known sample which was known standard. BCR-ABL with yellowish color has been detected.



**Graph 1.** Implication for house keeping gene  $\beta 2M$  by RQ-PCR method. Clear implication has been noted here which qualified the sample for further analysis of fusion quant BCR-ABL. The unknown  $\beta 2M$  is blue



**Graph 2.** The implication of the BCR-ABL major fragment by RQ-PCR method quantities by using known standards. BCR-ABL yellowish graph

**Table 6.** The determination of Philadelphia chromosome by Karyotyping and FIS. Identification of BCR/ABL by flowcytometry. RT-PCR, quantification of the BCR/ABL transcripts in Ph-positive CML according to age and sex of patients

Case	Age/sex	Karyotype	FISH	Flowcytometry MFI	RTPCR TT/HD
1	43/M	46,XY,t(9;22)(q34.1;q11)	93%	210	b2a2
2	48/M	46,XY,t(9;22)(q34.1;q11)	89%	177	b3a2
3	44/M	46,XY,t(9;22)(q34.1;q11)	62.5%	112	b2a2-b3a2
4	40/M	46,XY,t(9;22)(q34.1;q11)	95%	213	e1a2-b2a2-b3a2
5	41/M	46,XY,t(9;22)(q34.1;q11)	WM	160	b2a2
6	40/F	46,XX,t(9;22)(q34.1;q11)	66%	116	b3a2
7	49/F	46,XX,t(9;22)(q34.1;q11)	98%	217	b2a2
8	44/F	46,XX,t(9;22)(q34.1;q11)	84%	170	b3a2
9	46/M	46,XY,t(9;22)(q34.1;q11)	76%	133	b2a2
10	44/F	Without Metaphases	68%	119	b2a2-b3a2
11	48/M	Without Metaphases	71%	126	b2a2
12	48/F	Without Metaphases	69%	121	b3a2

F=female, M=male, WM=without metaphase

## DISCUSSION

### Karyotyping

Determination of the presence of the Ph chromosome by karyotyping at diagnosis is necessary as part of differential diagnosis and as prognosis. This is an entirely manual test with a turnaround time of 7 to 15 days. Chromosomes were extracted from erythrocytes of the patient's blood sample, and dropped on the slide with proper distance. If distance be less the dispread of chromosomes were not proper and many overlapping will be take place, in case of high distance, fraction of chromosome will be formed which detection will be very difficult. A band is an area of a chromosome which is clearly distinct from its neighboring area, but may be lighter or darker than its neighboring region. The standard methods of banding are the Q, G, R, and C banding techniques. In this study chromosomes have been treated to trypsin for taking different bands. The time has to be shorter regarding to high concentration of trypsin and longer in low concentration. Trypsin has specific on the chromosome for cleavage. If more detail is desired, the chromosomes can be treated with various enzymes in combination with stains to yield banding patterns on each chromosome. Three major effects of trypsin are as follow: 1) under digestion which the particular in this stage uptaking high amount of stain (hyper stain), 2) optimum

digestion which can uptake the best amount of stain, 3) over digestion which will not take stain.

Would there be advantages in replacing karyotype detection of Ph by FISH at diagnosis? The initial answer is no, since karyotyping and FISH were equal, as shown in the present work, except for the fact that FISH was faster. In cases that did not present metaphases by karyotyping, FISH was extremely useful in providing a result. In daily practice, FISH can be very helpful since index of karyotyping, when the patient is receiving myelotoxic drugs, there may not be enough metaphases for analysis. The FISH technique is especially valuable in these situations, as it is more sensitive even though it is faster. RT-PCR is still more sensitive but its clinical meaning has to be clarified. FISH allows later study using stored samples that is valuable. The fact that fixed cells were being utilized was useful in this work, although there were some limitations. Many samples from other patients that fitted the aims of this work were not included, often because the stored material was not in an adequate condition or there were not enough interphases for analysis.

### RT-PCR

Many PCR based protocols for the diagnosis of the BCR-ABL chimaera have been published. Most of them include the detection of the translocation at the level of the RNA transcript. The method usually consists of two steps. The first reaction is that of obtaining cDNA from RNA

by means of reverse transcription (RT). Subsequently, an aliquot of cDNA is transferred to another tube and subjected to the second reaction, that is, PCR amplification. Two different enzymes are used, namely, reverse transcriptase for the RT step and Taq polymerase for the PCR. RT-PCR can be simplified significantly if another thermostable enzyme, namely Tth, is used instead. Tth is known to possess both RT and DNA polymerase activity.<sup>2</sup> Thus, only minimal adjusting of the reaction mixture in the tube is necessary to switch the process from RT to PCR. Such “one tube” RT-PCR protocols have been developed already for many widely used procedures.<sup>3-4</sup> However, there are no published accounts of the potential benefits of this rapid RT-PCR strategy for the detection of BCR-ABL translocation. In this paper, we present a one tube protocol for BCR-ABL chimaera detection. The method is cheap and time saving. Also, the reduction in the numbers of manipulations minimises the risk of contamination. Finally, this modification combines both the specificity and sensitivity of traditional PCR procedures.

