Assessment of the Use of 8 color Flowcytometry in the Diagnosis of Acute Leukemia

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Abstract:
Background: Immunophenotyping has become a necessity in the diagnosis and classification of hematological neoplasms. It is a reliable, time saving and accurate method for not only the diagnosis but also for detection of minimal residual disease. There are ongoing developments in the field of flow cytometry in which more advanced flow cytometers have been developed allowing multicolor immunophenotyping. The question is; are they practically applicable or are they just for research purposes.
Methods: This study was conducted on 71 newly diagnosed acute leukemia patients. Samples were analyzed by using the 4 color FACS Calibur and 8 color FACS Canto II.
Results: There was significant difference between the sample volume required, monoclonal antibodies, number of tubes used and the turnaround time for each sample on comparing the 8 color flow cytometer to the 4 color flow cytometer.

Conclusion: It has been concluded that 8 multicolor flow cytometer definitely has the upper hand in time saving and reagents saving without the need to repeat the use of mono clonal antibodies. It also includes the leukemia associated phenotypes and aberrancies that would be missed when using the 4 color panel. However, there are certain drawbacks including technical difficulties and the requirement of high level of capability.
Keywords:
Acute leukemia, multicolor immunophenotyping, 8 color flow cytometry

Background:

Flow cytometric immunophenotyping has become essential for accurate diagnosis, classification, and disease monitoring in hemato-oncology. (1)

Leukemia's are the most common hematopoietic malignancies, and these disease categories represent various heterogeneous disease groups that include a large number of distinct biologic entities. While the diagnosis and classification of these malignancies were originally based primarily on morphologic features, at times supplemented by cytochemical studies, the diagnosis of hematopoietic malignancies now requires a complex series of specialized tools that include immunophenotyping and cytogenetic studies. (2)

Flow-cytometric immunophenotyping forms the basis of modern classification of acute and chronic leukemias. Finally, with multiparameter flow cytometry, it is now possible to identify routinely and reliably low numbers of leukemia and lymphoma cells (minimal residual disease) (3).

The use of multicolor techniques allows detailed characterization of various reactive and neoplastic populations, aiding in the diagnosis, classification, and prognostication of pediatric hematologic disorders (4).

It has also been proven that multiparameter flow cytometry has enhanced the differentiation between malignant cells and normal bone marrow cells in all cases, by using a single 8 color flow cytometry tube. It had a very high sensitivity to detection comparable to PCR at this level, however it had the upper hand in terms of simplicity and cost (5).

Shaver et al., 2015 concluded that constructing an immunophenotyping panel using objective,

specific and quantitative method allows the optimization and avoids problems of interdependence and redundancy in a large multi-antigen panel(6).

In this study we aim to evaluate the transition from the 4 color flow cytometer to the 8 color flow cytometer regarding their effectiveness in the diagnosis of acute leukemia. The main points of comparison were the lab tools and monoclonal antibodies used, the time to which a result was achieved and the sample volume that was required.

Methods:

This study was done on 71 patients of acute leukemia. Those patients were presented to South Egypt Cancer Institute Assiut University hospital in the period between December 2017 and June 2018.

All patients were subjected to a **complete clinical examination**, for assessment of the presence or absence of lymphadenopathy, splenomegaly, hepatomegaly, and manifestations of anemia, thrombocytopenia. **Other laboratory investigations were also carried out, including a complete blood picture with differential, bone marrow aspirate and immunophenotyping.**

Peripheral whole blood or bone marrow aspirate samples were collected in Ethylene Diamine Tetra Acetic acid (EDTA) vacutainer tubes.

Flowcytometry of the samples was performed on the FACS Calibur flow Cytometer (FC) (Becton Dickinson, BD, USA) and FACS Canto II FC (Becton Dickinson, BD, USA). The analysis was done using FACS DIVA software for the Canto II and Cell Quest Pro software for the FACS Calibur.

Lysis of erythrocytes was done by incubating with lysing solution at room temperature at a ratio of 1:9 (volume of sample: volume of lysing solution) for 10 minutes. The lysing solution is composed of 0.1 mM EDTA, 150 mM NH_4Cl, and 10 mM $KHCO_3$. After incubation, the sample was centrifuged (3500 rpm for 5 min at room temperature), the supernatant was aspirated, and the cells were washed twice in a phosphate-buffered saline solution.

