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Immunophenotyping and Immunoglobulin Heavy Chain Gene Rearrangement Analysis in Cerebrospinal Fluid of Pediatric Patients with Acute Lymphoblastic Leukemia

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ABSTRACT

Background: The diagnosis of CSF infiltration of hematologic malignancy has great prognostic and therapeutic implications both in symptomatic and asymptomatic patients. A diagnostic gold standard is not available and morphologic examination of CSF fails to demonstrate malignant cells in up to 45% of cases thought to be positive. Flow cytometric immunophenotyping and detection of clonal IgH genes rearrangement (IGHR) are considered to be more sensitive for detection of these malignant cells.

Aim of the Work: To assess the diagnostic accuracy of flow cytometric immunophenotyping and IGHR by real time PCR in comparison with classic cytology for diagnosing CNS infiltration in pediatric ALL.

Material and Methods: Forty five CSF specimens from pediatric patients with ALL were examined by flow cytometry (FCM) for immunophenotyping. Monoclonal antibodies were designed according to the BM or peripheral blood immunophenotyping at diagnosis. Twenty specimens were also examined for IGHR by light cycler. The results were compared with classic cytology.

Results: Twenty one samples were positive by FCM [21/45 (46.6%)]. Fifteen samples could not be analysed because of insufficient cell numbers. Twelve samples were positive by IGHR [12/20 (60%)] and only 10 samples were positive by cytology [10/45 (22.2%)].

Conclusions: The diagnostic value of FCM and IGHR are two to three times more sensitive than that of cytology. Therefore, immunophenotyping by FCM is recommended for routine diagnosis of CSF infiltration. Furthermore, IGHR analysis by real-time PCR appears to be a useful addition to morphological and FCM analysis of CSF in evaluation of CNS infiltration.

Key Words: *CSF – Flow cytometry – PCR – ALL.*

INTRODUCTION

Meningeal involvement is a frequent complication of hematological malignancies with an incidence of up to 25% in certain leukaemias and lymphomas **[1]**. The diagnosis of this involvement has great prognostic and therapeutic implications both in symptomatic and asymptomatic patients at high risk of such involvement **[2]**. A diagnostic gold standard is not available, and morphologic examination of cerebrospinal fluid (CSF) fails to demonstrate malignant cells in up to 45% of patients in whom meningeal involvement is thought to be present **[3]**. The major diagnostic problem in evaluating CSF involvement is distinguishing neoplastic infiltrates from inflammatory or infectious diseases **[4]**.

Flow cytometry (FCM) immunophenotyping is a valuable tool in the diagnosis and staging of lymphoproliferative disorders involving the lymph nodes, blood and bone marrow. While flow cytometric analysis is a standard procedure in the evaluation of blood and bone marrow cells, it is not generally applied to CSF samples in all clinical laboratories [5].

In addition, analysis of heavy chain gene (IgH) rearrangements by real-time polymerase chain reaction (PCR) is a powerful diagnostic tool for hematologists and oncologists. The detection of malignant cells by this technique has become the state of art for diagnosis, monitoring response to treatment and detection of minimal residual disease in leukemia and lymphoma **[6]**. The development of high technologies allows the application of real-time PCR assays in large prospective treatment studies for monitoring tumor cells in circulation as well as in bone marrow. Based on quantitative data

the kinetics of disappearance and reappearance of tumor cells can be followed up in "real-time". This allows developing new strategies to treat patients with an inadequate response to standard chemotherapy or at molecular relapse before symptoms or signs of clinical relapse occur **[7]**. However, this modality has not been widely applied to CSF specimens **[8]**.

In this study, we aimed to assess the diagnostic accuracy of flow cytometric immunophenotyping and IgH gene rearrangements (IGHR) analysis by real time PCR in comparison with classic cytology for diagnosing central nervous system (CNS) infiltration in acute lymphoblastic leukemia (ALL). In addition, we aimed at verifying the CNS status especially at first presentation, which is essential for risk stratification and proper treatment.

MATERIAL AND METHODS

Forty-five CSF specimens from pediatric patients with ALL were examined by FCM for immunophenotyping. In 12 patients, CSF analysis was performed because of neurological abnormalities (manifestation of increased intracranial tension, convulsion and cranial nerve palsy) and in 33 patients as part of their routine work up at first presentation; twenty-four patients at first presentation and nine patients at relapse.

