ACUTE LEUKAEMIA IMMUNOPHENOTYPING BY IMMUNOHISTOCHEMISTRY IN BONE MARROW TREPINE BIOPSY SECTIONS
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ABSTRACT

BACKGROUND
Classification of acute leukaemia is gaining importance as the treatment continues to evolve for specific genetic and pathogenetic subgroups. AIMS: A well organised panel of antibodies can be used for immunophenotyping of acute leukaemia by Immunohistochemistry (IHC) when only a Bone marrow trephine biopsy (BMTB) specimen is available for study. In other words when Flow cytometry (FCM) cannot be performed because of lack of availability of technology or inadequacy of bone marrow aspiration (BMA).

MATERIALS AND METHODS
The bone marrow biopsy taken from these patients were processed to obtain formalin-fixed paraffin-embedded tissue blocks. The sections taken were then processed for heat induced antigen retrieval and stained with primary antibodies of the IHC panel. The IHC panel consisted of CD45, CD34, CD117, TdT, PAX5, CD20, CD10, CD3, MPO and CD68.

RESULTS
A total of 115 cases were studied out of which 48 (47.9%) cases of AML and 60 (52.1) cases of ALL were identified. The specimens were from 53 (46.1%) females and 62 (53.9%) males, with average age of 24.6 years (ranging from 3 months to 80 years).

CONCLUSION
Further categorisation was done according to the differential expression of the markers in the panel of antibodies which finally resulted in the following subtypes, AML with Minimal Differentiation (AML MD – 6 (5.2%)), AML without maturation and with maturation (AML Myeloid - 19 (16.5%)), Acute Promyelocytic Leukaemia (APL - 20 (17.4%)), Acute Myelomonocytic Leukaemia (AML MM - 2 (1.7%)), Acute Monoblastic/ Monocytic Leukaemia (AML M - 1 (0.9%)), Acute Leukaemia Others (AL Oth - 7 (6.1%)), B cell Acute Lymphoblastic Leukaemia (B ALL - 58 (50.5%)) and T cell Acute Lymphoblastic Leukaemia (T ALL - 2 (1.7%)).

KEYWORDS
Acute, Leukaemia, Myeloid, Lymphoblastic, Immunohistochemistry, Immunophenotyping, Bone, Marrow, Biopsy.

HOW TO CITE THIS ARTICLE: Mishra DP, Behera B, John KE. Acute leukaemia immunophenotyping by immunohistochemistry in bone marrow trephine biopsy sections. J. Evid. Based Med. Healthc. 2018; 5(6), 517-521. DOI: 10.18410/jebmh/2018/105

BACKGROUND
Acute leukaemias (AL) comprises of a group of disorders which differ in aetiology, pathogenesis, morphology, course and prognosis, which leads to death if left untreated. They result from neoplastic proliferations of hematopoietic cells due to imbalance in stem cell proliferation, lineage commitment and differentiation in the hematopoietic system, when normal hematopoietic stem or progenitor cell sequentially acquires mutations which confer clonal growth advantage. Leukaemia constitutes about 2.5% of incidence of all the cancers worldwide, with a 5-year prevalence of 1.5% and a mortality of 3.2%. In India it constitutes 3.2% of incidence of all the cancers, mortality is 3.9% with a 5-year prevalence of 1.3%. The incidence, mortality and 5-year prevalence of leukaemia is more in males than in females both worldwide and in India. Classification and subtyping of acute leukaemias are important to assess the prognosis and institute a specific chemotherapy.

Two classification schemes are presently in use. These are French, American and British classification (FAB).1 and World health organization classification (WHO).2,3 A classification system must have relevance and importance in the clinical management, should be easily applicable and enable uniformity in reporting. Many centres with limited resources still follow the FAB classification system, because of the unavailability of flow cytometry (FCM) and molecular diagnostics. Lack of these resources limits the use of WHO classification system. Since the present WHO system should be followed for the best clinical practice, it is often not possible in the two-third of the world due to constraints in availability of resources.4 Diagnosis and classification of

Financial or Other, Competing Interest: None.
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DOI: 10.18410/jebmh/2018/105
hematopoietic neoplasms now are based on morphologic features, cytochemical studies, immunophenotyping, cytogentic studies and molecular genetics. A well organised panel of antibodies can be used for immunohistochemical (IHC) typing of acute leukaemia when only a bone marrow trephine biopsy (BMTB) specimen is available for study. The IHC on paraffin blocks of bone marrow is used when FCM cannot be performed because of lack of availability of technology or bone marrow aspiration (BMA) is not available or inadequate because of dry tap due to bone marrow fibrosis, hypocellular marrow aspirate. Immunophenotyping is now standard for acute leukaemias and it is most helpful in accurately diagnose acute lymphoblastic leukaemias (ALL) and some acute myeloid leukaemias (AML). Immunophenotyping results should always be interpreted along with morphology and relevant clinical and laboratory data.5-9

Aims and Objectives-
We conducted a study at the Department of Pathology, MKCG Medical College, Berhampur, Odisha, on 115 cases of acute leukaemia which consisted of both paediatric and adult age groups.