BD fixative and permeabilization agents were used for the cytoplasmic staining. The monoclonal reagents used were coated by different fluorochromes according to the acute leukemia panel documented by the South Egypt Cancer Institute Flow Cytometry Lab (SECI FCL) for samples analyzed on the FACS Calibur and the SECI FCL modified Euro Flow Panel (7) for diagnosis of acute leukemia:

1) **SECI Flowcytometry lab 4 color acute leukemia panel**:

a) First panel: (Table 1)

	FITC	PE	PerCp	APC
Tube 1	**CD 4**	**CD 8**	**CD 3**	**CD 45** (BD, USA, Code: 555485)
	BD, USA Tritest Code: 342414			
Tube 2	**CD 5**	**CD 10**	**CD 19**	**CD 45** (BD, USA, Code: 555485)
	BD, USA Tritest Code: 331357)			
Tube 3	**CD 13 (** DAKO, Carpinteria, CA**,** Code:F083101)	**CD 33** (BD, USA, Code: 347787)	**CD 34** (BD, USA, Code:347222)	**CD 45** (BD, USA, Code: 555485)
Tube 4	**CD 14** (BD, USA, Code: 347493)	**CD 117 (**BD, USA, Code: 332785)	**HLA-DR (**BD, USA, Code: 347402)	**CD 45** (BD, USA, Code: 555485)
Tube 5	**CD16 (**Bio legend, Code: 300306)	**CD 56 (**DAKO, Carpinteria, CA**, Code:** R725101)		**CD 45 (**BD, USA, Code: 555485)

b) Second panel:
A combination of any of the following along with one or more of the backbone markers: (Table 2)

Table 2: Second 4 color panel

Cyto: Cytoplasmic, BD (Beckton Dickinson)

Myeloid	B-ALL	T-ALL
Cyto MPO (FITC, **Beckman-Coulter, Brea, CA, Code: 50)**	**Cyto μ (FITC,** BD, USA, Code: 555782)	**CD 2 (FITC,** BD, USA, Code: 560777)
CD 36 (FITC, **Beckman-Coulter, Brea, CA, Code : 33)**	**CD 22 (APC,** BD, USA, Code: 562860)	**CD 7 (APC,** BD, USA, Code: 561604)
CD 64 (PE, **Beckman-Coulter, Brea, CA, Code: 54)**	**Anti κ (FITC) & Anti λ (PE) (**BD, USA, Code: 349516)	**CD 1a (APC,** DAKO, Carpinteria, CA, Code: 300110)
CD 235a (FITC, **BD, USA, Code:** 559943)		**Cyto CD 3(FITC,** Bio legend, Code:300306)
Cyto CD 61 (FITC, **BD, USA, Code:** 555753)		
Cyto CD 41a (FITC, **BD, USA, Code: 521956)**		
Cyto CD 42b (APC, **BD, USA, Code: 551061)**		

2) SECI flow cytometer lab 8 color acute leukemia panel (modified Euro flow panel):

a) Acute Leukemia Orientation Tube (Table 3)

	A LOT							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	cyCD3	CD45	CyMPO	CyCD79a	CD34	CD19	CD7	sCD3
Company	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	DAKO, Carpinteria, CA	BD, USA	BD, USA	BD, USA	BD, USA
Code	560351	560777	50	R715901	347222	557835	561604	560176

b) Myeloid panel: (Table 4)

S (surface), cyto (cytoplasmic), TDT (terminal deoxynucleotidy transferase), BD (Beckton Dickinson), CA (California), HLA-DR (Human Leukocyte Antigen)

	AML/MDS							
	Tube 1 (Neutrophil)							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	HLA-DR	CD45	CD16	CD13	CD34	CD117	CD11b	CD10
Company	BD, USA	BD, USA	DAKO, Carpinteria, CA	BD, USA	BD, USA	BD, USA	BD, USA	BD, USA
Code	655874	560777	302006	555394	347222	339217	550019	655404
	Tube 2 (Monocytic)							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	HLA-DR	CD45	CD35	CD64	CD34	CD117	CD300e(IREM2)	CD14

Company	BD, USA	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	BD, USA	BD, USA	BD, USA	BD, USA
Code	655874	560777	555452	33	347222	339217	656158	560180
	Tube 3 (Erythroid)							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	HLA-DR	CD45	CD36	CD105	CD34	CD117	CD33	CD71
Company	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	BD, USA	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	BD, USA
Code	655874	560777	54	560839	347222	339217	44	563671
	Tube 4 (Aberrant lymphoid markers)							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	HLA-DR	CD45	TDT	CD56	CD34	CD117	CD7	CD19
Company	BD, USA	BD, USA	BD, USA	DAKO, Carpinteria, CA	BD, USA	BD, USA	BD, USA	BD, USA
Code	655874	560777	332789	R725101	347222	339217	561604	560177
	Tube 5 (Stem cell markers)							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	HLA-DR	CD45	CD15		CD34	CD117	CD22	CD38
Company	BD, USA	BD, USA	BD, USA		BD, USA	BD, USA	BD, USA	BD, USA
Code	655874	560777	555401		347222	339217	562860	656646
	Tube 6 (Megakaryoblastic & mastocytosis)							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	HLA-DR	CD45	cytoCD41a	CD25	CD34	CD117	Cyto CD42b	CD9
Company	BD, USA	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	BD, USA	BD, USA	BD, USA	BD, USA
Code	655874	560777	521956	65	347222	339217	551061	655409

c) B-ALL panel: (Table 5)

S (surface), cyto (cytoplasmic), TDT (terminal deoxynucleotidyl transferase), TCR (T-cell receptor), BD (Beckton Dickinson), CA (California)

