An accurate and rapid flowcytomertic diagnosis of BCR ABL fusion protein an alternative way to detect the different phases of Chronic Myeloid Leukemia.

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Abstract:

Background: Chronic Myeloid Leukemia patients (90–95%) harbor the Philadelphia chromosome. BCR ABL plays a key role in disease pathogenesis of CML. Philadelphia Chromosome resulting in the fusion of the *BCR* and *ABL* (*bcr-Abl*) genes, which leads to the expression of the active tyrosine kinase BCR-ABL fusion protein.The correct diagnosis of Philadelphia Chromosome positive CML is critical in selecting the appropriate therapy and has a strong prognosis implication. Detection of BCR-ABL is very expensive and time consuming through various conventional methods. On the basis of that we are using a recently developed flow cytometric bead assay to detect the BCR-ABL fusion protein . Which is not only take 4 hours but also less expensive. **Materials and Methods**: During the study period of June 2009 to June 2012 total 338 CML patients were investigated. Among them 83 patients were newly diagnosed and 255 were follow-up patients and 298 patients were in chronic 36 in acute and 04 in blast crisis phase. We are using a rapid fluorescent immune bead assay to detect BCR ABL fusion protein. **Result:** Between them 223 (60%) were positive for BCR ABL fusion protein for flowcytometry bead assay and 115 (40%) individual patients were negative for same assay.**Conclusion:** This study has reveled that the BCR–ABL immunobead assay detects all types of BCR–ABL proteins in leukemic cells with high specificity and sensitivity.

Key word: immune bead assay, BCR- ABL, flowcytometry

Introduction:

Chronic myeloid leukemia (CML) is probably the most extensively studied human malignancy. It is a myeloproliferative disorder. CML patients (90–95%) harbor the Philadelphia (Ph)[1]. The Ph chromosome is created through the reciprocal translocation t(9;22)(q34;q11) of long arms of human chromosome 9 that contains the Abelson (ABL) kinase domain, fusing with a long arms

of breakpoint cluster region (BCR) on chromosome 22.The resulting fusion gene BCR-ABL encodes for an abnormal, non membrane bound oncoprotein.The mechanisms of action of the BCR ABL fusion protein are still not fully understood, but the onco-protein is a constitutively active tyrosine kinase that perturbs numerous signal transduction pathways resulting in uncontrolled cell proliferation, reduced apoptosis and impaired cell adhesion. Three fusion proteins of different sizes may be produced (i.e. p 190, p 210, and p 230) depending on the site of the breakpoint within the BCR gene (M- BCR, m-BCR, μ-BCR regions respectively) [2-4]. Registry data of CML patients from a number of Asian countries document a median age at diagnosis of 36–55 years .

BCR ABL plays a key role in disease pathogenesis of CML. So BCR ABL fusion protein detection is important for diagnosis, prognosis, disease monitoring, treatment outcome and follow-up. The development of inhibitors detected specifically against the BCR-ABL tyrosione kinase has opened innovative therapeutic avenues that have profoundly impacted on the management of the diseases harboring the genetic abnormality.These recent developments highlight the need for a rapid and reliable identification of this genetic lesion in patients with CML at presentation for whom several techniques, including cytogenetic analysis (Karyotyping and FISH), Southern Hybridization and real time quantitative polymerase chain reaction (RQ-PCR) are used to identify both the BCR-ABL gene transcripts and the BCR-ABL genes. Karyotying is performed on bone marrow samples, while FISH on both peripheral blood and bone marrow samples. The molecular detection of BCR ABL transcripts includes quantitative and qualitative techniques. Reverse transcriptase PCR (RT PCR) is used to detect BCR ABL transcripts while reverse transcriptase real time quantitative PCR (RQ PCR) to quantify BCR ABL transcripts[5-8]. However these techniques require the availability of specialized laboratories with skilled manpower, expertise and are often time consuming (1-2 days for FISH and PCR techniques and 1-2 weeks for karyotyping) and available only in a small number of laboratories [3,9-11].
On the basis of the above considerations in the present study we have tested a recently developed flow cytometric bead assay designed to detect the BCR-ABL fusion protein on chronic and acute phase of CML patients at Netaji Subhas Chandra Bose Cancer Research Institute in Kolkata in India and have determined the applicability, reliability and rapidity of this method. This is the first report of identifying the BCR-ABL transcripts at the translational level in CML patients by flow cytometric immunobead assay from India. For identification of presence of the BCR ABL fusion protein, we utilized the BCR ABLProtein Kit (BD Biosciences).The Cytometric Bead Assay (CBA) is an immunoassay that identifies semi quantitatively,the presence of BCR ABL fusion protein in the lysates of the leukemic cell population.