Twenty large samples were divided into two tubes to send the second for the molecular biology laboratory for IGHR analysis by light cycler, 3 of them with neurological abnormalities. All specimens tested for IGHR were from B-precursor ALL patients.

The results were compared with classic cytology routinely done for all samples. Medical ethical committee of Assiut University approved this study.

Flow cytometry:

Cell counting and preparation:

CSF was centrifuged at 100 x g for 10 min. within 2 h of obtaining the sample. Cells were counted by hemocytometer.

For flow cytometry analysis, a minimum of 1 ml CSF containing at least one cell/10µl was needed. Flow cytometry could not be accomplished for samples of insufficient cell numbers (one cell/10µl of CSF).

The supernatant was discarded and the cell pellet washed with phosphate buffered saline (PBS) and then by hemolysite.

Antibody cocktails were added to each tube according to the manufacturer's recommendations. Immunophenotyping was performed by standard three-colour immunofluorescent staining using fluorescence-labeled monoclonal antibodies, directed against the following surface markers [CD45, CD34, Terminal Deoxynucleotidyl Transferase (TdT) and CD33 conjugated with FITC from Caltage laboratories (Austria), CD14, CD19 and CD10 conjugated with PE from BD Pharmingen (Becton Dickinson, Biosciences), CD3 and CD19 conjugated with CyQ from IQ Products (Groningen, Netherlands) CD4/CD8 and kappa/lambda dual colored monoclonal Abs from Becton Dickinson (Biosciences, San Jose, CA)].

The antibody cocktails were selected according to the patients' baseline bone marrow immunophenotyping results and in combinations show atypical pattern of antigen expression.

The following mixes were used:

1- Isotypic control.

2- CD45/CD14.

3- CD4/CD8/CD3.

4- Cyt TdT/CD10/CD19.

5- κ/λ/CD19.

6- CD34/CD19.

7- CD33/CD19.

Mix 6 was used if the BM lymphoblasts in the base line immunophenotyping showed expression of CD34. Mix 7 was used only if the lymphoblasts in the BM base line immunophenotyping showed aberrant myeloid expression.

Flow cytometric analysis:

The flow cytometer (FACSCaliber; Becton Dickinson) was calibrated using CALIBRITE-3 beads FACSComp software. Data acquisition and analysis was performed using CellQuest software (Becton Dickinson).

Polymerase chain reaction:

DNA extraction from CSF was done by High pure template kit (Roche diagnostic, Mannheim, Germany).

Principle:

The extracted DNA was analysed for IgH chain gene rearrangements as follow:

First round PCR using consensus primers to amplify variable segments (VH)-joining segments (JH) of IgH gene to obtain sufficient product for second round PCR.

Second round PCR of the first round product using allele specific primers to amplify complementary determining region (CDR) of JH of IgH gene. This was performed to further specify the target gene.

Equipment:

Light Cycler Instrument (Roche Diagnostics, Mannheim, Germany).

Reagents:

A- Primers **[9]** are represented in Table (1) (a and b).

B- Light Cycler^{R} FastStart DNA Master^{plus} SYBR Green I.

Master mix component for IgH first and second round PCR amplification were done as manufacturer instruction.

Amplification was done using thermal cycler. The thermal profile includes:

• Initial denaturation step at 94ºC for 5 minutes.

• 40 cycles of amplification:

Denaturation at 94ºC for 30 seconds.

Annealing at 56ºC for 30 seconds.

Extension at 72ºC for 45 seconds.

PCR protocol on LightCycler for SYBR Green detection is represented in Table (2).

Quantification program: Amplification curves were obtained and the fluorescence values versus cycle number were displayed.

Melting curve program: Assessment of the specificity of the amplified product was achieved by performing a melting curve analysis. The resulting melting curve allows discrimination between primer dimmer and specific product.

Monitoring PCR with the SYBR Green 1 dye:

SYBR Green 1 dye binds to the minor groove of dsDNA. Fluorescence is generally enhanced by binding. During the various stages of PCR, different intensities of fluorescence signals can be detected, depending on the amount of dsDNA that is present. All DNA become single stranded after denaturation. At this stage of reaction SYBR Green 1 dye will not bind and the intensity of flouresence signal is low.

