Rapid Detection of Bcr-Abl Fusion Proteins by Immunobead Assay Flow Cytometry in Leukemia Patients

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Abstract: Philidalphia(Ph) chromosome [t(9;22)(q34;q11)] which results in the production of BCR-ABL fusion protein, with deregulated tyrosine kinase activity, is a hallmark of chronic myelod leukemia(CML) and present in a high risk group of acute lymphoblastic leukemia(ALL). This BCR-ABL tyrosine kinase has been specifically targeted by tyrosine kinase inhibitors (TKI) which have profoundly modified the treatment and prognosis of the diseases harboring this genetic abnormality. Consequently, the rapid and accurate detection of BCR-ABL is of utmost importance in the diagnosis and optimal management of leukemias. Currently applied techniques are RT-PCR and cytogenetic analysis, which are costly, time-consuming, and require specialized laboratories. We utilized a recently developed immunoassay that qualitatively identifies the presence of BCR-ABL proteins in the leukemic cell lysate. The BCR-ABL is captured and detected by a cytobead assay and analysed by flow cytometry. We aimed at evaluating the effectiveness of this technique in detecting BCR-ABL in ALL patients and identifying CML cases among CML-suspected patients. The assay was conducted on 100 peripheral blood and bone marrow samples of 20 healthy controls, 55 patients suspected of having CML and 25 ALL patients. Results were compared to those obtained by conventional karyotyping and fluorescence in situ hybridization (FISH). BCR-ABL protein was positive in 35/55(63.6%) of CML suspected cases and 15/25(60%) ALL cases with 100% concordance with cytogenetic analysis data. The procedure was simple and feasible and proved to be reliable in rapidly identifying CML cases and Ph+ALL allowing for their prompt management.

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1-Introduction

The molecular analysis of recurrent chromosomal abnormalities (e.g. rearrangements, deletions) has provided valuable insight into the pathogenesis of hematological malignancies. A paradigm is Philidalephia (Ph) chromosome, caused by the reciprocal translocation between chromosome 9 and 22, t(9;22)(q34;q11), resulting in the BCR-ABL fusion gene which encodes a cytoplasmic protein with deregulated tyrosine kinase activity responsible for leukemogenesis.[1,2] Three fusion proteins of different sizes may be produced (p190, p210 and p230) depending on the breakpoint site within the BCR gene. The oncoprotein product phosphorylates many cellular targets, leading to the activation of intracellular signaling pathways such as Ras[3] ,Jak/STAT[4] and Akt/PI-3kinase[5] pathways, which induce abnormal proliferation, resistance to apoptosis, modification of cellular adhesion and genetic instability.

The Ph chromosome represents a hallmark of chronic myeloid leukemia (CML) as it can be found in almost all cases (95%). Furthermore, the introduction of the use of BCR-ABL tyrosine kinase inhibitor (TKI) (imatinib mesylate), that specifically targets the ATP-binding site of ABL kinase domain, in the treatment of CML has modified the natural history of the disease.[6,7]

As far as acute lymphoblastic leukemia (ALL) is concerned, the new 2008 WHO classification now recognizes B-ALL with t(9;22)(q34;q11) as one of seven genetic entities.[8] It can be identified in 2-5% of childhood, in 25-30% of adult and about 40% of older ALL patients. It is incorporated into risk stratification as its presence is associated with a very unfavorable prognosis[9,10] ,as well as risk- adapted therapy as TKI, in combination with chemotherapy or alone, is nowadays utilized upfront as first-line therapy for Ph+ALL.[11-14]

Therefore, it is of utmost importance in the diagnosis of both CML and ALL to demonstrate the presence of Ph chromosome or its transcripts. Molecular techniques, by real time qualitative polymerase chain reaction (RT-PCR) and cytogenetic analysis by conventional karyotype and fluorescence in situ hybridization (FISH) to identify BCR-ABL oncoproteins and Ph+ translocation respectively, represent the methods currently used.[15] These techniques, although reliable, are costly, time consuming and require specialized staff; criteria which are usually confined to specialized regional laboratories. A novel method for the detection of BCR-ABL fusion proteins using bead-based flow cytometric immunoassay has been produced and is available for research use only. The method utilizes a principle very similar to a classical ELISA test, where

the BCR-ABL proteins are immunocaptured on bead coupled with anti-BCR capture antibody, subsequently detected using a secondary anti-ABL1 detection antibody, producing a Sandwich complex comprised of both capture bead and detection fluorophore, to be read out by flow cytometry. The assay was performed on a cell lysate to allow for the detection of the intra-cellular proteins.[16]