	B-ALL							
	Tube 1							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	CD20	CD45	CD58	CD66c	CD34	CD19	CD10	CD38
Company	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	DAKO, Carpinteria, CA	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	BD, USA
Code	655872	560777	IM1218U	342304	347222	557835	B92400	656646
	Tube 2							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**

	SmIgκ	CD45	Cytoμ	CD33	CD34	CD19	sIgM	SmIgλ
Company	BD, USA	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	BD, USA	BD, USA	BD, USA	BD, USA
Code	561327	560777	555782	53, 54	347222	557835	551062	562893
	Tube 3							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
		CD45	TDT	CD13	CD34	CD19	CD22	CD9
Company		BD, USA	BD, USA	BD, USA	BD, USA	BD, USA	BD, USA	BD, USA
Code		560777	332789	555394	347222	557835	562860	655409

d) T-ALL panel: (Table 6)

S (surface), cyto (cytoplasmic), TDT (terminal deoxynucleotidyl transferase), TCR (T-cell receptor), BD (Beckton Dickinson), CA (California)

T-ALL								
	Tube 1							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	CytoCD3	CD45	TDT		CD5	CD10	CD1a	sCD3
Company	BD, USA	BD, USA	BD, USA		BD, USA	BD, USA	DAKO, Carpinteria, CA	BD, USA
Code	560351	560777	332789		341109	341112	300110	560176
	Tube 2							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	CytoCD3	CD45	CD2	CD117	CD4	CD8	CD7	sCD3
Company	BD, USA	BD, USA	BD, USA	Beckman-Coulter, Brea, CA	BD, USA	BD, USA	BD, USA	BD, USA
Code	560351	560777	555326	51	332772	557746	561604	560176
	Tube 3							
	V450	**Horizon V500c**	**FITC**	**PE**	**PercpCy5.5**	**PECy7**	**APC**	**APCH7**
	CytoCD3	CD45	TCRγδ	TCRαβ	CD33	CD56		sCD3
Company	BD, USA	BD, USA	DAKO, Carpinteria, CA	DAKO, Carpinteria, CA	BD, USA	BD, USA		BD, USA
Code	560351	560777	465238	306708	333146	557747		560176

As for the Statistical Analysis; data was collected and analyzed by using SPSS (Statistical Package for the Social Science, version 20, IBM, and Armonk, New York). Continuous data was expressed in form of mean (range) while nominal data was expressed in form of frequency (percentage).

*Chi*²-test was used to compare the nominal data of different groups in the study and ANOVA test for more than two groups in case of normally distributed data and to also calculate the *p value.*

P value of ≤ 0.05 was considered statistically significant.

Results:

Mean age of enrolled patients was 19.21 years with range between 1 and 70 years. Majority (51%) of patients were males and 35 (49%) of them were females. As regarding type of leukemia; 36 (51%) had AML, 32 (46%) had ALL and 2 (3%) had biphenotypic acute leukemia (BPAL). (Fig.1)

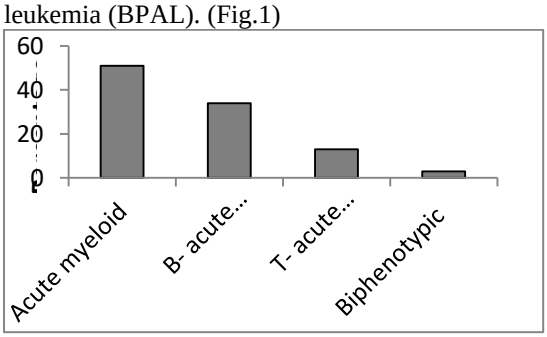


Fig. 1: Types of acute leukaemi

As for the subtypes of acute myeloid leukemia (AML) (Table 2), they were classified into AML (M1/M2) represented 13 cases (36.1%), Acute promyelocytic leukemia (APL-AML), (AML M3) represented 6 cases (16.7%), AML (M4/M5) 11 cases (30.5%), AML M6 1(2.8%) case, AML M7 4 (11.1%) cases and finally AML M0 1 (2.7%) case. **(Fig. 2)**