Materials and Method:

Patients:

During the study period of June 2009 to June 2012 total 338 CML (Male: 227; female: 111) patients with median age of 40±1.47 (6yrs-80yrs) were investigated for the status of BCR-ABL protein in translational level in a tertiary cancer research institute named Netaji Subhas Chandra Bose Cancer Research Institute in Kolkata, India. Among them 83 patients were newly

diagnosed and 255 were follow-up patients (under Imatinib therapy).Written consent was taken from individual patient and institutional ethical committee had approved the study. Among 338 patients 298 patients were in chronic phase, 36 in acute phase and 04 in blast crisis phase.

Sample Collection:

Three to 5 ml of peripheral blood or 2 to 3 ml of bone marrow samples were obtained into EDTA vial from patients for routine slide studies and flowcytometric technique. The diagnosis of CML was established according to morphological, cytochemical and immunological criteria according to FAB and WHO classifications. Histopathological analysis of bone marrow had been done for all patients also.

Specimen Characteristics:

Bone Marrow and Blood are samples.

Positive and negative control: Lysates prepared from 24 normal healthy blood donors' peripheral leukocytes (WBC) and HL60 cell line were used as negative controls whereas K562 cell line and 20 positive samples (already detected positive by Real Time PCR) were used as positive controls.

Assay Methods:

Principle of the assay: The flowcytometric immunobead assay [BCR ABL protein kit BD Bioscience] for BCR ABL fusion protein detection is used for qualitative and semi-quantitative detection of the BCR-ABL protein in CML cell population lysates. The capture beads are coated with an antibody specific to one epitope of the BCR-ABL fusion protein. The detector reagent is a PE conjugated antibody specific to a different epitope. The capture beads and detector reagents are incubated with a prepared sample obtained through lysis of the leukemic cells. If BCR ABL protein is present in the tested sample, sandwich complexes are formed which can be detected by their mean or median fluroscence intencity level[12,13].

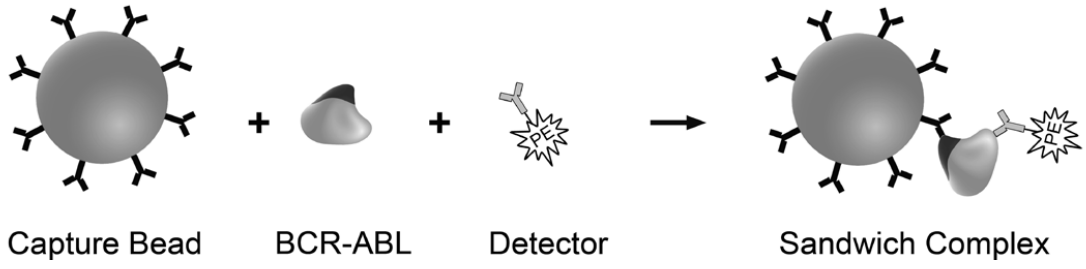

Fig. 1: Principle of the assay

Study design:

Analysis of BCR-ABL protein:

For identification of presence of the BCR ABL fusion protein, we utilized the BCR ABLProtein Kit (BD Biosciences).The Cytometric Bead Assay (CBA) is an immunoassay that identifies semi quantitatively,the presence of BCR ABL fusion protein in the lysates of the leukemic cell population. By lysis of the leukemic cells, the fusion proteins are released and they are recognized by an anti-BCR antibody coupled to a bead and a PE (Phyco-Erithin) labeled anti ABL antibody[14].