The rapid-turnaround time, ease and specificity of this assay, coupled with the availability of flow cytometry in many laboratories and its established role as a cornerstone in the diagnosis and classification of hematologic malignancies, render this method very attractive for evaluation as it can be run in parallel to routine immunophenotyping. On the basis of the above considerations, we aimed, in the present study, at testing the effectiveness of the BCR-ABL proteins immunobead-based flow cytometric assay to rapidly and reliably identify Ph+ ALL cases among ALL patients and CML patients among CML-suspected cases.

2. Subjects and Methods

The presence of BCR-ABL protein was investigated in 100 freshly obtained peripheral blood (PB) or bone marrow (BM) samples. Those consisted of 20 healthy controls, 55 patients representing with neutrophilia and/or thrombocytosis suspected of having CML (age range=35-65 years) and 25 newly diagnosed ALL cases (17 adults and 8 children) attending Hematology/Oncology Units of Armed Forces Hospitals, Eastern Province, KSA over a period of 24 months.

All patients were subjected to thorough history taking, complete physical examination and radiological investigations (U/S and CT-scanning).

Peripheral blood and/or bone marrow samples were drawn from the 55 patients suspected of CML for complete blood count (CBC), peripheral smear and BM aspirate microscopic examination, neutrophil alkaline phosphatase (NAP) scoring (Sigma, St Louis, USA), followed by detection of Ph chromosome by conventional G-banding and FISH analysis and the flow cytometric immunobead assay for the presence of BCR-ABL proteins.

Diagnosis of the 25 denovo ALL patients was established by morphological, cytochemical and immunological criteria according to FAB and WHO classifications. PB and BM aspirate samples were used for CBC, microscopic smears examinations and cytochemical staining. Immunophenotypic analysis was performed on Coulter Epics-XL flow cytometer (Coulter electronics, Hielaeh, FL, USA) by staining BM/PB cells with various combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin cyanin 5 (PC5) labelled monoclonal antibodies against the following antigens: CD2, CD3, CD5, CD7, CD10, CD13, CD14, CD19, CD20, CD33, CD34, CD45, HLADR and MPO (Coulter electronics, Hielaeh, FL,USA).[18] Conventional G-banding and FISH analysis were used to detect t(9;22)(q43;q11), followed by immunobead assay for BCR-ABL proteins.

It should be noted that care was taken to include all the cases proved to be Ph+ by cytogenetic analysis in the study.

Detection of BCR-ABL oncogene or its transcript using:

I. Cytogenetic analysis by: A) conventional G-banding, which was done by classic techniques and interpreted according to International System of Human Cytogenetic Nomenclature (ISCN19) using chromoscan, applied imaging system (CytoVision). B) FISH analysis, using fluorophore-labeled single stranded DNA sequence probe homologous to t(9;22)(q34;q11) (TelVysion).[17]

II. Flow cytometric immunobead assay (CBA): was done utilizing the BCR-ABL protein kit (BD-Biosciences) which qualitatively identifies the presence of BCR-ABL fusion proteins in the cell lysate of examined sample. By lysis of leukemic cells , the oncogene proteins are released and are recognized by anti- BCR antibody coupled to a bead and a PE-labeled anti-ABL antibody, to be detected as the bead population mean fluorescence intensity (MFI) using a Coulter Epics-XL flow cytometer.