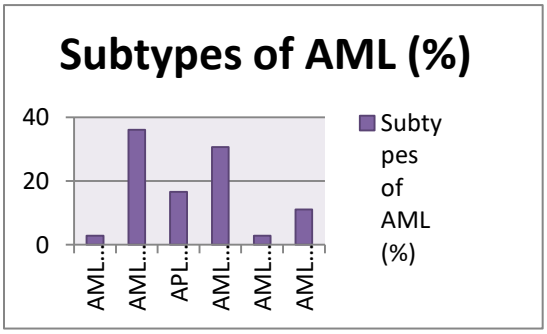


Fig. 2: Subtypes of AML

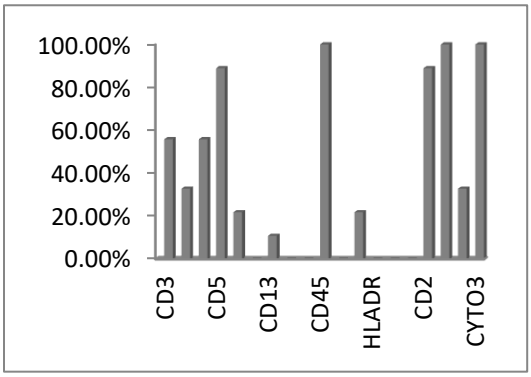
As for subtypes of ALL: B-ALL was diagnosed in 24 cases (34% of total AL cases and 73% of ALL cases) while T-ALL was in 9 cases (12% of total AL cases and 27% of ALL cases). Within the T-ALL cases 2 (22%) cases were diagnosed as early T- precursor ALL (ETP-ALL) while the remaining 7 (78%) cases were classified precursor T-ALL.

Finally the 2 BPAL cases were classified, one of them was B&T ALL and the other one was myeloid/B AL.

I. Phenotypic Results of the Cases:

A. Phenotype of T-ALL

As for the cases diagnosed T-ALL by the 4 color (FACS Calibur); the T-cell markers surface CD 3 was positive in 67% of the cases while it was positive in 100% of patients when done cytoplasmic, CD 4 was positive in 33% of the cases and CD 8 in 56% of the cases. CD 5 was positive in 89%, CD 2 89%, CD 7 in 100% and CD 1a in 33% of the cases. The B-cell marker CD 19 was negative in all cases while CD 10 was positive in 22% of the cases. The myeloid markers CD 13 was positive in 11% of cases and CD 33 was negative in all cases. While the



marker CD 117, was positive in 22% of the cases, suggesting the diagnosis of ETP-ALL. (Fig. 3)

Fig.3: Diagnosis of T-ALL by four colors

Using the FACS Canto II, 8 colors flow cytometer the results were as follows (Fig. 4). For the T-cell markers: surface CD 3 was positive (67%), CD 8 was positive in (56%), CD 4 was positive (33%), CD 7 was 100% positive, CD 2 was positive in (89%), cytoplasmic CD 3 was positive in (100%), TdT (terminal deoxynucleotidyl transferase) was positive in (22%), CD 5 in (89%), CD 1a in (22%). The precursor marker CD 34 was negative in 100% of cases, the stem cell marker used as a leukemia associated marker (LAP); CD 123 was positive in 22% of cases. The myeloid markers CD 33 was negative in all cases while CD 117 was positive in 22% of cases. The B cell markers CD 19, cytoplasmic CD79a were negative in all cases while CD 10 was positive in 22% of cases.

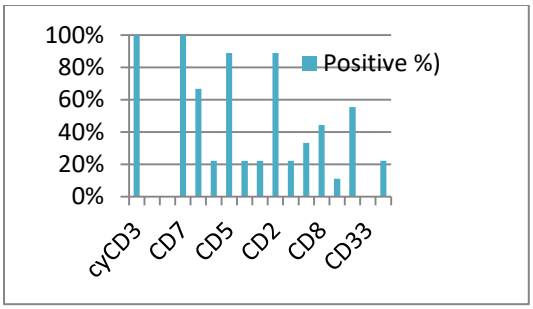


Fig.4: Diagnosis of T-ALL by eight colors

B. Phenotype of B-ALL

In the diagnosis of B-ALL by the FACS Calibur, the following was observed: CD 19, CD 10, CD 22 and HLA DR were positive in 100% of cases, while Cytoplasmic µ was positive in 62% of cases. The rest of the panel; markers for T-lymphocytes (CD 3, 4 &8), markers for NK cells (CD16 & CD56) were negative. As for the myeloid markers CD 13, 117, cytoplasmic myeloperoxidase enzyme (MPO) were negative, however CD 33 was aberrantly expressed in 25% of the cases. The precursor marker (CD 34) was positive in 67% of the cases and CD45 was dimly expressed in 67% of cases. (Fig. 5)

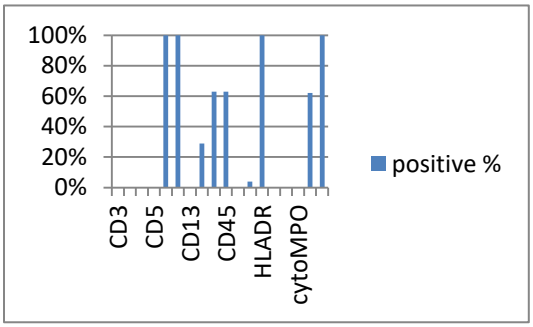


Fig.5: B-ALL by four colors

Upon the use of the FACS Canto II, the following was noticed. The markers for B-cells CD 19 and CD 10 were positive in 100% of the cases, cytoplasmic 79a was positive in 58% of patients, cytoplasmic µ was positive in 62%, CD 22 was positive in 100% of patients and CD 9 was positive in 92% of the cases. The proliferation markers used for minimal residual disease (MRD) CD 38 was positive in 75%, CD 58 was positive in 96% and CD 66c was positive in 58% of patients. The myeloid marker CD 33 was aberrantly expressed in 25% of cases. The remaining B-cell markers: CD 20 was positive in 33%; surface Immunoglobulin heavy chain µ(s Ig M), light chain kappa and light chain lambda were negative in all cases. CD 34 and CD 45 (dim) were positive in 67% of cases. Finally the T-lymphocyte marker CD 7 and the myeloid lineage specific marker cytoplasmic MPO were negative in all cases. (Fig. 6)