According to the manufacturer's instruction, 1-2 ml of whole blood or marrow specimens containing 25*106 cells were incubated with 50 ml of a 1x stock of BD Pharm lyse lysing buffer (BD Biosciences), for ten minutes at room temperature with occasional mixing to lyse red blood cells. PBS (Phosphate Buffer Saline) with 5% of FBS was used twice to wash the cells. Subsequently, at least 250 µl of the Pretreatment Buffer obtained by diluting the 1X stock of BCR ABL Pretreatment A (BD Biosciences) and the 1X stock of BCR ABL Pretreatment B (BD Biosciences) were added to each sample and controls; samples were then incubated on ice for ten minutes and washed once by adding PBS with 5% FBS. Thereafter, samples and controls were incubated for 15 minutes with 100 µl of the 1X stock of the BD Lysate treatment Reagent (BD Biosciences) and then centrifuge at 20,000 g for ten minutes at 4^0C. Fifty micro litter of the cell lysates, from both samples and controls, were incubated for two hours in dark with 50 µl of an anti-BCR antibody coupled to a bead (BD Biosciences) and 50 µl of the PE labeled anti-ABL antibody (BD Biosciences). After washing with the CBA Wash Buffer (BD Biosciences Pharmingen), samples were re-suspended in 300 µl of the CBA Wash Buffer and acquired on flowcytometer (FACS Calibur, BD) using the cellquest Pro software (BD Biosciences), after FACS caliber instrument setting using the cytometer setup with specific template and tracking (CS&T) Bead system according to the manufacturer's guidelines (BD Biosciences). There are 300 beads/ 300 µl of bead suspension. The presence of sandwich complexes (capture beads BCR-ABL protein detector) was expressed as the bead population median fluorescence intensity (PE MFI) generated by the detector reagent. The absence of the BCR-ABL fusion protein was defined utilizing the mean ± 0.02 SD of PE MFI of normal peripheral leukocytes.

Results:

Patients were represented with the symptoms such as fatigue and weight loss with greater degrees of leucocytosis and splenomegaly and lower haemoglobin levels as well as bone pain. Most bleeding patients had normal or elevated platelet counts, suggesting that platelet dysfunction was the primary cause of hemorrhage. Although thrombocytosis was common, thrombosis were not seen.

The bone marrow aspirate showed hypercellularity with a markedly increased myeloid elements. On biopsy the bone marrow was strikingly hypercellular with pronounced granulocytic hyperplasia, thick paratrabecular Cluff of immature myeloid cells and numerous small hypolobulated megakaryocytes. The karyotype of the bone marrow was 46XY,t(9;22)(q34;q11).

Table: 1 Clinical features of the studied patients:

Symptoms:

Fatigue (%)	Joint pain or bone pain (%)	Weight Loss(%)	Sweating(%)	Infection(%)	Weakness(%)	Nausea(%)	Fever(%)	Swollen feet(%)	Spleen Enlargement(%)	Liver Enlargement (%)
43.75	43.75	57.5	20	26.25	56.25	35	45	23.75	90	30

Table: 2 CBC Profile of CML Patients:

Characteristics	No. of patients	Mean ± S.E
WBC Count	83 (newly diagnosed)	1448096±11341.00/ Cu.mm
	255 (Follow-up patients)	14,000±22.10/ Cu.mm
Hb	338	10.85±0.46 gm/dl
Platelet Count	83 (newly diagnosed)	97.32±6.35 *10^3/µl
	255 (Follow-up patients)	257.15±29.70*10^3/µl
Cellularity in Bone Marrow	338	Hypercellular
Percentage of Blast	338	7.48 ±2.90
Percentage of Promyelocytes	338	9.16 ±3.25
Percentage of Metamyelocyte	338	17.19 ±3.07