During annealing the PCR primers hybridize to the target sequence, resulting in small parts of dsDNA to which SYBR green 1 dye can bind thereby increasing fluorescence intensity.

In the elongation phase, the PCR primers are extended and more SYBR Green 1 dye can bind. At the end of the elongation phase all of the DNA has become double stranded and a maximum amount of dye is bound. The fluorescence is recorded at the end of the elongation phase and increasing of amount of PCR product can be monitored from cycle to cycle.

Melting curve analysis of amplicons with SYBR Green 1 detection:

Each dsDNA product has its own specific melting temperature (Tm) which is defined as the temperature at which 50% of the DNA becomes single stranded and 50% remains double stranded, the most important factors that determine that Tm of dsDNA are the length and the GC content of that fragment.

In going from low to high GC content a difference of up to 40ºC can be measured in Tm. Checking the Tm of a PCR product can thus be compared with analyzing a PCR product by length in gel electrophoresis.

RESULTS

In this work, 45 CSF-samples were examined. Twenty-one samples were positive by FCM [21/45 (46.6%)]. Fifteen samples [15/45 (33.3%)] could not be analysed because of insufficient cell numbers. Twelve samples were positive by IGHR [12/20 (60%)] and only 10 samples were positive by cytology [10/45 (22.2%)]. A total of 26/45 positive samples were detected; 8 samples were positive for both FCM and cytology, five samples were positive for both IGHR and cytology (Table 3). Thirteen samples [13/45 (28.9%)] were positive by FCM and negative by cytology and three cases were

positive by IGHR and not by cytology or FCM (two could not be analysed due to insufficient cell numbers and one was negative) (Table 4). Two samples were positive for both cytology and FCM and not by IGHR and two cases were positive for cytology but not by FCM (Table 4). The first was negative for all markers by FCM and positive by PCR and the cells in the second were reactive T lymphocytes and negative for malignancy by FCM.

The gated cells in the FCM analysis varied greatly from 20 to 9808 cells; analysis of cases with large number of cells (Fig. 1) was not difficult as those cases with very low count number that needs aberrant phenotype to ensure the malignant involvement (Fig. 2).

The percentages of positive cases by the three methods in relation to the clinical manifestations are show in Table (5). All the 15 samples that had no sufficient cells to analyse were from cases at presentations. There were two patients with CNS manifestation; one showed negative results with cytology and FCM and the other was positive by cytology and negative for malignancy (all the cells were reactive T lymphocytes) by FCM. These two were cases diagnosed as toxic encephalitis by MRI.