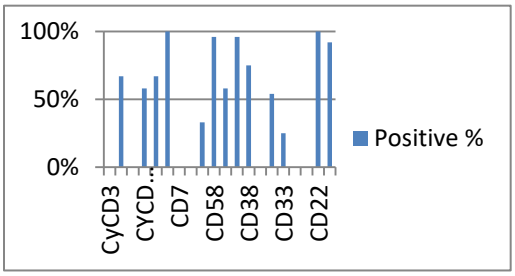


Fig.6: B-ALL by 8 colors FACS Canto

C. Phenotype of AML:

In the AML cases, according to FACS Calibur, the markers for T-lymphocytes (CD 3, 5, & 8) were negative in all cases, while the T-lymphocyte marker (CD 4) was positive in 19% of acute myeloid leukemia patients as a monocytic marker. The B-lymphocyte marker (CD 19) was aberrantly expressed on 11% of cases and the other B-lymphocyte markers (CD 22 & cytoplasmic µ& CD 10) were negative in all cases. CD 34 was positive in 72%, HLA-DR was positive in 69% of cases and CD 45 (dim) was positive in 86% of cases. The marker for natural killer (NK) cells (CD 56) was aberrantly expressed in 25% of the cases and CD 16 was positive in 19% of cases. The myeloid lineage specific antigens Cytoplasmic MPO was positive in 67% of cases, CD 33 was positive in 94%, CD13 was positive in 69 % of cases and CD 117 was positive in 86% of cases. The monocytic marker CD 36 was positive in 47% of cases and CD 64 was positive in 50% of cases. The markers of megakaryoblasts (cytoplasmic 41a was positive in 8% of cases, cytoplasmic 42b was positive in 11% of cases and CD 61 was positive in 6% of cases. Finally the anti glycophorin A (CD235a) was positive in 3% of cases. (Fig.7)

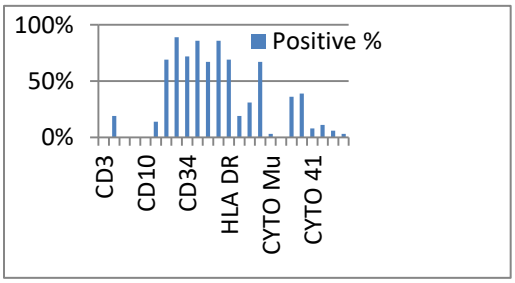


Fig.7: AML by four colors

By the FACS Canto, the AML cases were analyzed accordingly. The T- lymphocyte marker (surface and cytoplasmic CD 3) was negative in all cases while the CD 7 marker was aberrantly expressed in 14% cases. The B lymphocyte markers (cytoplasmic CD79a and CD 22) were negative in all cases, the CD 19 was aberrantly expressed in 11% of cases and CD 9 was also aberrantly expressed in 11% of cases, all of which could be used as marker for detection of minimal residual disease along the NK marker CD 56 which was positive in 25% of cases and CD 7 which was discussed previously. The precursor marker CD 34 was positive in 69% of cases, HLA-DR was positive in 75% of cases and CD 45 was positive in 89% of cases. The myeloid markers CD 117 was positive in 83% of cases, CD 13 was positive in 69% of cases and CD 33 was positive in 94% of cases. The neutrophil markers CD 11b was positive in 36% of cases, CD 10 was negative in all cases, CD 35 was positive in 36% of cases. The monocytic markers CD 64 was positive in 50% of cases, CD 300e was positive in 25% of cases, CD 14 was positive in 28% of cases, CD 36 was positive in 53% of cases. The erythroid markers CD 105 was positive in 3% of cases and CD 71 was positive in 14% of cases. The stem cell marker CD 15 was positive in 6% of cases, CD 38 (proliferation marker) was positive in 81% of cases. The megakaryocytic marker cytoplasmic CD41a was positive in 8% of cases and cytoplasmic CD 42b was positive in 14 % of cases. (Fig. 8)

