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ABSTRACT

***Objective*: The aim was to determine relative frequencies of acute leukemia immunophenotypes using commonly expressed markers and to describe the clinicopathological characteristics.**
***Design*: This was a prospective cross-sectional study.**
***Setting*: The study was based at Aga khan clinical laboratory department.**
***Subjects*: One hundred and thirty two (132) consecutive blood and bone marrow specimens from patients suspected to have acute leukemia were analysed for cytomorphological characteristics and immunophenotyping. The clinical-pathological characteristics were also recorded. Immunological category was assigned using the EGIL criteria.**
***Results*: There were 88 AML and 42 ALL patients analysed for immunophenotypes. Only tw cases of biphenotypic leukemia were found. The commonest overall AML morphological sub-type was AML-M2, 26 (29.5%). Majority of ALL cases were B-cell immunological sub-type (96.6%). Early pre-B phenotype constituted 62.07% and Common B-cell ALL 37.93%. There were only 4 cases of T-cell ALL. Majority of patients presented with anaemia with a median hemoglobin of 7.5g/dl (range 2-15g/dl). The median platelet count was 55 (range 4-462 $\times$ 10^9/L).**
***Conclusion*: Immunophenotyping of acute leukemia is beneficial in accurate diagnosis of patients with these malignancies in this setup. T-cell ALL, AML-M6 and M7 are less frequent than what has been reported in most studies in Africa.**
***Key words*: acute leukemia, immunophenotype, WHO, EGIL, CD markers**

INTRODUCTION

Hematological malignancies are becoming major causes of morbidity and mortality in all age groups. The Leukemias have been shown to be a major health problem over the years with an incidence that is on the rise (1). Part of the reason for increase in incidence is due to improved diagn≠≠osis that exists and increased awareness of the condition .Availability and invention of new diagnostic modalities such as molecular techniques, immunological techniques to confirm suspected cases of any hematological malignancy have also made diagnosis and therapeutics of these conditions easier(2). Acute leukemias have been re-classified by the World Health Organization (WHO) and this supersedes the earlier FAB criteria (18-22). A basic principle of WHO system is that the classification of hematopoietic and lymphoid neoplasms should utilize not only morphological findings but also genetic, immunophenotypic, biologic and clinical features to define specific disease entities(4-5).

Ensuring accuracy in diagnosis is always the first goal towards correct treatment for patients with leukemia. Further more, standardisation, validation and reproducibility in diagnostic techniques are essential as a way of quality assurance in the laboratory(6). However, in resource poor countries like Kenya, all the diagnostic modalities of leukemia are not available and therefore the prevalence of the various subtypes of acute leukemia (AL) is not known. There is paucity of data on the prevalence of the various subtypes and immunophenotypic characteristics of the acute leukemia in Kenya. The authors sought to characterize the immunophenotypic profiles of acute leukemia and their clinical –pathological characteristics in all age groups.

MATERIALS AND METHODS

The study was carried out between June 2009 and August 2010. The aim was to determine relative frequencies of acute leukemia immunophenotypes using commonly expressed markers and to describe their clinicopathological characteristics. All cases of

confirmed acute leukemia by cytomorphology were eligible for inclusion. Cases of Chronic lympho/ myeloproliferative disorders, Myelodysplastic disorders, Acute leukemia with antecedent hematological malignancies and poor quality of bone marrow aspirate or peripheral blood film material that would make interpretation difficult(i.e. poorly preserved material resulting in hemolysis, clotting or freezing; use of formalin or other fixative; sample specimens showing degenerative changes) were all excluded. Cases of acute leukemia already on cytotoxic drugs and/or radiotherapy were also excluded.

Bone marrow (BM) aspirates and blood films were assessed for cytomorphology. The pathological features were determined from full blood counts levels and this determined pancytopenia, leukocytosis, thrombocytopenia, and anemia. Bone marrow aspirates and peripheral blood films were stained with Leishman stain for morphological analysis. The morphological characteristics and cytochemical staining were reviewed independently to classify the acute leukemia according to the FAB criteria. Diagnostic interpretation and sub-typing as recommended by the FAB criteria was utilized. Clinical features (lymphadenopathy, splenomegaly, clinical CNS disease, hepatomegaly, anemia, bleeding-included any petechiae, purpura, gum bleeding, epistaxis, per vaginal bleeding, gastrointestinal bleeding and hematuria) were recorded.

Bone marrow and blood samples were immunophenotyped by using the multiparametric five-colour flow cytometer (Beckman Coulter FC 500) , utilizing the panels of monoclonal antibodies using the European Group for the Immunological Characterization of Leukemia (EGIL) criteria. Cell suspensions were stained with multiple panels of three monoclonal antibodies and a two-step strategy was used, labeled with fluorescein isithiocyanate (FITC), Phycoerythrin (PE) and R-phycoerythrin-Texas red (ECD) or Peridin chlorophyll Protein (PerCP). Cell suspensions were also stained with panels of identically conjugated isotype controls for the antibodies of each panel. Leukemic samples were considered positive for a particular antigen if 20% or more of leukemic cells reacted with a particular monoclonal antibody. Co- expression of the surface markers was analysed when antibodies of more than one lineage were present and scoring for bilineage undertaken using the EGIL criteria. The panels of antibodies included the following: CD34, HLA-DR, CD117, CD13, CD14, CD33, CD11c, CD19, CD10, CD20, cCD3, TdT, CD7, cMPO, sIg, CD15, CD56,CD45. The intracytoplasmic markers such as cCD3, cMPO, TdT and cIg antigens were evaluated by fluorescent conjugated monoclonal antibodies after fixation and permeabilisation of leukemic cells using IntraprepTM solution.

The cases were classified into six main ALL immunophenotypes and eight AML immunophenotypes. A case was considered pro-B ALL if it expressed (CD19+, CD34-,HLADR+,TdT -,lack of surface immunoglobulin),Pre B-ALL expressed same positive markers as pro-B ALL and (CD34+,TdT+,CD10+/- CD19+),Common B-ALL expressed similar positive markers with a high positivity for (CD10 ,CD19+,CD34-/+,and cytoplasmic and surface Ig).

The T-cell ALL were considered when the markers for T-cell were positive i.e.,(cCD3+ ,CD7+CD34+ ,TdT+,CD4- ,CD8-) as precursor T-cell ALL) and T-cell ALL with (cCD3+,CD7+,TdT+). Myeloid cases expressed progenitor antigens (HLA-DR, CD7, CD34, CD117), as well as myeloid antigens (CD13, CD33, MPO), other myeloid lineage antigens (CD14, CD15) were found in more mature myeloid immunophenotypes.

Data Analysis: Data was analysed using SPSS version 15.0. Patterns of categorical data (lymphadenopathy, haepatomegaly, Clinical CNS disease, and splenomegaly) were described graphically inform of tables and graphs.

Analysis of Variance (ANOVA) was used to test the differences of hemoglobins, WBC, platelets count among the phenotypes. The association of immunophenotypes and the clinical pathological presentation was tested with Chi-Square test or Fishers exact test where numbers were below 5. A P-value of less than 0.05 was considered statistically significant.

RESULTS

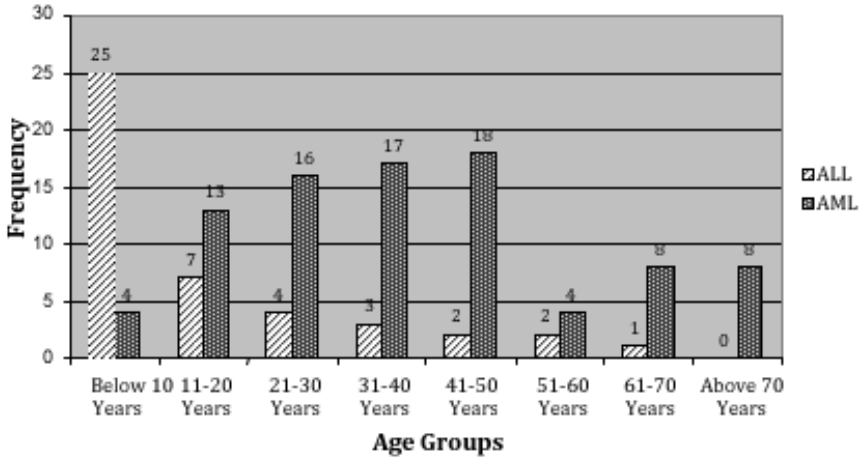
There were 138 cases of acute leukemia as diagnosed morphologically out of which 132 were subjected to immunophenotyping. Six of the cases were rejected since they did not meet the inclusion criteria as outlined and specimen recollection was not feasible. Out of the 132 analysable specimens, 42 patients (31.8%) were less than fifteen years of age and classified as children, while 90 were adults (68.2%). These also comprised of 67(50.8%) males and 65 (49.2%) females with M: F ratio of 1.03:1. The ages ranged from two years to 87 years with a median age of 29.5 years as seen in (Table 1).

Table 1
Demographic characteristics of patients

Age category	Male (%)	Female (%)	Total
Below 10 years	16 (55.2%)	13 (44.8%)	29
11-20 years	8 (40.0%)	12 (60.0%)	20
21-30 years	3 (15.0%)	17 (85.0%)	20
31-40 years	8 (40.0%)	12 (60.0%)	20
41-50 years	14 (70.0%)	6 (30.0%)	20
51-60 years	4 (66.7%)	2 (33.3%)	6
61-70 years	8 (88.9%)	1 (11.1%)	9
Above 70 years	6 (75.0%)	2 (25.0%)	8
Total	67 (50.8%)	65 (49.2%)	132

Eighty eight (67%) cases were acute myeloid leukemia and 42 (31.8%) were acute lymphoblastic leukemia. The peak age range for AML patients was 41-50 years. There was a notable rise of AML cases from the 6th decade. There were 42 cases of ALL and two cases of biphenotypic leukemia (BAL). ALL was the commonest form of acute leukemia in young children with the relative frequencies reducing with age as illustrated in the bar graph in (Figure 1).

Figure 1
Distribution of cases of Acute leukemia in different age categories (n=132)



The commonest AML in all patients was AML-M2 accounting for 26 (29.5%) with 19 (21.6%) cases seen in adults and 7(7.95%) in children. The median age of adults with acute myeloid leukemias was 39.5 years (range 16-87). The peak age range for adult patients with the AML-M2 immunophenotype was 35 to 45 years as shown.

Forty two cases were ALL and two cases were biphenotypic leukemia. Majority of ALL cases were seen in patients below 15 years of age and were of the B-cell phenotype 29 (69.04%) with the dominance of early pre-B phenotype at 62.07%, followed by Common B- ALL in 37.93% of patients, while the T- cell ALL was seen in only one child. T-cell ALL (3 cases) were seen in adults. Only two cases of biphenotypic leukemias were seen in this series and were found in adults.

cMPO was the highest expressed cytoplasmic marker at 85.2% ,CD 117, CD13 and CD33 were the most commonly expressed surface markers for myeloid leukemia with 70.5% (95% CI: 60.3-79.7), 81.8% (95% CI: 73.2-89.6) and 73.9 % (95% CI: 63.9-82.6) positivity respectively. HLADR and CD34 were also significantly expressed at 79.5% and 80.7% respectively. HLADR was negative in almost all the promyelocytic AML (aml-m3/v3) save for two cases which had dim positivity. CD7 (19.3%) and CD19 (19.7%) were the most common aberrantly expressed

lymphoid markers on the myeloid blasts. CD11c and CD14 were the most commonly expressed markers for defining AML-M4/M5 as illustrated in (Table 2).

Table 2
Distribution of antigens against AML immunophenotypes (n=88)

	AML M0 (n=18)	AML M1 (n=9)	AML M2 (n=26)	AML M3 (n=9)	AML M4 (n=17)	AML M5 (n=7)	AML M6 (n=0)	AML M7 (n=0)	AML 3V (n=2)	Total (n=88)
cCD3	3	-	2	-	-	1	-	-	1	7 (7.9%)
CD5	3	-	3	-	-	-	-	-	1	7 (7.9%)
CD7	3	2	5	4	2	1	-	-	-	17(19.3%)
CD10	2	-	3	2	-	-	-	-	-	7 (7.9%)
CD11c	6	1	9	2	15	4	-	-	-	37(42%)
CD13	11	9	23	5	15	7	-	-	2	72(81.8%)
CD14	4	1	7	3	14	4	-	-	-	33(37.5%)
CD15	-	-	1	1	1	5	-	-	-	8 (9%)
CD19	4	0	4	3	5	1	-	-	1	18(20.5%)
CD20	-	-	-	-	-	-	-	-	-	-
CD33	6	8	21	8	14	6	-	-	2	65(73.9%)
CD56	-	-	1	-	4	1	-	-	-	6 (6.8%)
CD117	14	6	22	6	11	2	-	-	1	62(70.5%)
TdT	-	-	-	-	-	-	-	-	-	-
HLADR	15	9	24	2	15	5	-	-	-	70(79.5%)
MPO	18	8	23	8	11	5	-	-	2	75(85.2%)
CD45	18	9	26	9	17	6	-	-	2	87(98.9%)
CD34	17	8	19	8	15	3	-	-	1	71(80.7%)
CD 61/41	-	-	-	-	-	-	-	-	-	-
CD235a	-	-	1	-	-	1	-	-	-	2 (2.3%)

CD19 was the most expressed marker for B lymphoid leukemia (81.8% positivity). Other markers that were highly expressed in the B ALL phenotypes were HLADR, TDT, and CD10 with 93.2%, 77.5% and 77.3 % positivity. Overall, CD10 was expressed in 77.3% of cases and especially in B-ALL cases. CD7 was universally expressed in the T-ALL cases as well as cCD3 at 20.3 %;(95%CI: 9.3-34.6).
CD13 and CD 33 were both aberrantly expressed in 20.5 % of the ALL cases as shown in (Table 3).

Table 3
Distribution of antigens against ALL immunophenotypes (n=44).

	PRO B ALL (n=2)	PRE B ALL (n=21)	COMMON B ALL (n=15)	PRE T ALL (n=2)	T CELL ALL (n=2)	Biphenotypic (n=2)	Total (n=44)
cCD3	1	3	1	2	1	1	9 (20.3%)
CD5	0	4	2	2	2	2	12 (27.3%)
CD7	0	8	0	2	2	2	14 (31.8%)
CD10	0	17	14	0	1	2	34 (77.3%)
CD11c	0	1	0	0	0	0	1 (2.3%)
CD13	0	3	4	1	0	1	9 (20.5%)
CD14	0	1	1	0	0	1	3 (6.8%)
CD15	0	0	2	0	0	1	3 (6.8%)
CD19	1	20	15	0	0	0	36(81.8%)
CD20	0	5	5	0	0	1	11(25%)
CD33	0	4	3	1	0	1	9 (20.5%)
CD56	0	0	0	0	0	0	0 (0%)
CD117	0	0	0	0	0	1	1 (2.3%)
TdT	1	17	13	1	2	0	34(77.5%)
HLADR	1	20	15	2	1	2	41(93.2%)
cMPO	0	0	0	0	0	1	1 (2.3%)
CD45	2	21	15	2	2	2	44(100%)
CD34	1	11	4	1	0	2	19(43.2%)

Majority of the patients with acute leukemia presented with anemia as evident in the low hemoglobin median levels, 7.45g/dl (range 2-15g/dl); the median WBC count of 27 X 109/L (range 0.1-587) and platelets level of 55.1 X 109/l (range 4-462). The lowest median hemoglobin level was noted in AML-M2, while the lowest median platelets count was evident in AML-M3.
AML-M3 showed the lowest median for WBC counts while the highest median was seen in AML-M2 as illustrated in (Table 4).

Table 4
The median values and ranges of WBC, Hb, platelet counts among the different AML immunophenotypes.

	AML M0 Median (range)	AML M1 Median (range)	AML M2 Median (range)	AML M3 Median (range)	AM3V Median (range)	AML M4 Median (range)	AML M5 Median (range)	P value
White Blood cells (WBC)	18.85 (3 to 171)	23.1 (5 to 82)	23.5 (1 to 226)	10.2 (0.1 to 191)	18.5 (18 to 19)	27.0 (2 to 192)	44 (20 to 77)	0.770
Hemoglobin (Hb)	8.05 (3 to 12)	6.8 (5 to 15)	6.55 (4 to 12)	8.5 (2 to 11)	6.3 (3 to 9)	9.5 (6 to 12)	7.3 (4 to 11)	0.135
Platelets	35 (7 to 460)	39 (15 to 104)	43.4 (4 to 210)	36.4 (11 to 80)	108.3 (14 to 203)	77.0 (51 to 298)	58 (20 to 124)	0.041

Note: The Kruskal Wallis test was applied to establish the differences in median levels of WBC, Hb and platelet count across all the AML immunophenotypes).

Thrombocytopenia was associated more with AML-M0 and AML-M3. The highest median WBC counts were seen in AML-M5 and AML-M4 while the lowest counts were seen in AML-M3.There was a significant difference in the medians of platelets counts across the immunophenotypes (P=0.041). In AML, the lowest hemoglobin was seen in patients with AML-M3V at 6.3 g/dl (range 3 to 9 g/dl) as shown. The lowest median hemoglobin was seen in Pre B common ALL and was 5.8 g/dl (range 3 to 10 g/dl). Thrombocytopenia was more associated with Pro B-ALL with a median platelets level of 18(range15 to 21 × 10^9/l). The highest median WBC counts were seen amongst the Pre T-cell ALL patients 44.2 (range 2 to 86 × 10^9/l). There was no significant difference in the clinical and hematological parameters across various ALL immunophenotypes.

DISCUSSION

This work gives the largest series of acute leukemias in Kenya providing data on immunophenotypic distribution of acute leukemias in adults and children. The five color flowcytometric immunophenotyping in this study pioneered use of the technique for acute leukemia in Kenya. The study comprised of 67(50.8%) males and 65 (49.2%) females with M: F ratio of 1.03:1. Acute leukemias have been shown to occur at equal frequencies in males and females in Sub-Saharan Africa probably indicating the etiological causes to be associated with common environmental factors (7-9). The age ranged from two years to 87 years with a median age of 29.5 years.

The commonest AML immunophenotype in all patients was AML-M2 accounting for 26 (29.5%) cases with 19 (21.5%) cases seen in adults and 7(7.95%) in children which is slightly higher than the frequency of 27-29% reported in the literature in all age groups. Advani et al has also observed a higher frequency of AML-M2 in both pediatric and adult population in India(15). The proportion of AML-M4 in this study was 19.3%. This falls in the same frequency as 16-25% reported in the literature. The incidence of AML-M3 in this study was 12.5% and was slightly higher than reported frequency in the literature at 5-10%. An immunoprofile of CD34-, HLADR-, CD14- and CD33+ strongly favored AML-M3, as was seen in our patients. AML is a genetically and phenotypically heterogenous disease and these findings could just be due to the different spectrum of mutations that has occurred in this population of patients and the effect of other factors involved in leukemogenesis. Future studies involving cytogenetics and molecular analysis to determine if any differences at the molecular levels in our patients exist should be carried out. There were no cases of AML-M6 yet a frequency of 3-5% has been reported in the literature. There was no case of AML-M7 in this study. Generally, this would imply that the AML-M6 and AML-M7 are of low frequency and the study would need a higher number of patients to document them even if they are of low frequency. Majority of cases were B-ALL at 29(96.6%) with the dominance of early pre-B phenotype in 62.07%, followed by Common B- ALL in 37.93%. Majority of ALL cases were seen in children<15 years and were of the B-cell phenotype 29 (69.04%), while the T- cell ALL was seen in only one child.

There was a close association between T-cell phenotype with older age and higher leukocyte count, however, the cases were very few (four cases) to make any statistical inferences. The marked preponderance of childhood B-ALL in our series is, at least partly, attributable to the age distribution of the population and the general view that ALL is a disease of children and so these results were expected.

The majority of patients presented with anaemia in this study with the median hemoglobin of 7.45g/dl (range 2-15 g/dl). Bleeding was most commonly seen in the promyelocytic and the monocytic leukemia. The median platelet count level in this study was 55.1 (range 4-462 × 109/L). This indicates generally that patients in this series had low platelets levels hence the high rate of anaemia and bleeding observed. Although lymphadenopathy is not often seen in AML this was present in 9% of the cases in this study. It is possible that patients could have had coexisting viral and mycobacterial infections presenting with adenopathy and a high index of suspicion for AML was needed. However, as expected there were more cases of ALL presenting with adenopathy at 75% compared to those seen in AML.The phenotype associated with most cases of adenopathy was the Pre B-cell ALL but this was not statistically significant (P=0.193). Dakka et al has reported the presence of splenomegaly in >60% of his cases with ALL(11). In this study, however, splenomegaly was seen in 34% of patients with AML, a rate lower than in some studies in literature. In ALL, splenomegaly was noted in 12 (27.7%) of cases (p = 0.381).The low frequency in this study could be due to the fact that none of the patients were subjected to radiological investigation for splenomegaly or hepatomegaly.

In conclusion, the relative frequencies of the immunological subtypes in this study are similar to that reported by most researchers in the world, except for the low frequency of T-ALL, AML-M6 and AML-M7. This study utilized flow cytometry to make a diagnosis of acute leukemia, a technology that has not been utilized here before. Immunophenotyping of acute leukemia is beneficial in accurate diagnosis of these malignancies in this setup and routine use of immunological typing would be recommended to benefit both the patients and clinicians.

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Authors:

Kabera B: Contributed to research design, acquisition, analysis, interpretation of data and wrote the paper.

Riyat M: Contributed to research design and gave expert advice in data interpretation.

Macharia WM: Contributed to research design, and supervised writing the manuscript draft.

Pamnani R: Contributed to research design, and interpretation of data.

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