MATERIALS AND METHODS
BMTB specimens from 115 adult and paediatric patients with acute leukaemia, diagnosed on examination of bone marrow aspirate smears and cytochemical tests were obtained. All were newly diagnosed cases of acute leukaemia. The 115 cases studied included all age groups. Hammersmith protocol or Intermediate turnaround time protocol was used in BMTB fixation and decalcification.10 These tissues were then processed to obtain formalin-fixed paraffin-embedded tissue blocks. Representative sections were chosen for immunostaining after review of tissue sections that confirmed the diagnosis. Sections were cut at 2 to 3 μm thickness, mounted on amino pro-ethoxy silane (APES) coated glass slides, and allowed to dry at room temperature overnight. Immunohistochemical staining was done after the heat-induced epitope retrieval. All the primary antigens used were DAKO Flex, ready to use antigens. The antigens used included hematopoietic lineage marker CD45 (2B11+PD7/26), markers of immaturity CD34 (QB End 10), myeloid lineage marker CD117 (c-kit) and lymphoid lineage marker TdT (EP266). T cell lineage marker CD3 (Anti Human-CD3) and B cell lineage marker CD10 (56C6) and Pax5 (DAK-Pax 5). Other markers used were CD20 (Clone L26), CD68 (KP1) for monocytic lineage and MPO (Anti Human-Myeloperoxidase) for myeloperoxidase enzyme. Ready to use, Dako EnVisionTM Flex/HRP 5M802 was used as secondary antibody. Dako EnVisionTM Flex DAB+ Chromogen was used.10-14

Staining for each individual antigen was considered positive if more than 10% of leukemic cells were stained. Blast cells with typical morphologic features showing large central nuclei, dispersed chromatin, and prominent nucleoli were identified. The percentages of positive blast cells were based on a 200-cell differential count and were examined under oil immersion. Positive controls were used for each antibody. The results of immunohistochemistry analysed along with Wright-Giemsa-stained smears of peripheral blood and/or bone marrow aspirates and cytochemistry for MPO enzyme.15,16

RESULTS
A total of 115 cases were studied out of which 48 (47.9%) cases of AML and 60 (52.1) cases of ALL were identified. The specimens were from 53 (46.1%) females and 62 (53.9%) males, with average age of 24.6 years (ranging from 3 months to 80 years).

All the blast cells of ALL (60/60 – 100%) and AML (48/48 – 100%) showed diffuse weak positivity by CD45 the marker for hematopoietic lineage.

Next group comprised of maturation specific markers such as CD34, CD117 and TdT. CD34 was positive in all cases of ALL, both T ALL (2/2 -100%) and B ALL (58/58 – 100%). But in AML MD (3/6) and AML Myeloid (12/19) only few cases were positive. It was negative in all cases of APL (0/20). It was 100% positive in all cases of AML MM (2/2) and AML M (1/1). CD117 found to be more sensitive than CD34 in AMLs. It was 100% positive in AML MD (6/6), AML MM (2/2) and AML M (1/1). Only some cases were positive for CD117 in AML Myeloid (10/19) and APL (12/20). It was negative in all cases of ALL, both B ALL (58/58) and T ALL (2/2). TdT was 100% positive in all cases of ALL, i.e. T ALL (2/2) and B ALL (58/58). But it was negative in most of the cases of AML, like AML Myeloid (0/19), APL (0/20), AML MM (0/2), and AML M (0/1), except AML MD (6/6) where all cases were positive.