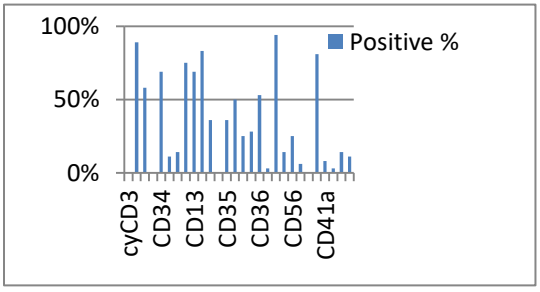


Fig.8: AML by eight colors

D. Phenotype of the BPAL:

It has been mentioned earlier that there were 2 cases of biphenotypic acute leukemia representing 3% of the total. One of the cases was mixed B & T ALL and the other one was myeloid/B-cell acute leukemia. Upon using the 4 color flow cytometer, the first case was positive for the T-lineage markers (CD2, CD 7, CD 5 and cytoplasmic CD 3) and negative for the remaining T-lineage markers (CD 4, CD8, CD 1a, and surface CD3.) As for the B-lineage markers; it was positive for (CD 19, CD 10, CD 22 & cytoplasmic μ). In addition to the precursor marker CD 34, CD 45^{dim} and HLA-DR, all were positive. However all myeloid lineage markers were negative (CD33, CD13, CD 117 & CD 14) and also the NK cell markers (CD 56 & CD 16.)

On the other hand, when using the 8 color flow cytometer for the same case, the phenotypic results were as follows. The T-lineage markers were: (cyto CD3, CD7, CD2 & CD5) were positive and the (CD3, CD4, CD8, CD 1a, TCR alpha/beta & TCR gamma/delta) were negative. The B-lineage markers were: the positive markers being CD19, CD10, CD 22, cyto μ and CD 9, while the negative markers being cyto 79a, CD20, surface heavy μ chain and light chains (κ & λ). The myeloid lineage markers (cyto MPO, CD 13, CD 33, CD117) were negative. The precursor markers CD 34 & cyto TdT were positive. The remaining markers were; CD 58 was negative, CD 38 was positive, CD 66c & CD 123 were also negative.

The other case was myeloid/B acute leukemia which when analyzed by the 4 color flow cytometer the result was like this. The T- lineage markers were negative (CD3, CD4, CD8 & CD5). The B-lineage markers: CD 10, CD 19, and CD 22 were positive while cyto μ was negative. The myeloid markers: CD 13, CD 33, CD 117, CD 64 and cyto MPO were positive while CD 36 and CD 14 were negative. 45, CD 34 and HLA-DR were positive while CD 16 and CD 56 were negative.

As for the results when analyzed by the 8 color flow cytometer; starting with T-lineage markers (cyto & surface CD3, CD 7) were negative. The B-lineage markers: cyto 79a, CD 19, CD 10, and CD 22 were positive while CD 20, CD 9, surface Ig M, Ig κ and Ig λ were negative. CD 66 c, CD 38, CD 58 were positive as well as CD 34, HLA-DR and CD 45. The myeloid markers that were positive were CD 13, CD 33, CD 117, CD 64, CD 11b and cyto MPO while the rest of the myeloid panel markers were negative.

E. Comparative Results between 4-color and 8 –color Flow cytometers:

Reagents used in 4- Color and 8-Color Flow Cytometry in Case of T-Lymphocytic Leukemia:

Table 7 shows reagents used in 4- color and 8- color flow cytometry in case of T-ALL. Number of monoclonal antibodies was 26 antibodies in all patients with T-ALL in case of 4- color flow cytometry while it was 21 antibodies in case of 8- color flow cytometry. Number of tubes was 4 in all patients with T-ALL in case of 8- color flow cytometry while number of tubes ranged between 5 and 14 tubes in case of 4- color flow cytometry.

Sample volume was 200 µl in all patients in case of 8- color flow cytometry and turnaround time ranged between 5 and 8 hours but in case of 4- color flow cytometry, sample volume ranged between 250 and 750 µl and range of turnaround time was between 24 and 32 hours.

Concerning the number of monoclonal antibodies, the number of tubes, the sample volume and the TAT required to diagnose patients with T-ALL were significantly lower in case of usage of 8-color cytometry in comparison to 4- color cytometry ($P< 0.05$).

Reagents used in 4- Color and 8- Color Flow Cytometry in Case of B- Lymphocytic Leukemia: (Table 8)

It was noticed that number of monoclonal antibodies was 23 in all patients with B- ALL in case of 4- color flow but with 8- color, number of monoclonal antibodies ranged between 20 and 21 with insignificant differences between 4-color and 8-color as regarding the number of monoclonal antibodies ($P= 0.65$).

Number of tubes that were used in patients with B-ALL was significantly higher in case of 4- color flow than 8-color flow (10 (4- 19) versus 4 (4- 4); $P= 0.04$). Also, both of the sample volume and turnaround time (TAT) were significantly lower in case of 8- color flow in comparison to 4- color flow.

Sample volume with 4- color ranged between 200 and 950 µl while it was 200 µl in all patients in case of 8- color flow. Turnaround time (TAT) ranged between 5 and 48 hours in case of 4- color flow and between 4 and 8 hour in case of 8- color.

Reagents used in 4- Color and 8- Color Flow Cytometry in Case of Acute Myeloid leukemia: (Table 9)

Number of monoclonal antibodies ranged between 33 and 40 in patients with AML in case of 4- color flow and this was significantly higher in comparison 8- color flow where the range of antibodies was between 20 and 32. Also, number of tubes was significantly higher in case of 4- color flow (11 (2- 25) versus 7 (4- 7); $P= 0.04$).