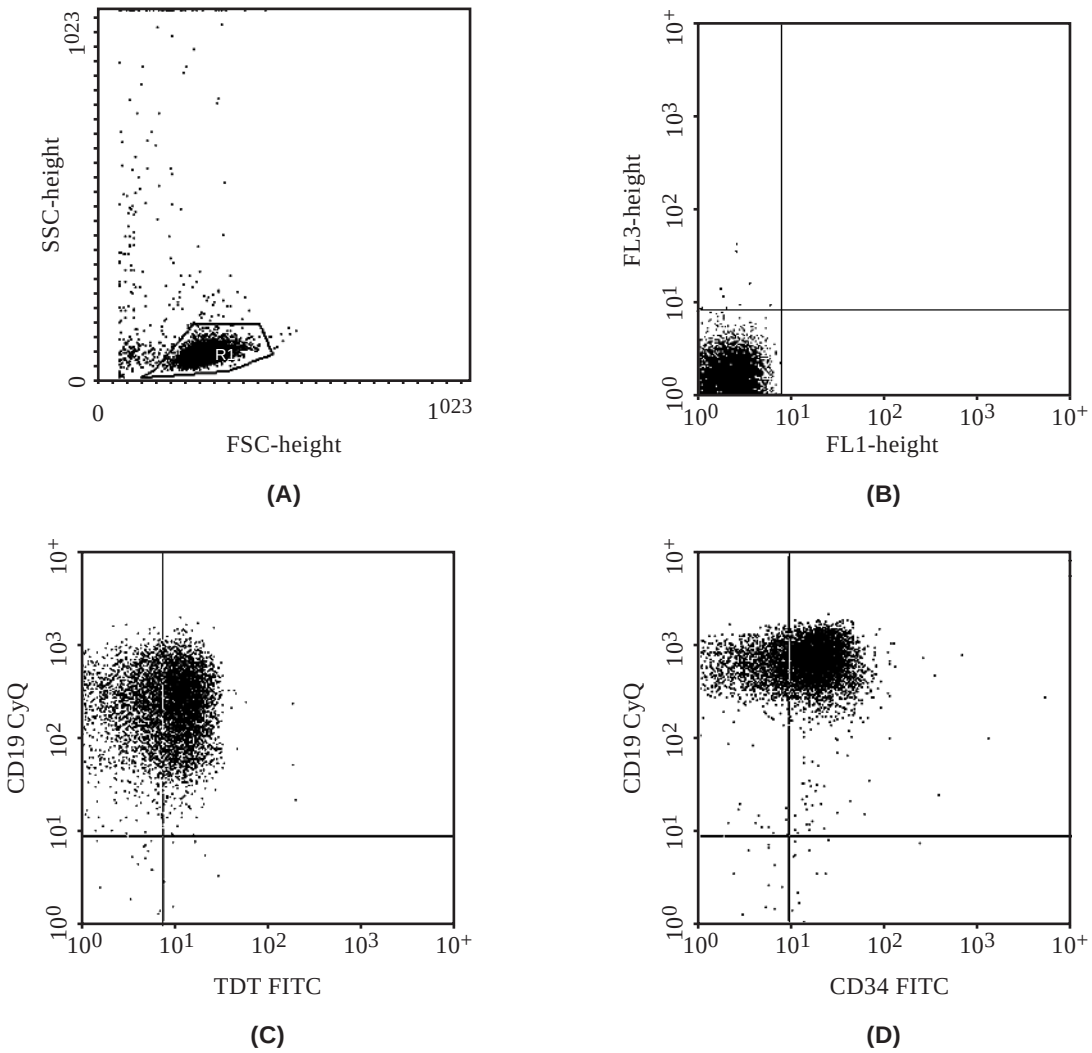

Fig. (1): (A) Dot plot of FSC versus SSC showing a distinct population (R1). (B) Dot plot of FL1 versus FL3 gated on R1 showing clear isotypic control (C) Dot plot of TdT-FITC versus CD19-CyQ gated on R1, showing that the gated cells co-express both markers. (D) Dot plot of CD34-FITC versus CD19-CyQ gated on R1 showing that the gated cells express CD34 also.