We used lineage specific markers such as PAX5, CD20, CD10, CD3, MPO and CD68. The markers for ALL are PAX5, CD20, CD10 and CD3. The AML markers are MPO and CD68. PAX5 which is a lineage specific marker for B lymphoblasts were typically positive in B ALL (58/58 – 100%). The T ALLs (0/2) and all the cases of AML (0/48) were negative for PAX5. CD20 is also a B lineage marker but less sensitive than PAX for B ALL (33/58) cases. T ALL (0/2) cases and AMLs (0/48) were negative for CD20. CD10 was also positive in B ALL (55/58), with absent expression in T ALL (0/2) and all AMLs (0/48). The T lineage marker for lymphoblasts was CD3. CD3 was positive only in T ALL (2/2) and was negative in B ALL (0/58) and AML (0/48). Myeloid lineage markers used were MPO and CD68. The B ALL and T ALL were negative for both MPO and CD68; it was only expressed in the myeloid series. MPO was found to be variably positive in AMLs. MPO was found to be positive in AML Myeloid (19/19), AML MM (2/2) and APL (20/20) with strong positivity in APL. It was negative in AML MD (0/6) and AML M (0/1). MPO was found to be positive in AML Myeloid (19/19), AML MM (2/2) and APL (20/20) with strong positivity in APL, and CD68 which is a monocytic marker was positive in AML MM (2/2) and AML M (1/1).

There is third group of cases with miscellaneous IHC results. This may include cases of undifferentiated leukaemia, biphenotypic leukaemia, mixed phenotypic acute leukaemia, pure erythroid leukaemia, acute
megakaryoblastic leukaemia and acute basophilic leukaemia. Their IHC phenotypes were as follows. All were weakly positive for CD45 (7/7), CD34 (2/7), CD117 (2/7), TdT (1/7), PAX5 (0/7), CD20 (1/7), CD10 (1/7), CD3 (0/7), MPO (0/7) and CD68 (1/7). The antigen profile in this study is given in Table-1.

Evaluation of lymphadenopathy, organomegaly, PS studies (PS), complete blood count (CBC), BMA cytology (BMA), cytochemistry (MPO), BMTB (BMTB) and IHC allowed the subtyping of acute leukaemias into the following groups as given in Table-2 and Figure-1. AML with Minimal differentiation (AML MD – 6 (5.2%)), AML without maturation and with maturation (AML Myeloid - 19 (16.5%)), Acute Promyelocytic Leukaemia (APL - 20 (17.4%)), Acute Myelomonocytic Leukaemia (AML MM - 2 (1.7%)), Acute Monoblastic/ Monocytic leukaemia (AML M - 1 (0.9%)), Acute Leukaemia Others (AL Oth - 7 (6.1%)), B cell Acute Lymphoblastic Leukaemia (B ALL - 58 (50.5%)) and T cell Acute Lymphoblastic Leukaemia (T ALL - 2 (1.7%)).

**AML MD-AML with Minimal differentiation, †AML Myeloid-AML without maturation and with maturation, ‡APL-Acute Promyelocytic Leukaemia, §AML MM-Acute Myelomonocytic leukaemia, ¶AML M-Acute Monoblastic/ Monocytic leukaemia, **B ALL-B cell Acute Lymphoblastic Leukaemia and ††T ALL-T cell Acute Lymphoblastic Leukaemia, ‡‡AL Oth-Acute Leukaemia Others.

### Table 1. Phenotypic Profile of Acute Leukaemia in the Present Study

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<th>Total No (%)</th>
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### Table 2. Distribution of Acute Leukaemia Subtypes

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DISCUSSION

A number of studies,1,17-21 have been done previously to assess the feasibility of immunophenotyping by immunohistochemistry on paraffin embedded bone marrow trephine biopsy sections. Many of them compared the immunophenotyping by FCM with immunohistochemistry. The immunohistochemistry has at least taken the back stage in immunophenotyping of acute leukaemia because of the advances in flow cytometry and molecular and cytogenetics. But immunohistochemistry still holds its importance when institutions have limited machinery and funds, because it can utilize the basic histotechnique machinery already available for immunophenotyping with the addition of several markers.

Even though it is not possible to definitely classify AML, it is possible to differentiate it into myeloid, monocytic, erythroid or megakaryocytic lineage. The panel of antibodies used consisted of CD45, CD117, CD34, TdT, CD20, PAX5, CD10, CD3, MPO and CD68. A combination of clinical evaluation, CBC, PS studies, BMA, cytochemistry, and additional BMTB and IHC provides a more efficient approach to acute leukaemia diagnosis because we could go a step further in the diagnosis of acute leukaemia by subtyping it, and not merely dividing it into ALL or AML. And few cases of undifferentiated leukaemia could also be further sub typed according to IHC results. Out of the total 115(100%) cases which were diagnosed on the basis of BMA and cytochemistry were AML – 47 (40.9%), ALL – 57 (49.6) and undifferentiated Acute Leukaemia – 11 (9.5%). IHC on BMTB paraffin section subtyped this further. The AML cases were further subtyped into AML with minimal differentiation – 3 (2.6%) (Image-1), AML with and without maturation – 19 (16.5%), Acute promyelocytic leukaemia – 20 (17.4%), Acute myelomonocytic leukaemia– 2 (1.7%), Acute monocytic/monoblastic leukaemia– 1 (0.9%), and other Acute leukaemias – 2 (1.7%). The ALL cases were further subtyped into B ALL – 56 (48.7%) and T ALL 1 (0.9%). Undifferentiated Acute leukaemias were subtyped into AML with minimal differentiation – 3 (2.6%), B ALL – 2 (1.9%) (Image-2), T ALL 1 (0.9%) and other leukaemias – 5 (4.3%).

Acute Leukaemia Others (AL Oth - 7 (6.1%)) – The other leukaemias of NOS type like Pure erythroid leukaemia, Acute megakaryoblastic leukaemia, Acute basophilic leukaemia and Acute leukaemias of ambiguous lineage were considered under Acute Leukaemia Others because of the inconsistency of immunohistochemical markers and lack of specific markers for such classification. The patients belonged to different age group from 2 years to 68 years. Physical examination did not show any lymphadenopathy or organomegaly in adult patients, while paediatric patients showed organomegaly and/or lymphadenopathy. Anaemia and thrombocytopenia was present in all the cases, while TLC ranged from 2,810 cells/cmm to 1.2 Lakh cells/cmm, with an average of 23,910 cells/cmm. PS and BMA also showed the presence of blasts mostly of undifferentiated morphology constituting >20% of marrow nucleated cell population. So, these were classified under the heading of leukaemia. BMTB showed hypercellular marrow with presence of undifferentiated blasts. IHC was done on BMTB. CD45 showed weak positive staining (7/7) confirming their hematopoietic origin, with variable positivity for CD117 (3/7), CD34 (4/7) and TdT (3/7), which further confirms immaturity in lymphoblastic and/or myeloid lineage. The markers TdT, CD20, CD10, CD3, CD68 and MPO were negative. The additional markers that can be used for further subtyping these leukaemias are HgA/GPA for Pure erythroid leukaemia, CD41/CD61/FVIII for Acute megakaryoblastic leukaemia. Acute leukaemia of ambiguous lineage is better confirmed and categorised by molecular genetic analysis. Rare acute undifferentiated leukaemias does not express any markers specific for lymphoid or myeloid lineage, display only positive CD34 and weak CD45. Mixed phenotype AL shows lymphoid and myeloid lineage markers in single or two separate blast populations. Diagnosing this form of AL by IHC alone is difficult (certain markers such as CD19, CD13, and CD33 are not routinely available by IHC). Sensitivity and specificity of antibodies are more for monoclonal antibodies used by FCM. Ancillary testing such as cytogenetics and molecular genetics are important in these situations.

Diagnosis and classification of hematopoietic neoplasms now are based on morphologic features, cytochemical studies, immunophenotyping, cytogenetic studies and molecular genetics. In spite of these advances in ancillary studies, morphologic evaluation plays a major role in the pathological diagnosis of haematological malignancies. As the number of molecular genetic tests increases in this field, morphological evaluation still plays a major role in cost-effective tissue processing and deciding ancillary tests. In this context our study further underlines the role of IHC on bone marrow paraffin sections in the diagnosis of acute leukaemias as a cost effective and practical method. Moreover, the combination of clinical evaluation, CBC, PS studies, BMA, cytochemistry, BMTB and IHC provides a more efficient approach to acute leukaemia diagnosis. Many studies were conducted in this regard but the insurgence of FCM, molecular genetics and cytogenetics, forced IHC to take a back stage. But IHC still holds value in places with economic and technological constraints. Keeping this in mind we undertook this study of IHC as a cost effective and practical method to subtype acute leukaemia.

CONCLUSION

This study indicates that immunohistochemistry adds value to the diagnosis of acute leukaemia and its subtyping by confirming the lineage when other methods of immunophenotyping like FCM are not available. The
selection of antibody panel is always done based on peripheral smear and bone marrow aspiration morphology and cytochemistry. This antibody panel used helps to determine lineage associated with leukemia and this will serve as a meaningful pointer towards the direction of treatment or further investigations.

REFERENCES