**Stage 1:** The first step is reverse transcription (RT), in which RNA is reverse transcribed to cDNA using reverse transcriptase. This step is very important in order to perform PCR since DNA polymerase can act only on DNA templates. The RT step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used. In this paper I have used of 25°C for this stage.

**Stage 2:** involves the denaturation of the dsDNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction. Duration of this stage was 5 minutes and the enzyme has to be resistance to the high temperature like “Tag Polymerase” which in RTPCR is Tth, it possess both RT and DNA polymerase activity.

**Stage 3:** Then, the temperature is decreased until it reaches the annealing temperature which can vary depending on the set of primers used, their concentration, the probe and its concentration, and the cations concentration. The main consideration when choosing the optimal annealing temperature is the melting temperature (T<sub>m</sub>) of the primers and probes. The annealing temperature chosen for a

PCR depends directly on length and composition of the primers. This is the result of the difference of hydrogen bonds between A-T (2 bonds) and G-C (3 bonds). An annealing temperature about 5 degrees below the lowest T<sub>m</sub> of the pair of primers is usually used, in this experiment I have used of 48°C for 30 minutes. T<sub>m</sub> can calculate according to the below formula (*Stephen A. BUSTIN and Reinhold MUELLER, 2005*):

$$T_m = 4(G+C) + 2(A+T)$$

The final step of PCR amplification is DNA extension from the primers. This is done with thermostable Tth polymerase, usually at 72°C, the temperature at which the enzyme works optimally. The length of the incubation at each temperature, the temperature alterations, and the number of cycles are controlled by a programmable thermal cycler. The analysis of the PCR products depends on the type of PCR applied. If a conventional PCR is used, the PCR product is detected using agarose gel electrophoresis and ethidium bromide.

#### **FISH**

The accuracy for FISH analysis was very and close to 100%. It was possible to compare the two methods (FISH and karyotyping) and it is clear that FISH has a well-defined use. FISH and karyotyping are not mutually exclusive methods but complementary. Karyotyping is an entirely manual test with a turnaround time of 7 to 15 days. Based on results of conventional cytogenetics (karyotyping) in my study, I acknowledge that conventional cytogenetics (karyotyping) has its limitations. I could not obtain any metaphase spread in one of my samples and in another sample, only very few cells were present. The proportions of metaphases identified in cytogenetics depend on several factors, such as the number of metaphases analysed and the method used for obtaining metaphases. These metaphases only represent a small proportion of the total cells of a sample. However, conventional cytogenetics (karyotyping) has its strength because it is able to detect additional nonrandom chromosomal changes. Cheaper and faster methods would be ideal. FISH is a feasible technique, faster (usually 48 hrs) but still expensive due to the cost of probes. A small quantity (1ml) of sample is sufficient for FISH. The FISH test is thus rapidly gaining importance as a useful diagnostic tool in oncology, as more probes are now available for common chromosome abnormalities.

### Flow cytometry

Flow cytometry is a technique for analyzing large populations of single cells. For this method, the diagnostician has to analyze multiple bivariate dot plots from the cytometer. I developed a simple flow cytometric bead assay for detection of the BCR-ABL fusion protein in cell lysates, using a bead-bound catching antibody against one side of the fusion protein and a fluorochrome-conjugated detection antibody against the other side of the fusion protein. The flow cytometric immune bead assay is a fast and easy technique for specific detection of BCR-ABL proteins in leukemic cells. It is not dependent of the breakpoint position in the BCR gene, does not need special laboratory facilities other than a routine flow cytometer, provides results within several hours and it can be run in parallel to routine immunophenotyping (no extra technician time needed).

### CONCLUSIONS

To conclude since karyotyping and FISH were equal, as shown in the present work, except for the fact that FISH was faster. In cases that did not present metaphases by karyotyping, FISH was extremely useful in providing a result. With karyotyping Philadelphia chromosome can be detected in a clear pattern. In daily practice, FISH can be very helpful since index of karyotyping, when the patient is receiving myelotoxic drugs, there may not be enough metaphases for analysis. The FISH technique is especially valuable in these situations, as it is more sensitive even though it is faster. RT-PCR is still more sensitive but its clinical meaning has to be clarified. By RT-PCR b3a2 breakpoint has been detected and by RQ-PCR method house keeping gene  $\beta$ 2M has been detected and also by PCR method BCR-ABL can be recognize which is highly sensitive, but still no demonstrable Ph chromosome and no M-bcr rearrangement in this method. The flow cytometric immune bead assay is a fast and easy technique for specific detection of BCR-ABL proteins in leukemic cells. It is not dependent of the breakpoint position in the BCR gene, within several hours provide results. But still no detection of particular gene is in this method, so more suitable for diagnostic. 26

### ACKNOWLEDGMENTS

With grateful thanks to Dr. Amit Verma for his helpful aid and Triesta Reference Lab at HCG hospital to providing facilities, equipments and material requirements as well as supplying a perfect area to work. Also with special thanks to Dr. Morteza Atri for giving his attention in order to edition of this paper.

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