Cutoff PE-MFI values: The same procedure was done with randomly selected 24 normal samples of blood and the median fluorescence (PE MFI) was measured. The slightest fluorescence reflected from the normal uncoated beads are measured and this gives a date which is below 1.3±0.02 fluorescence (maximum value of fluorescence:1.34 and minimum value of fluorescence :1.26) and accepted for normal cutoff value for this laboratory set-up. Any value up to 1.32 has been accepted as negative for BCR ABL detection.

Positive control: A suspension of the cell line was made and the cell count of the suspension was done.A normal blood sample was taken where we know the cell count of leukocytes. By simple mathematical calculate the volume of the cell line suspension was added to the normal blood samples in such a manner that 3 blood samples contain BCR-ABL positive cells in 1%,10%,and 50% respectively.The fluorescence of the bead immunoassay by flowcytometry were done.The lysates prepared from the Real- Time PCR positive CML patients were also run in flowcytometry immune bead assay and the fluorescence (M.F.I) was found much more than 1.3±0.02. The mean MFI value of positive cell line and cell lysates of already detected (by RQ PCR) CML patients were 88.36 and 17.94 respectively.

We have reported the flowcytometric determination of the fusion protein of 5 positive patients after 24,48,72 and 96 hours of sample collection at room temperature and have found a positive signal though with less intensity compared to the signal obtained from fresh cells of those samples when processed within 24 hours. Similar results have been achieved positive on cell lysates when stored at -80^0c and processed after 15 and 20 days. Whereas the normal cell lysates showed the same PE MFI in above all conditions.

Eighteen samples including 7 newly diagnosed CML cases who showed negative result in flowcytometry were outsourced for Real Time PCR as we do not have any Real Time set up in our lab. 11 samples (positive in flow cytometry) showed positive results in Real Time PCR and 7 newly diagnosed patients who were negative in flowcytometry showed negative result in Real Time PCR also. No discrepancy was found between two setups in our study. Among 11 positive samples 9 were positive for M-BCR (p 210) and two were positive for (p190).

Table3: Result of analysis of BCR-ABL protein:

No. of Samples tested	Positive samples		Negative samples	
338	**223**		**115**	
	Follow-up cases (mean follow up period 2 yrs)	Newly Diagnosed	Follow-up cases(mean follow up period 4.5 yrs)	Newly Diagnosed
Blood samples	147	76	108	7
Bone Marrow samples	147	76	----------	----------

Table:4 Mean Values of positive PE M.F.I for different phases of CML patients:

	Chronic Phase	Accelerated Phase	Blast Crisis
No. of Samples	183	36	04
Mean MFI Value	2.19±0.052 (Range:1.36-4.61)	7.69±0.734 (Range:3.8-17.15)	35.18±16.078 (Range: 13.2-99.1)

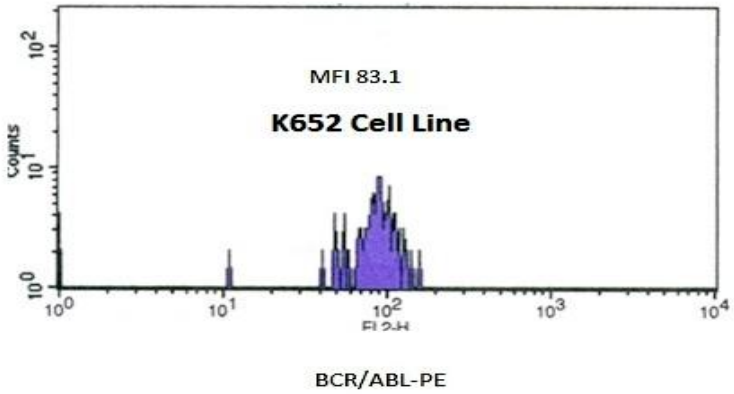

Fig. 2: BCR - ABL Positive Cell line

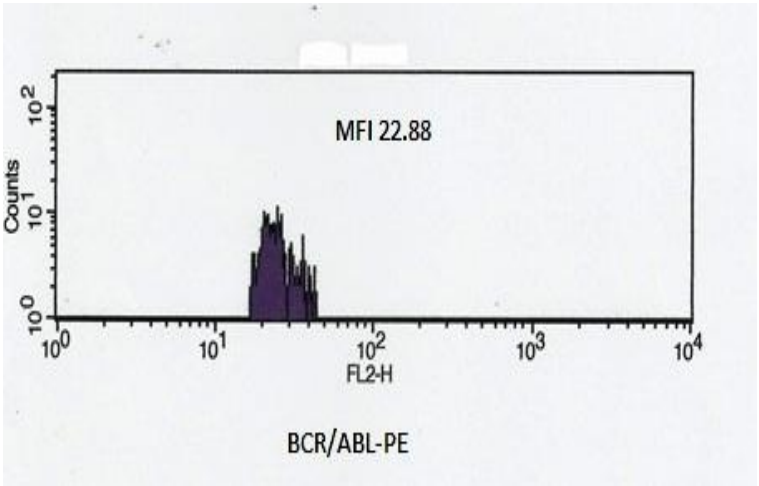

Fig. 3: Positive Patients (Blast Phase of CML)

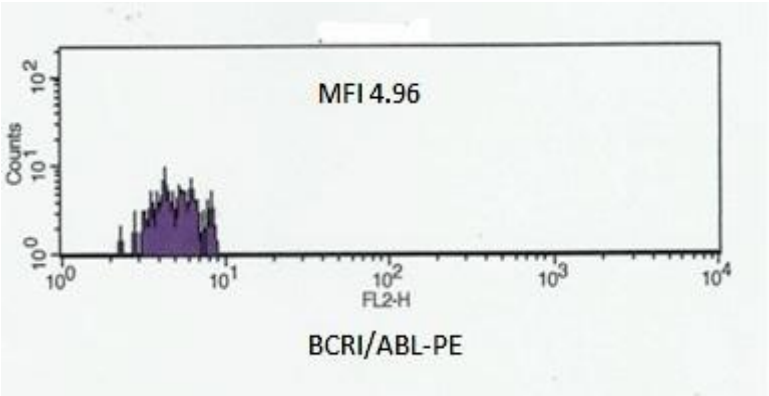

Fig. 4: Positive Patients (Accerelated Phase of CML)

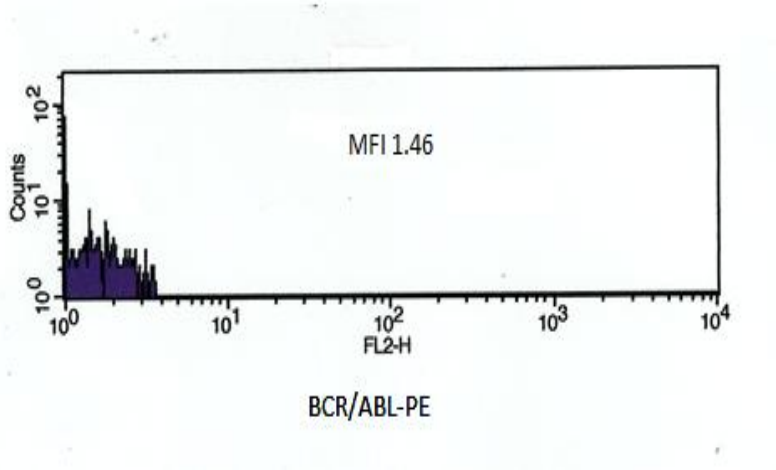

Fig. 5: Positive Patients (Chronic Phase of CML)

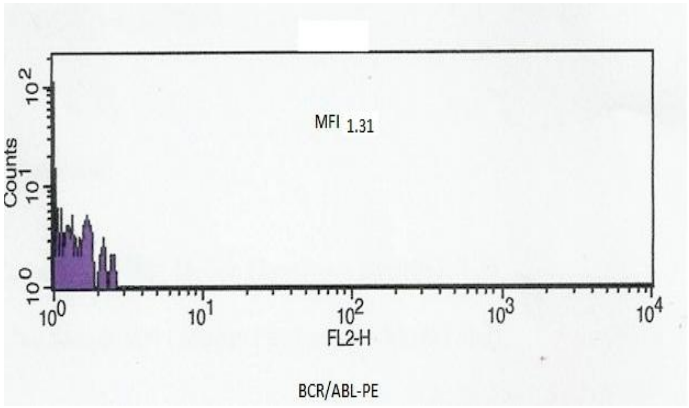

Fig. 6: Negative Control

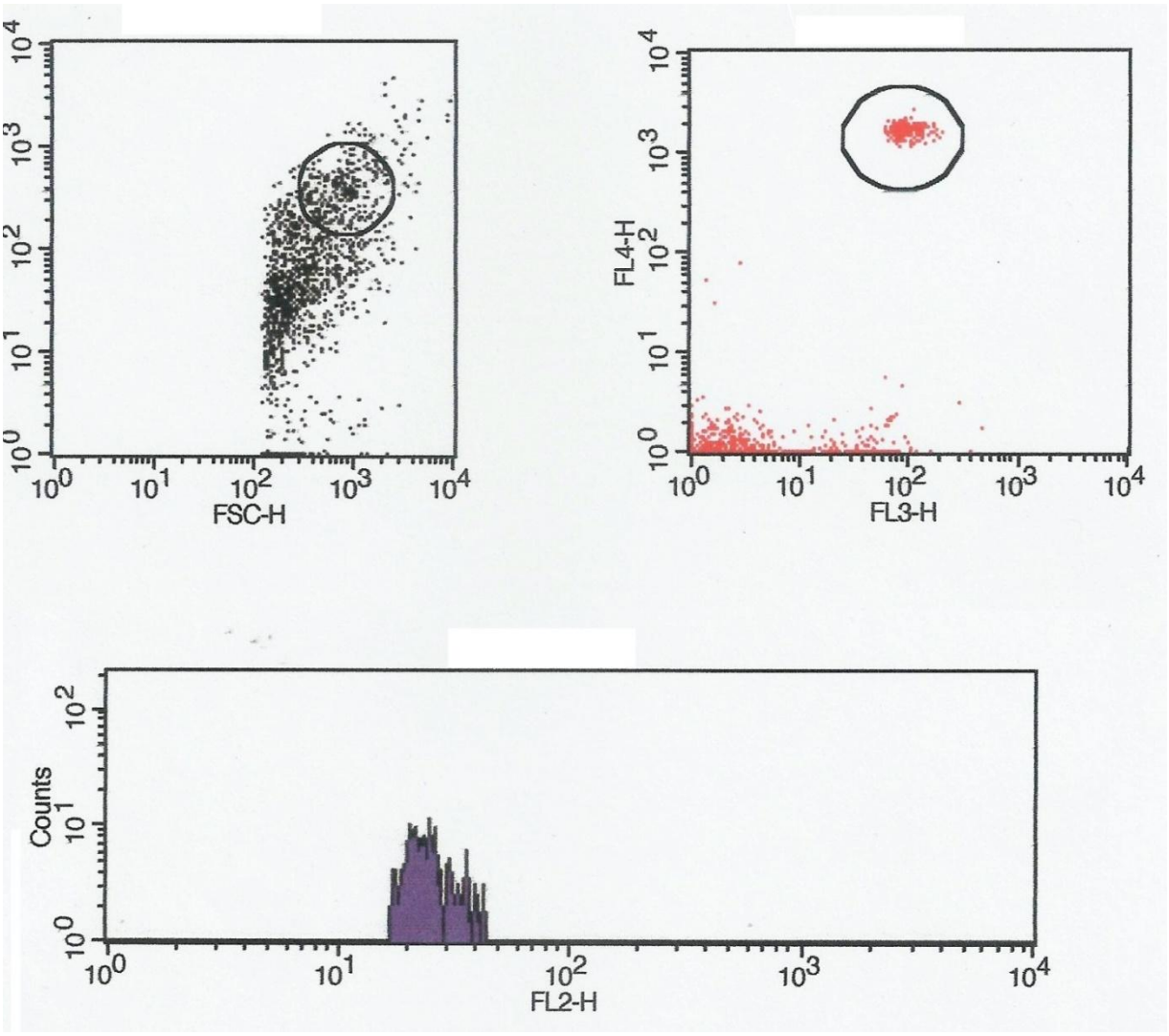

Fig. 7: Total representation of a positive sample

Discussion

Administration of tyrosine kinase inhibitors have shown efficacy in the treatment of Philadelphia positive CML patients.But these therapy requires the detection of the t(9;22) or BCR ABL fusion gene.The conventional methods like Karyotyping, FISH or PCR are suitable for the investigation of this genetic disorder but they are laborious, time consuming, expensive and can only be carried out in a restricted number of highly qualified laboratories.Very few investigators have used the newly developed flowcytometry immunobead assay to detect the BCR-ABL fusion gene in translational level in ALL patients. We have also investigated the fusion protein BCR-ABL

using this flowcytometry technique but in CML patients. The data of our 338 patients showed the reliability and specificity of this technique. Moreover the result shown within 4 hours indicates its rapidity which is an extra advantage of this technique.

In our study, out of 83 newly diagnosed patients, more than 90% patients showed the presence of BCR-ABL fusion protein. Seven newly diagnosed patients showed the negative result for BCR-ABL fusion protein (both in RQ PCR and flow cytometric assay) and they were sent for other mutation study.

There was no as such study from eastern Indian Chronic Myeloid Leulamia patients by flowcytometric bead assay. We have screened 338 Chronic Myeloid Leukemia patients and 24 normal individuals .Where in chronic leukemia patient who was detected by histopathological analysis. 223 (60%) were positive for BCR ABL fusion protein for flowcytometry bead assay and 115 (40%) individual patients were negative for same assay. Imatinib therapy csuses the cytogenetic as well as molecular response in 40% patients completely and that's why they showed negative signal in flowcytometric immune bead assay.

According to a study conducted by Weerkamp et. al BCR-ACL bead immune-assay showed a good correlation with Q-RT PCR method for detection of BCR-ABL positive samples. This bead immunoassay system showed a 92.3% concordance rate to QRT PCR. This system offers a convenient approach to development of multiplex assay for fusion protein involved in leukemia disease[15].

In another study, Greenberg. D et.al described that BCR ABL bead immuno system demonstrated a 100% correlation with conventional methods (conventional cytogenetics or molecular methods) for identification of Philadelphia chromosome positive cells. Also this assay can provide valuable sample information within 4 hours[16,17].

In 2009 S. Raponi et.al showed in a institutional study in Italy that in comparison to real time PCR flowcytometric bead assay of BCR ABL fusion method proved reliable, reproducible, of simple execution and it was successfully completed within four hours, from the marrow or blood collection on $25*10^6$ total cells containing at least 10 % of leukemic cells as the manufacturer's instruction. The availability of a method capable of highlighting the presence of the BCR ABL protein (p^{190}, p^{210} and $p^{190/210}$) through a flowcytometric analysis and thus to reliably offer proteomic information to investigators researching targeted anti-tyrosine kinase treatment. This identification can translate into timely implementation of targeted therapeutic strategy.