The manufacturer's instructions were followed:

1) Preparation of mononuclear cells concentrate (MNCs) using ficole-hypaque (in cases of suspected CML) or whole blood/marrow specimen lysate using lysing solution (in cases of ALL) containing at least $25x10^6$ cell/mL.
2) Performing of cell pretreatment: 250 µL of pretreatment buffer (obtained by dilution of1X Stock pretreatment A and Stock pretreatment B) were added to each sample, incubated on ice for 10 minutes and washed once using phosphate buffer saline (PBS).
3) Lysing the leucocytes (to release the intra-cellular BCR-ABL protein): 100µL of lysing solution (prepared by diluting 50X Stock BD lysate treatment reagent in BD pharmagin cell lysis buffer) were added to each sample, incubated for 15 minutes and centrifuged at20,000g for 10 minutes at 4°C. The supernatant (cell lysate) was not discarded.
4) Performing the bead immunoassay: 50µL of cell lysate were combined with 50µL of capture beads (ant- BCR antibody coupled) and 50µL of detector reagent (PE-labeled anti-ABL antibody), incubated for 2 hours in the dark at room temperature with shaking sufficient to maintain

constant agitation. Washing was performed with CBA wash buffer and samples were resuspended in 300µL of the wash buffer.

5) Acquiring the data: on Coulter Epics XL flow cytometer after performing the cytometer set up according to the manufacturer's guide lines. The average MFI from normal healthy subjects was calculated, and a sample was considered positive for the presence of BCR-ABL fusion protein if its MFI was >2SD of PE-MFI of normal peripheral leucocytes.

3. Results

The results of the present study are presented in Tables 1-2 and figure 1.

This study was conducted on 100 subjects divided as follows: 20 healthy controls, 55 patients suspected of having CML and 25 denovo ALL cases.

The 55 patients suspicious of CML were 34 males and 31 females (M: F ratio=1.6:1) with age ranging from 35-65 years. Their clinical data are shown in (Table1). Complete clinical, radiological and laboratory investigations proved them to be 20 non-CML and 35 CML patients. According to clinical and laboratory criteria, the CML patients were further subdivided into 31(88.6%) chronic phase (CP) and 4 (11.4%) accelerated phase (AP) cases. The presence of AP cases accounts for the high upper level of blast count ranges noted in the PB and BM of CML cases. All 35 CML cases were Ph+ by conventional karyotyping and FISH analysis.

Regarding the 25 denovo ALL patients, they were 17 adults and 8 children with age ranging from2-52 years and M: F ratio of 1.5:1. In the samples collected from these patients, the leucocyte count (TLC) ranged from 18.2-60.8 $x10^9$/L with leukemic blast cells constituting 19-88% in the PB (absolute blast count=6.7-48.9 $x10^9$/L) and 45-95% in the BM (Table 1). Immunophenotypically, only one patient was T-ALL and the remaining 24 were B-ALL, which were further subdivided into 13 common ALL (cALL), 5 pre-B ALL and 6 pro-B ALL. By cytogenetic analysis (G-banding and FISH analysis) 15 cases were Ph+ (14 adults and 1 child). Of note, 2 of the adult B-ALL cases were proved to be originally CML cases in blast crisis (BC).

As previously mentioned, care was taken to recruit all CML and ALL cases established to be Ph+ in this study.

The 20 healthy control subjects were cytogenetically examined for Ph+ chromosome by G-banding and FISH analysis and were proved negative (Table 2). The binding of the peripheral leucocytes of those 20 healthy subjects to the immunocapture beads was used to determine the analytical detection limit of the presence of BCR-ABL oncoproteins by flow cytometric immunobead assay (CBA). The MFI value from the normal cellular samples was 12.2±3.4, giving a cut-off value between positive and negative set at 24.3±2.2, as calculated by mean±2SD (Figure 1A).

When the 55 patients suspected of CML were tested by CBA for BCR-ABL fusion proteins, the results were 100% concordant with those of cytogenetic analysis, being negative in the 20 non-CML cases and positive in all 35 CML cases (Table 2). The CML cases expressed varying MFI, 21 with low MFI and 14 with high MFI, the highest being noted in the accelerated phase (AP) cases and the newly diagnosed patients (Figure 1B and 1C).

Similarly, fully concordant cytogenetic analysis and CBA results were observed in the 25 ALL cases included in the study (Table 2). 15 of the 25 (60%) studied cases proved to express Ph+ by G-banding and FISH, as well as being positive for BCR-ABL fusion protein by CBA, all with high MFI, including the B-ALL blast crisis of CML (Figure 1D). The CBA positive cases were 14/17 (82.3%) adults, and only 1/8 (12.5%) childhood cases. Regarding immunophenotypic distribution, 10/13 (77%) cALL, 2/5 (40%) pre-B ALL and 3/6 (50%) pro-B ALL expressed the BCR oncoprotein, while the single T-ALL case studied was negative for it (Table 2).

Table (1): Clinical and laboratory data of CML-suspected cases and ALL patient

	Non-CML (n=20)	CML (n=35)*	ALL (n=25)
Age (years)	35-58	38-65	2-52
Sex (M: F)	14:8 (1.5:1)	22:13 (1.7:1)	15:10 (1.5:1)
TLC ($x10^9$/L)	30.1-63.0	35.2-230.4	18.2-60.8
Hb (g/dL)	9.8-14.9	8.4-12.6	8.5-12.1
Platelets ($x10^9$/L)	263-845	108-634	18-201
%Blasts in PB	0	1-18**	19-88
%Blasts in BM	0-1	5-21***	45-95
Absolute blast count ($x10^9$/L)			6.7-48.9
NAP score	11-201	0-56	-
IPT: B-ALL T-ALL		2 (BC)	24 1
Ph+ FISH	0	35	15

*: CML cases were 31 CP (chronic phase); 4 AP (accelerated phase) which account for the high blast count in PB (**) and BM (***).

Table (2): Results of flow cytometric immunobead assay (CBA) compared to Ph+ by FISH analysis in all studied samples

		Ph chromosome (FISH)		BCR-ABL (CBA)		
		Negative	Positive	Negative	Positive	
					Low MFI	High MFI
Healthy controls(n=20)		20	0	20	0	0
Non-CML (n=20)		20	0	20	0	0
CML (n=35)	CP (n=31)	0	31	0	21	10
	AP (n=4)	0	4	0	0	4
ALL (n=25)	cALL (n=13)	3	10	3	0	10
	Pre-B ALL (n=5)	3	2	3	0	2
	Pro-B ALL (n=6)	3	3	3	0	3
	T-ALL (n=1)	1	0	1	0	0

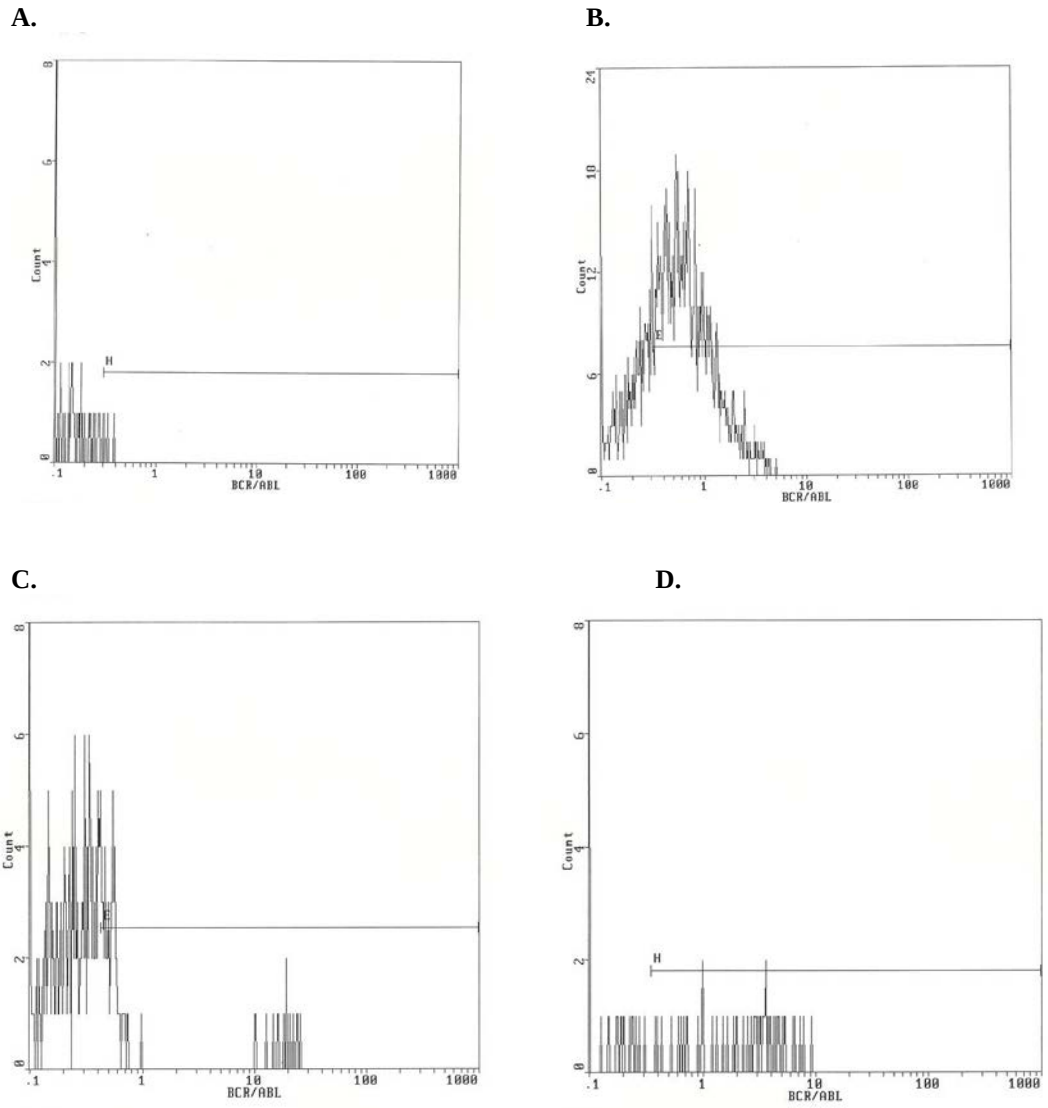

Figure (1): Representative data of BCR-ABL protein flow cytometric immunobead assay in healthy controls (1A); low MFI CML case (1B); high MFI CML case (1C) and B-ALL case (1D)

4. Discussion

Philadelphia (Ph) chromosome or t(9;22)(q34;q11) results in the BCR-ABL fusion gene, which encodes cytoplasmic proteins with constitutive tyrosine kinase activity. It is present in 95% of patients with CML and in a high risk subset of patients with ALL (2-5% of childhood ALL and 25-30% of adult ALL).[18,19] The BCR-ABL fusion protein has been successfully targeted for therapy by tyrosine kinase inhibitor (TKI), imatinib mesylate, which selectively induces growth inhibition and apoptosis of BCR-ABL positive cells.[20] Consequently, the detection of BCR-ABL Aberration is of utmost importance for the diagnosis, classification and treatment of leukemia patients.

Conventional approaches to BCR-ABL detection are limited to karyotyping and FISH analysis or RT-PCR techniques, all of which are time consuming and require specialized facilities.

A novel simple flow cytometric immunobead assay for the detection of BCR-ABL fusion proteins in cell lysates has been developed, which utilizes a bead bound anti-BCR catching antibody and a fluorophore conjugated anti-ABL detection antibody.[16] We aimed at evaluating this technique in detection of BCR-ABL protein in ALL patients and its differentiating power among CML suspected cases by comparing its results with those of conventional karyotyping and FISH analysis.

As far as CML is concerned, Ph+ is a hallmark of the disease, and its definitive diagnosis requires t(9;22)(q34;q11) demonstration. In the present study, 55 patients suffering from neutrophilia and/or thrombocytosis suspected of having CML were investigated. 35/55 of those patients were cytogenetically proved to be Ph+, and expressed BCR-ABL proteins positivity by flow cytometric immunobead assay (CBA), displaying 100% concordant results between the two techniques (35/35 cases). Our data are similar to those obtained by **Weerkamp *et al.*** [16], who demonstrated the positivity of BCR-ABL proteins in 19/19 CML cases studied by CBA and RT-PCR, and those of **Lucas *et al.*** [21], who studied 110 suspected CML cases and 70/110 were positive for BCR-ABL proteins by CBA and RT-PCR (70/70 cases, 100% concordance).

Pandey *et al.*[22], were able to detect BCR-ABL by CBA in 65 CML patients out of 75 cases proved Ph+ by FISH analysis, (65/75) with 88% concordance value. This difference in resullts could be due to the fact that in cell samples containing high frequencies of mature myeloid cells, protein instability problems may be encountered as a result of protease activity.[21] In an attempt to reduce this proteolytic activity present within mature neutrophils which could influence the CBA assay sensitivity, we chose to use MNCs of CML patients rather than lysate. Another step to decrease this interference could be the addition of protease inhibitors at several steps of the assay.[16]

We noticed a difference in the levels of MFI of BCR-ABL protein detected by CBA in the CML patients included in our study; 14 expressed high MFI while 21 expressed lower MFI. This variability in the degree of BCR-ABL expression is also present in previous studies.[16,21] Of interest, this high positivity was noted in all 4 AP cases and newly diagnosed CML patients, which could be attributed to the fact that the transcript and protein levels of BCR-ABL are elevated in primitive CML progenitors relative to more mature cells.[23] Furthermore, it has previously been shown that the degree of decrease in BCR-ABL transcript level within the first 3 months of TKI treatment can predict subsequent clinical outcome.[24] Therefore, this observation regarding the BCR-ABL MFI variability needs further evaluation, as it might pave the way to the use of a feasible, rapid and readily available test for monitoring disease course, response to therapy and prediction of clinical outcome.

Fifteen (14 adults and 1 child) of the 25 ALL studied cases proved positive for BCR-ABL by both cytogenetic analysis and CBA, with 100% concordance. Similar results were described by **Weerkamp *et al.***[16] However, **Raponi *et al.*** [25] demonstrated lower sensitivity of the CBA, as they were unable to detect the fusion protein (proved positive by RT-PCR) in 2 steroid treated patients due to the very low leukemic cell count (blast cells constituting 2-2.3% of total cell count). Practically, this should not be a hindrance to the denovo diagnosis and classification of ALL, as virtually, no ALL patients at diagnosis has very low number of marrow or blood leukemic cells.

Therefore, in the study at hand, we have documented an absolute correlation between the expression of BCR-ABL protein using CBA and Ph chromosome detection by cytogenetic analysis. CBA successfully differentiated CML patients from cases with neutrophilia and/or thrombocytosis suspected of having CML. Since most patients with neutrophilia and/or thrombocytosis do not have CML, a rapid and simple screening test for BCR-ABL protein could be clinically useful. In ALL cases, CBA was able to identify the higher risk group of Ph+ ALL patients. The assay was reliable, applicable, relatively rapid, being successfully completed within 4 hours. It could easily be run in parallel to routine immunophenotyping.The availability of such a method capable of detecting the presence of BCR-ABL protein has important implications as it can document the effective transduction of the molecular transcript.

Furthermore, the BCR-ABL antibody was developed against a non homologous region of ~80 amino acids, encoded by exon-1 in order to detect all known BCR-variants (p190, p210 and p230) irrespective of the breakpoint in BCR-gene.[16]

The development of specifically targeted TKI against BCR-ABL kinase has had profound impact on the management of diseases harboring this genetic aberration. Indeed, TKIs have modified the natural history of CML.[6,7] Moreover, recent studies were able to induce hematologic remission with oral TKI, no chemotherapy and partly at home in ALL patients with Ph+.[11-14] This therapeutic approach to CML and Ph+ ALL patients requires the accurate and rapid identification of BCR-ABL fusion proteins in order to allow timely implementation of a targeted therapeutic strategy. Criteria currently unmet in many places, as the already utilized molecular techniques are expensive and require specialized staff. Furthermore, FISH largely depends on the availability of intact and informative interphase nucleii, and RT-PCR is hindered by the inherent variability in amplification and standardization of quantitation.[26] On the other hand, flow cytometry has long been established as a cornerstone in the diagnosis and classification of hematologic malignancies. Therefore, the possibility of utilizing such a rapid, simple, reliable and readily available technique opens the way to offering much required approach for diagnostic and therapeutic causes.[27]

In conclusion, the flow cytometric immunobead assay is a fast, easy and reliable technique for the specific detection of BCR-ABL protein in leukemic cells, with highly concordant results with currently used molecular methods. It can be used as a rapid and simple screening test for CML suspected cases, which could economize time and cost, as most patients will be negative and will not require further detailed investigation. In acute leukemia, it could contribute to its faster classification. Therefore, CBA could allow, in both diseases, CML and Ph+ ALL, for earlier diagnosis, prompt clinical and therapeutic management. It awaits further evaluation regarding its possible role in monitoring of clinical outcome and minimum residual disease.

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