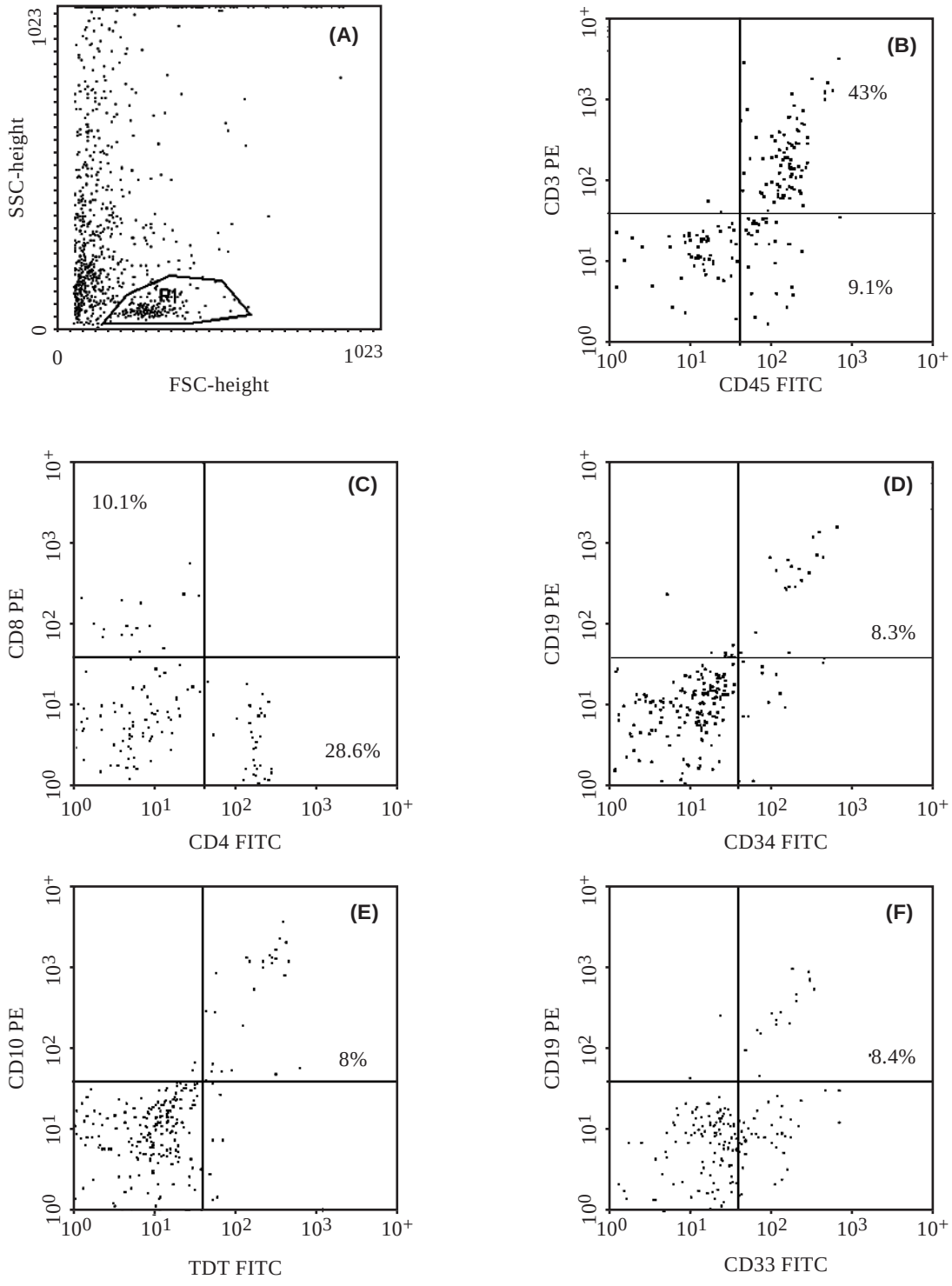

Fig. (2): (A) Dot plot of FSC versus SSC showing a distinct population (R1). (B) Dot plot of CD45-FITC versus CD3-PE gated on R1 showing showing 2 population groups the first is of T lymphocytes (43%) that coexpress CD45 and CD3 and the second is of malignant population that express CD45 and not CD3 (9.1%). (C) Dot plot of CD4-FITC versus CD8-PE gated on R1 showing that the T lymphocytes are reactive and distributed into CD4 cells (28.6%) and CD8 cells (10.1%). (D) Dot plot of CD34-FITC versus CD19-PE gated on R1 showing that 8.3% of gated cells co-express both markers (lymphoblast cells) which are the malignant population. (E) Dot plot of CDTdT-FITC versus CD10-PE gated on R1 showing that 8% of the gated cells co-express both markers (lymphoblast cells) which are the same malignant population. (F) Dot plot of CD33-FITC versus CD19-PE gated on R1 showing that the same population 8.4% of the gated cells express aberrant myeloid marker.

Table (1-A): Sequences of the consensus primers for IgH used in the first round PCR.

Sequences of the consensus primers for IgH	Size	Name	Length	GC %	Tm ºC
5GCC CAG GAC TGG TGA AGC3	376bp	VH4/6 outer	18	66.7	65
5ACC TGA GGA GAC GGT GAC3	376bp	JH	19	63.2	65.1

Table (1-B): Sequences of the allele specific primers for IgH used in the second round PCR.

Sequences of the allele specific primers for IgH	Size	Name	Length	GC %	Tm ºC
5ATC TAT TAT AGT GGG AGC ACC3	177bp	VH4/6 inner	21	42.9	57.9
5ACC CCG TAC CAG CTG CCT CC3	177bp	JH	20	65	68.6

IgH : Heavy chain gene.
PCR: Polymerase chain reaction.
Tm : Melting temperature.
bp : Base pair.
VH : Variable segments in heavy chain gene.
JH : Joining segments in heavy chain gene.

Table (2): PCR protocol on Light Cycler for SYBR Green detection.

Analyis mode acquisition mode	Cycles	Segment	Temperature	Hold time
Pre-incubation:				
None	1		95ºC	10 min.
Amplification:				
Quantification none	45	Denaturation	95ºC	1s
None		Annealing	60ºC	10s
Single		Extension	72ºC	20s
Melting curve:				
Melting curve none	1	Denaturation	95ºC	0s
None		Annealing	65ºC	15s
Continuous		Melting	95ºC	0s
Cooling:				
None	1		40ºC	30s

PCR: Polymerase chain reaction.

Table (3): Detection of CNS infiltration by IGHR and FCM in comparison to cytology in ALL patients.

	IGHR analysis		Immunophenotyping by FCM		
	Positive	Negative	Positive	No cells	Negative
Cytology:					
Positive	5/20	2/20	8/45	0/45	2/45
Negative	7/20	6/20	13/45	15/45	7/45

IGHR: Heavy chain gene rearrangements.
FCM: Flow cytometry.

Table (4): Positive CSF samples for malignant involvement by the three methods in 45 ALL patients.

No	Clinical manifestation	Cytology	FCM	IGHR
1	CNS (M)	-ve	+ve	ND
3	CNS (M)	+ve	+ve	+ve
5	RW up	-ve	+ve	ND
6	CNS (M)	-ve	+ve	ND
8	RW up	+ve	+ve	+ve
9	CNS (M)	-ve	-ve	+ve
10	CNS (M)	-ve	+ve	ND
11	Relp	-ve	+ve	ND
13	Relp	+ve	+ve	ND
14	Relp	+ve	+ve	+ve
16	CNS (M)	-ve	+ve	ND
22	CNS (M)	+ve	+ve	ND
23	RW up	-ve	No cell	+ve
26	Relp	+ve	+ve	+ve
27	CNS (M)	-ve	+ve	+ve
28	Relp	+ve	+ve	-ve
29	CNS (M)	+ve	-ve	ND
33	RW up	-ve	No cell	+ve
35	RW up	-ve	+ve	+ve
36	RW up	+ve	+ve	-ve
38	CNS (M)	-ve	+ve	ND
40	Relp	-ve	+ve	ND
41	Relp	-ve	+ve	ND
42	CNS (M)	+ve	-ve	+ve
44	Relp	-ve	+ve	+ve
45	Relp	-ve	+ve	+ve

CNS (M) : Central nervous system manifestation.
RW up : Routine work up of the ALL patient at first presentation.
Relp : Relapsed ALL.
FCM : Flow cytometry.
IGHR : Heavy chain gene rearrangements.
+ve : Positive.
-ve : Negative.
ND : Not done.
No cell : Insufficient cell number.

Table (5): Distribution of the CSF positive cases according to the clinical manifestation in ALL patients.

	Cases at presentation	Relapsed cases	CNS manifestation	Total
Cytology:	2/24 (8.33%)	4/9 (44.44%)	4/12 (33.33%)	10/45 (22.2%)
FCM	4/24 (16.66%)	9/9 (100%)	8/12 (66.66%)	21/45 (46.6%)
IGHR	4/11 (36.36%)	4/5 (80%)	4/4 (100%)	12/20 (60%)

CNS : Central nervous system.
IGHR : Heavy chain gene rearrangements.
FCM : Flow cytometry.

DISCUSSION

Hematological malignancies comprise many prognostically distinct subtypes, thus, a uniform approach to therapy would be inappropriate **[10]**. Instead, emphasis is placed on a strict assessment of risk at the time of diagnosis, so that only patients at high risk for relapse are treated with more intensive therapy, while at those lower risk may have less toxic treatment. CNS involvement is important for the prognosis and treatment; it requires CNS-directed therapy including irradiation and high dose chemotherapy **[11]**. Leukemic blasts in CSF can be found in one third of patients at diagnosis, the majority have no neurological symptoms **[12]**. Accurate CNS assessment at presentation or relapse is very essential for treatment stratification. Recent treatment protocols offer high dose chemotherapy and CNS radiotherapy only for patients with CNS infiltration and reduce treatment for patients without CNS infiltration to decrease the toxicity of treatment and late effect of systemic chemotherapy and CNS radiotherapy **[13]**.