Our data of 80 patients also demonstrated the 100% co relation with real time quantitative PCR. Grigorian et.al showed the discrepancies in the ALL cases between flowcytometric assay and real time PCR, Sara Raponi also showed two such cases with discripencies .In both studies they have shown either very negligible leukemic cell count of the sample or the steroid treatment of the patients resulted into the negativity in flowcytometric bead assay technique but actually they were positive in PCR technique. They recomended an absolute correlation between the presence or absences of BCR-ABL prtotein kit and the positivity or the negativity of the transcripts assessed according to conventional PCR techniques[3,4]. In this regard, our PCR data is too

small in comparison to flowcytometry data (80 samples vs 338 samples), so we have to perform more PCR studies to conclude anything.

Bead-based immunoassays such as the CBA are powerful tools to measure multiple analytes (e.g., proteins) within individual samples. Multiparameter analysis of the large amounts of data generated by bead-based assays. This data must be quickly and efficiently reanalyzed and converted from a general list of characteristics about the sample to specific, useful, and comparable results for individual analytes. Without a rapid and simple system to reanalyze archived data files to extract the information about each bead population, all advantages of the bead-based assay's multiplex capabilities would be lost. The data collected using a flow cytometer include information about the physical and spectral parameters of the beads such as size and the fluorescence emission characteristics of each bead population. These fluorescence emission characteristics include the fluorescent emission of the dyed beads and the potential fluorescent emissions of the detection fluorochrome (e.g., Phycoerythrin). When samples are analyzed using a flow cytometer in conjunction with a typical data acquisition and analysis package (e.g., BD CellQuestk software), a list-mode data file is saved using a flow cytometry standard file format, FCS. The data stored in the FCS files can be reanalyzed to determine the median fluorescence intensities (MFI) of the various bead populations, defined by their unique physical and spectral characteristics, to then compare reference samples with unknowns. Analyte levels within individual samples can then be calculated from calibration curves generated by serial dilutions of standard analyte solutions of known concentration. An automated or semiautomated analysis method is necessary for rapid reanalysis of the data stored in each FCS file. For the CBA assays, this analysis method is a unique software package[18].

Analysing reported data and our data we can conclude that flowcytometric method for detection of BCR ABL fusion protein is thought to be a sensitive, effective, less time consuming and easy to perform technique which can meet up the necessity of detecting BCR-ABL fusion protein in CML as well as ALL patients.

The flow cytometric immunobead assay ensures recognition of all variants of the BCR-ABL fusion protein independently of the break point position (16 of E G) because the anti BCR antibody was designed as unique epitope upstream of all BCR gene break points , and anti ABL antibody was designed against an epitope downstream of the large ABL break point region. Our data also suggest that this technique can identify all the BCR-ABL fusion protein irrespective of their break point region. Because 9 patients with p210 proteins and 2 patients with p190 protein (RQ PCRdata) both showed the positive result in Flowcytometric immunobead assay technique. We believe this assay might identify the rare fusion proteins such as p195, p200 or p225 though they need to be proved experimentally.

Further we can say, that the BCR–ABL immunobead assay detects all types of BCR–ABL proteins in leukemic cells with high specificity and sensitivity. This assay can document the effective transaction of the molecular transcript. It allows rapid identification of the presence of the BCR-ABL protein (p190, p210, p190/210) through a flowcytometric analysis and thus to reliably offer proteomic information to investigators researching targeted anti-tyrosine kinase treatment. This identification can translate into the timely implementation of a targeted therapeutic strategy. Moreover it does not need specialized laboratory facilities other than a flow

cytometer, provides results within 4 hours, and can be run in parallel to routine immunophenotyping. It may be a good alternative assay system for rapid and reliable detection of BCR-ABL fusion protein in chronic or acute leukemia cases.

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