Range of sample volume was between 100 and 1250 µl in case of 4- color flow and between 200 and 350 µl in case of 8- color flow while range of turnaround time (TAT) was between 24 and 48 in case of 4- color flow and between 5 and 8 in case of 8- color flow.

Each of monoclonal antibodies, number of tubes, sample volume and turnaround time (TAT) were significantly lower in case of 8- color flow in comparison to 4- color flow ($P= 0.03$, 0.04, 0.04 and 0.02 respectively).

Aberrant markers detected by the 4 color flow cytometry panel compared with the 8 color flow cytometry panel:

It was noticed that nine patients with AML had aberrant expression of either CD7 or CD9. These CDs were detected with the usage of 8- color flow cytometry where 4- color flow cytometry failed to detect theses aberrant expressions. However, the 4 color flow cytometry would have been able to detect them but only if additional monoclonal antibodies, tubes and sample volume were added. Aberrant expression of CD9 was detected in 4 patients with AML and in 5 patients CD7 aberration was detected.

Discussion

Flow cytometry is an indispensable tool for the diagnosis of acute leukemia. With increasing numbers of high quality monoclonal antibodies that recognize the various hematopoietic cell markers becoming commercially available, routine flow cytometry panels are expanding in many diagnostic laboratories. Accompanied by this development is a concomitant increase in reagent cost and labor necessary to perform these panels.

As noted, flow cytometry is a very complex laboratory procedure that requires both great technical skill and very knowledgeable and experienced interpretation. Because of the variations from laboratory to laboratory, it is extremely difficult to standardize the procedures and antibody panels. In the last decade, many flow cytometry laboratories in the world have endeavored to find the minimal number of antibodies that could correctly diagnose acute leukemia. (8)

In this study we aim to evaluate the transition from the 4 color flow cytometer to the 8 color flow cytometer regarding their effectiveness in the diagnosis of acute leukemia. The main points of comparison were the lab tools and monoclonal antibodies used, the time to which a result was

achieved and the sample volume that was required.

As for the antibody selection panel:Haycocks, Lawrence (8) stated an obvious problem with using clustering patterns to guide antibody panel selection was that the interpretation of these patterns required a degree of subjective analysis and experience. The patterns themselves may also be affected by instrumentation, choice of fluorochrome, the characteristics of individual antibody preparations, and by sequential adjustments made to a single analyzer. With practice and consistency in reagent selection, some of these potential difficulties can be lessened and that was addressed by the Euro flow consortium (9). Van Dongen, Lhermitte (7) explained how the Euro flow panel was created, the steps for choosing each monoclonal antibody and their value and that was our reference to creating our 8 color flow cytometry panel.

This study was done on 70 patients who were diagnosed with acute leukemia. There was a male predominance (51%) in the cases of acute leukemia which agreed with Omran, Elsharkawy (10), who described higher incidence in males (55%).

The cases of acute leukemia were classified into AML (51%), B-ALL (34%), T-ALL (13%) and MPAL (3%). While according to Das A, *et al.* (2) B-A LL accounted for (50%) of the cases and was more common than AML (40%) while T-ALL represented 7% and MPAL was also 3%. However in their study they didn't include the erythroid and megakaryocytic lineages markers and therefore weren't able to diagnose subtypes of acute myeloid leukemia.

The results of this study are also comparable to a study that was also carried out in Egypt, Salem and Abd El-Aziz (11) agreed with the current study results in that a higher fraction was AML (69%) than ALL, and B-ALL was higher than T-ALL. However in the subtypes, Salem et al, found that APL AML represented 23% of cases versus the 17% in the current study. In his study there was predominance of AML M4/M5 34.5% (31% in the current study) in contrast to the present study there was predominance of AML M1/M2 (36%.) Their study was conducted on 164 cases of acute leukemia as opposed to 71 cases in this study.

San Miguel, Martínez (12) reported that 46 (87%) of 53 AML cases had an aberrant phenotype; this figure was confirmed by the same authors in another report on 126 AML cases (13). Whereas in the current study, the cases of AML with aberrant lymphoid markers were 30.5% using the 4-color flow cytometry panel. In the meantime there was a noted increase of aberrancies in AML cases up to 55.6% using the 8 color flow cytometry panel. As for these aberrant markers in AML, the most common lymphoid marker was CD 56 (25%), followed by CD 7 (14%) then CD 9 & CD 19 both (11.1% each.) According toAl-Mawali, Gillis (14), CD 7 was the most common aberrant lymphoid marker and the least was CD19.

As for the presence of aberrant markers in B-ALL, CD 33 was expressed in 25% of the cases opposing to Seegmiller, Kroft (15) who found it in 43% of cases of B-ALL, however their study was done on a larger group of 200 B-ALL cases and they used CD 33 PE (clone P67.6) versus the current CD 33 was APC (clone WM53).