The cohort of patients characterized by risk factors as high risk seems to be 4-5 folds larger than the subgroup, which will actually develop CNS disease. More sensitive and specific laboratory methods would be crucial to detect occult CNS infiltration and if validated in clinical trials, to ensure optimal treatment while reducing unnecessary therapies [5,14]. The early detection of CSF involvement allows targeted approaches and the use of intrathecal drugs represent a critical step in the treatment of the disease **[15]**.

Until now, no absolute standard has been established to diagnose the involvement of CNS and all methods conventionally applied are associated with problems. All methods used for detection of leptomeningeal seeding including cytology, flow cytometry and the DNA based examination have pitfalls that need to be considered. Conventional cytology is positive only when large numbers of neoplastic cells are present in the CSF. The interpretation of the results is based on morphology and therefore, poor fixation or cell debris may lead to negative findings at time when the malignant cells are actually present in the CSF. In addition, some examiners may consider atypical morphology as inconclusive evidence for malignancy while others may read it as positive cytology **[8]**. In our study only 10 cases were positive by cytology, one of them was false positive; the cells were reactive as proved by FCM. That indicates that cytology alone cannot asses the CSF infiltration.

Many studies were published about the superiority of FCM in detecting CSF residing abnormal cells as compared to conventional cytomorphology **[2,4,5,14-19]**. Our data are in accordance with them and show high sensitivity of FCM in detecting CSF infiltrating malignant cells even in the absence of positive cytomorphology, which were about 29% in our study and between 27 and 78% in the others. In spite of differences as regards conventional cytology performances, all studies clearly showed that FCM is able to increase dramatically the detection of occult CSF infiltration. Taking in consideration that the previous studies were based upon analysis of heterogeneous cohorts of cases or focused on lymphomas.

As regards the percentages of positive cases by FCM in relation to the clinical manifestations, we noticed that they were twice those detected by cytology in cases at first presentation and reached the level reported in the literature **[12]**.

Taken together, our and literature results indicate FCM as the first choice technique, probably due to its intrinsic capability of exploring a large series of cell specificities at a single cell level. It can offer a unique and objective method by the combination of different strategies as aberrant immunophenotype **[15]**. It is a simple, quick and reliable technique. Results were available within 2h, which can speed up the therapeutic management; this may be a cost-reducing factor **[18]**. The advantages of flow cytometry include the ability to enrich for possible malignant cells via gating techniques in samples with mixed cell populations and the rapidity of the analysis **[5]**. One of the strengths of FCM is its sensitivity. In our results, CSF involvement could be demonstrated by FCM and not by cytology in cases with very low cell counts that accounted for only 0.86% of the total number of cells. This is similar to the 1.4% reported by Frensh et al. **[17]** and 0.9% reported by Finn et al. **[16]**. This can be attributed to the simultaneous evaluation of multiple surface markers on each cell.

However, FCM could still be falsely negative in some cases that can be detected by other

methods. This may be explained by lack of CD34 or CD10 in some cases, loss of some antigens in cases of relapsed ALL or dilution of malignant B cells by large numbers of polyclonal normal B-lymphocytes **[14]**. It is also important to recognize, that while FCM could detect 13 cases of CSF infiltration with negative cytology, a total of 15 cases (33.3%) could not be analysed due to insufficient number of cells. This figure is slightly higher than that reported by French et al. **[17]** (29%). This may be due to a higher number of cases at presentation in relation to relapsed and symptomatic ones in our study.

The DNA-based molecular techniques do not require intact cells. DNA is stable and can be recovered from CSF even after tumor cell lysis, probably making it a more sensitive indicator of malignancy than FCM and cytology requiring presence of intact tumor cells **[20]**. The detection of clonal Ig gene rearrangements using PCR technique offers an alternative because of its high sensitivity **[21-22]**. The rearrangement of variable, diversity and joining segments (VDJ) of IgH genes generates unique DNA junctional sequences that are specific by its size and sequence to each B cell clone **[23]**.

Many attempts have been made to use PCR for identifying IGHR in cells from CSF **[8,20,21, 24,25,26]**. Several factors contribute to this diversity, including the presence of somatic hypermutations that prevent binding of the PCR primers and the fact that the consensus primers for any particular assay are not complementary to all V regions. Finally, as with all PCR protocols, false-positive results due to technical problems as contamination are possible **[20]**.

Some authors used one round of PCR amplification to determine the clonality of the B-cell population in the CSF **[21,22]**. However, the relatively low sensitivity of this method can produce false negative results because of the small number of cells usually present in most CSF samples. So others applied the highly sensitive semi-nested PCR **[27]** in the search for IgH gene rearrangements in CSF sample cells. This technique has proved to be sufficiently sensitive to produce a detectable band from a single B cell **[24]**. However to achieve a reliable detection of monoclonality, previous methods require the presence of approximately >1% of clonal B cells to show a clearly visible rearranged band **[28]**. The sensitivity of PCR methods for Ig gene rearrangement is limited by the separating power of the gel and the discriminating power of the eye to recognize a faint band of clonal B cell superimposed upon a diffuse smear, which is generated by the reactive polyclonal B cell population present in all tissues **[29]**.

PCR analysis of the IgH gene typically involves the use of a consensus primer pair, with the upstream primer being homologus to a V segment and the downstream primer annealing to one of the J segments **[30]**. Usually a single J region primer is sufficient to recognize all six possible J segments, but no single V region primer recognizes all V segments, since there are many more V segments that are more heterozygous as compared with J segments. This is the primary explanation for the lack of a 100% diagnostic sensitivity of a single primer pair IgH PCR assay **[31]**.

In this study, we used a real time PCR assay to evaluate infiltration of CSF by leukemic cells. This method was based on LightCycler technology and SYBR green dye for detection of the gene rearrangements. Real-time PCR permits accurate detection of PCR products during the exponential phase of the PCR amplification process, which is in full contrast to the classical PCR end point detection. Owing to the real time detection of fluorescent signals during or after each subsequent PCR cycle, detection of PCR data can be obtained in a short time and no post-PCR processing is needed, thereby drastically reducing the risk of PCR product contamination **[32]**.

Few studies have compared the sensitivity of IGHR with morphological analysis of CSF; all of them in lymphoma. Only one showed that IGHR analysis did not appear to be more sensitive than morphological evaluation, in which a low incidence of lymphomatous spread to spinal fluid was found (eight from 76 patients) **[25]**. Possibly, because of pretreatment of the majority of patients with steroids prior to lumbar puncture and the small CSF volume analyzed **[22]**.

Our results, which showed 60% infiltration of CSF by malignant cells, agree with the results

of Baehring et al. **[8]** who has indicated a sensitivity of IGHR analysis of 58%, however they worked on large B cell lymphoma not ALL. Our results also agree with the result of Ekstein et al. **[20]**, who has reported that in 60% of patients with active CNS lymphoma, the CSF was positive for the presence of IgH gene rearrangement, while in 95% of patients responding to treatment, the test was negative.

It is clear that the sensitivity of real-time PCR is superior to both cytology and FCM especially for those at first presentation and those with CNS manifestations. However, there were two cases (10%) positive for cytology and FCM and negative by real-time PCR. False negative results in PCR were reported in small samples, and in the presence of a low cell count. This may be due to difficulty to obtain DNA of sufficient quality. It was difficult to obtain enough DNA to run in 14% of CSF samples in Ekstein et al. **[20]** study. Others reported an even higher rate of inability to extract DNA from CSF (29%) **[25]**.

False positive results in real-time PCR could be eliminated by optimization conditions. The design of clone-specific primers and the annealing temperature are important steps for achieving accurate data. Melting curve analysis was found to be an essential tool for characterizing the PCR products. The sensitivity, specificity and predictive value of positive or negative results of IgH gene rearrangement by real-time PCR in the CSF require further evaluation.

In conclusions: The diagnostic value of FCM and IGHR are two to three times more than that of cytology. Malignant cells in CSF can be classified according to the immunological surface profile by FCM. Therefore, immunophenotyping by FCM is recommended for routine diagnosis of CSF infiltration combined with cytology to increase the diagnostic yield. Furthermore, IGHR analysis by real-time PCR appears to be a useful addition to morphological and FCM analysis of CSF in the evaluation of CNS infiltration in ALL. It is reliable; relatively sensitive and highly recommended if there are no sufficient cell numbers for FCM analysis in cases at presentation, in relapsed cases or in those with CNS manifestations with negative results for both cytology and FCM.

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