Although investigative flow cytometry will discover more interesting immunological markers for leukemia cells, clinical flow cytometry faces a dilemma to balance a limited budget and to maintain a highly accurate diagnosis of acute leukemia. With the increasing pressure on cost-effectiveness of clinical laboratories, reagents consumed must be put into consideration. As concluded, that the monoclonal antibodies and the number of tubes used were significantly lower in 8 color flow cytometry than the 4 color flow cytometry.

As for the difference between the cytometers themselves, Ashman, Sachdeva (16) concluded that the difference in the type of flow cytometers and soft wares influences results and analyses process. During the different analyses of the cases, it was observed that FACS Diva software provided with FACS CANTO II (8 color flow cytometer) had much more options than Cell Quest Pro software provided with the FACS Calibur (4 color flow cytometer). For example the ability to perform biexponential gating to identify negative and dim populations was only available in the FACS Diva software.

Conclusion

Multipara meter flow cytometry is an essential tool for diagnosis and classification of acute leukemia. It has a very important role in detecting minimal residual disease (MRD.) All of those combined contribute to the tailored medical management for each leukemic patient. On comparing 4 colors to 8 color flow cytometers, we conclude that 8 multicolor flow cytometer definitely has the upper hand in time saving and reagents saving without the need to repeat the use of mono clonal antibodies. It also

includes the leukemia associated phenotypes and aberrancies that would be missed when using the 4 color panel. Finally, it ensures that each acute leukemia case has a complete baseline to be able to build upon it the MRD study.

However, there are certain drawbacks to upgrading our flow cytometer. First of all, there were technical difficulties; like forgetting to put the monoclonal antibodies due to using too many tubes at the same time, the frequent need to compensate the settings of the flow cytometer and the consumption of much more amount of flow cytometer solutions. Secondly, it requires a much higher level of expertise to be able to analyze and deal with all the different variables.

List of Abbreviations

AL	Acute Leukemia
ALL	**Acute Lymphoblastic Leukemia**
AML	**Acute Myeloid Leukemia**
APC	Allophycocyanin
APCH-7	Allophycocyanin-Hilite 7
APL-	**Acute Promyelocytic Leukemia**
BPAL	**Biphenotypic Leukemia**
CD	**Cluster of Differentiation**
Cyto	**Cytoplasmic**
EDTA	**Ethylene-Diamine-Tetra-Acetic Acid**
ETP	**Early T Precursor**
FACS	**Fluorescence Activated Cell Sorting**
FC	**Flowcytometry**
FITC	fluorescein isothiocyanate
MRD	**Minimal Residual Disease**
NH4Cl	**Ammonium Chloride**
NK	**Natural Killer**
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanine7 conjugate
PerCP	Peridinin chlorophyll-A protein complex
SECI FCL	**South Egypt Cancer Institute Flow Cytometry Lab**
TAT	**Turn Around Time**

Competing Intere s: st

There are no competing interests.

Authors' Contributions:

Y.M. have carried out the preparation of samples, acquisition of data, analysis and interpretation of data and drafted the manuscript.

N.S. & M.Z. have contributed to designing the work.

D.S. has contributed by supervising and revising the work.

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Table 7: Reagents were used in 4- color and 8- color flow in case of T- ALL

	4- color flow	8- color flow	*P* value
No. of monoclonal antibodies	26 (26- 26)	21 (21- 21)	**0.03**
No. of tubes	8 (5- 14)	4 (4- 4)	**0.02**
Sample volume (µl)	400 (250- 750)	200 (200- 200)	**0.02**
Turnaround time (TAT) (hour)	27 (24- 32)	6 (5- 8)	**0.03**

Data is expressed in form of median (range). *P* value was significant if < 0.05. **ALL**, acute lymphocytic leukemia

Table 8: Reagents used in 4- color and 8- color flow in case of B- ALL

	4- color flow	8- color flow	*P* value
No. of monoclonal antibodies	23 (23- 23)	20 (20- 21)	0.65
No. of tubes	10 (4- 19)	4 (4- 4)	**0.04**
Sample volume (µl)	500 (200- 950)	200 (200 -200)	**0.04**
Turnaround time (TAT) (hour)	26 (5- 48)	6 (4- 8)	**0.02**

Data is expressed in form of median (range). *P* value was significant if < 0.05. **ALL**, acute lymphocytic leukemia

Table 9: Reagents used in 4- color and 8- color flow in case of AML

	4- color flow	8- color flow	*P* value
No. of monoclonal antibodies	33 (33- 40)	32 (20- 32)	**0.03**
No. of tubes	11 (2- 25)	7 (4- 7)	**0.04**
Sample volume (µl)	550 (100- 1250)	350 (200- 350)	**0.04**
Turnaround time (TAT) (hour)	27.5 (24- 48)	7 (5- 8)	**0.02**

Data is expressed in form of median (range). *P* value was significant if < 0.05. **AML**, acute myeloid leukemia

List of Figures: