RAPID AND SAFE ALTERNATIVE FOR CONVENTIONAL MYELOPEROXIDASE STAIN- A STUDY

Anita Choudhury[1], Manoj Kumar Patro[2], Dinabandhu Bishoi[3], Debi Prasad Mishra[4]

[1]Assistant Professor, Department of Pathology, MKCG Medical College and Hospital, Brahmapur, Odisha.
[2]Associate Professor, Department of Pathology, Bhima Bhoi Medical College and Hospital, Balangir, Odisha.
[3]Laboratory Technician, Balaji Diagnostics, Brahmapur, Odisha.
[4]Professor and HOD, Department of Pathology, MKCG Medical College and Hospital, Brahmapur, Odisha.

ABSTRACT

BACKGROUND

Myeloperoxidase (MPO) staining is a cytochemical stain was the first step in morphological characterization of two basic types of Acute leukemia.[1,2] Until recently when the procedure was eliminated from the diagnostic protocol because of the proven carcinogenic potentiality of Benzidine. In advanced laboratories the procedure is now replaced by immunocytochemical demonstration of MPO. The main disadvantage of immunocytochemical stain is cost associated with the procedure, hence it is not possible for use in developing and under-developed countries. Besides the carcinogenic threat, the routine MPO staining procedure is also time consuming.

The present study was aimed at, evaluating the new method that, successfully removes the use of the benzidine by DAB which has uncertain carcinogenic potentiality.

MATERIALS AND METHODS

130 blood smears both peripheral blood smears and bone marrow aspirate smears were stained by the routine benzidine method and new DAB method and evaluated for stain intensity, cell morphology and background by two pathologists and each parameter was graded from 1 to 3. The total score for each case was calculated and compared using statistical formulae.

RESULTS

The new test has the additional advantage of being simple and less time consuming with excellent staining properties.

CONCLUSION

The newer technique using DAB was found to be a better procedure with good staining qualities, easy to perform as the liquid solution is stable and it is not an established carcinogen.

KEYWORDS

Cytochemistry, Myeloperoxidase (MPO), DAB, Safe and rapid method.

HOW TO CITE THIS ARTICLE: Choudhury A, Patro MK, Bishoi D, et al. Rapid and safe alternative for conventional myeloperoxidase stain- a study. J. Evid. Based Med. Healthc. 2018; 5(49), 3392-3396. DOI: 10.18410/jebmh/2018/690

BACKGROUND

Leukaemia is one of the malignancies prevalent all over the world at all ages. Cytochemical stains play a valuable role in lineage identification to myeloid and lymphoid lineages which is pivotal for diagnosis, management and prognosis.[3] Many stains are used in practice such as Myeloperoxidase (MPO), Periodic acid Schiff (PAS) and non-specific esterase. Prior to availability of immunological markers these cytochemical stains were used to classify leukaemia, FAB Classification 1976. Myeloperoxidase is a cytochemical staining for polymorphonucleocytes in blood or bone marrow films. The staining characteristics of polymorphonucleocytes the are used to distinguish acute myeloid leukaemia from other type of leukaemia.[4]

Financial or Other, Competing Interest: None.
Submission 09-11-2018, Peer Review 17-11-2018,
Acceptance 26-11-2018, Published 01-12-2018.
Corresponding Author:
Dr. Manoj Kumar Patro,
Associate Professor, Department of Pathology,
Bhima Bhoi Medical College and Hospital,
Balangir, Odisha.
E-mail: manojkpatro@gmail.com
DOI: 10.18410/jebmh/2018/690

Among all the cytochemical stains MPO plays a vital role in determining the lineage i.e. whether the differentiation is towards the myeloid lineage or not. Though Sudan Black B (SBB) has been used for the purpose it has its limitations. Benzidine used for routine MPO staining has been proved as a carcinogenic[5,6] and hence the test is banned globally and is replaced by immunocytochemical demonstration of the same in centres where facilities are available. But in developing countries like India the immunocytochemical demonstration of MPO is limited to advanced centres. This creates a need for a safe alternative for the routine MPO stain.

Besides the carcinogenicity of Benzidine the other demerits with the usual staining procedure were enzyme inhibition, cellular distortion, time-consuming, etc. In an attempt to replace benzidine some authors used benzidine dihydrochloride, but it was found that this compound of benzidine is also carcinogenic.[7] Some authors proposed the use of Diaminobenzidine (DAB) as a substitute of benzidine in MPO stain.

Aims and Objectives

The present study was undertaken to compare this new rapid method of detecting MPO activity in blood and bone

marrow smears using DAB with the routine method using benzidine.

MATERIALS AND METHODS

This study was conducted at MKCG Medical College and Hospital, Brahmapur during the period March 2017 to Feb 2018. Peripheral blood smear and bone marrow aspiration smears of both leukemic and nonleukemic individuals were included in the study. In each case two smears were prepared air dried and stained with routine MPO stain and the new technique using DAB. Both the stained smears were evaluated by two pathologists who were blinded for the procedure adopted for staining to avoid bias. The smears were evaluated for (a) intensity of staining, (b) Cellular morphology and (c) cleanliness of the background.

Principle of DAB Method- MPO present in the granules of myeloid lineage oxidizes DAB in presence of hydrogen peroxide resulting in change of colour of the DAB from blue to brown.

Reagents Required

- **Fixative**
  95% ethanol: 90 mL
  40% formaldehyde: 10 mL
- **Diamino Benzidine Solution**
  3.3 Diaminobenzidine - 0.05 Gm
  Sodium bicarbonate – 0.1 Gm
  Distilled water - 100 mL
- **3% hydrogen peroxide**.

Procedure

- Fix the dried peripheral smears of blood or bone marrow smear in fixative for 1 minute.
- Rinse the fixed smears in the distilled water.
- Add the mixture of reagent (1 ml of DAB solution + 0.02 mL of H_2O_2) on the slide to completely cover the smear.
- Incubate at 37^0C for 5 minutes.
- Rinse in water for 30 seconds.
- Counterstain with haematoxylin for 4 minutes.

Observations

- Positivity was demonstrated by the presence of brownish black granules in the cells.
- Neutrophils and Eosinophils in all cases stained positive and in eosinophils the staining was darker.
- Myeloblasts were variably positive.
- Lymphoblasts and Monocytoid cells were negative.

RESULTS

Each case was assessed by observing the three parameters, staining characteristics, cellular morphology and background cleanliness and for each parameter a score of 1 to 3 was awarded i.e. for best staining it was 3 and poor or no staining it was 1, well preserved cell morphology after staining it was 3 and distorted morphology it was 1 and similarly the background was completely clean it was 3 and dirty background it was 1.

Sl. No.	DAB				MPO			
	Stain Intensity	Cell Morphology	Background	Total Score	Stain Intensity	Cell Morphology	Background	Total Score
1	2	3	3	**8**	2	2	2	**6**
2	3	3	2	**8**	3	2	1	**6**
3	2	2	1	**5**	3	2	1	**6**
4	3	2	3	**8**	2	2	1	**5**
5	3	2	3	**8**	3	2	1	**6**
6	3	3	2	**8**	2	2	2	**6**
7	3	3	2	**8**	2	1	1	**4**
8	2	2	2	**6**	2	1	1	**4**
9	3	2	2	**7**	2	2	1	**5**
10	2	2	1	**5**	2	1	1	**4**
11	2	2	2	**6**	1	2	2	**5**
12	3	2	2	**7**	2	2	2	**6**
13	1	3	3	**7**	3	2	1	**6**
14	3	2	3	**8**	2	2	2	**6**
15	2	2	1	**5**	2	2	1	**5**
16	2	2	2	**6**	2	2	1	**5**
17	1	3	3	**7**	2	1	1	**4**
18	3	2	3	**8**	2	1	1	**4**
19	3	3	3	**9**	2	2	1	**5**
20	2	2	1	**5**	2	2	1	**5**
21	3	3	3	**9**	1	2	2	**5**
22	3	2	3	**8**	3	2	1	**6**
23	3	3	3	**9**	3	2	1	**6**

24	3	2	2	**7**	3	2	1	**6**
25	1	3	3	**7**	2	2	2	**6**
26	1	3	3	**7**	2	2	1	**5**
27	3	2	3	**8**	2	2	2	**6**
28	3	2	2	**7**	2	1	1	**4**
29	3	3	3	**9**	2	2	1	**5**
30	3	2	3	**8**	2	1	1	**4**
31	1	3	3	**7**	3	2	1	**6**
32	3	3	2	**8**	2	2	1	**5**
33	3	3	3	**9**	2	2	2	**6**
34	3	3	3	**9**	2	2	1	**5**
35	3	3	2	**8**	2	1	1	**4**
36	2	3	3	**8**	1	2	2	**5**
37	3	2	3	**8**	2	2	2	**6**
38	3	3	3	**9**	2	1	1	**4**
39	3	2	3	**8**	2	2	1	**5**
40	2	2	1	**5**	2	2	1	**5**
41	2	2	2	**6**	2	1	1	**4**
42	3	3	3	**9**	2	2	2	**6**
43	3	3	3	**9**	3	2	1	**6**
44	3	2	2	**7**	2	2	1	**5**
45	3	3	3	**9**	3	2	1	**6**
46	3	2	3	**8**	2	2	1	**5**
47	3	3	3	**9**	2	1	1	**4**
48	3	2	2	**7**	3	2	1	**6**
49	2	2	1	**5**	2	1	1	**4**
50	3	3	2	**8**	2	2	1	**5**
51	3	2	3	**8**	3	2	1	**6**
52	2	2	2	**6**	2	2	1	**5**
53	2	3	3	**8**	2	1	1	**4**
54	3	2	3	**8**	1	2	2	**5**
55	3	3	2	**8**	2	1	1	**4**
56	2	2	2	**6**	1	2	2	**5**
57	3	3	3	**9**	2	2	1	**5**
58	2	2	2	**6**	2	2	1	**5**
59	2	2	2	**6**	2	1	1	**4**
60	2	2	1	**5**	3	2	1	**6**
61	1	3	3	**7**	2	2	1	**5**
62	1	3	3	**7**	2	2	2	**6**
63	3	2	3	**8**	2	2	2	**6**
64	1	3	3	**7**	2	2	1	**5**
65	3	2	3	**8**	3	2	1	**6**
66	3	2	3	**8**	2	1	1	**4**
67	2	3	3	**8**	2	2	2	**6**
68	3	3	3	**9**	1	2	2	**5**
69	3	3	3	**9**	2	2	2	**6**
70	1	3	3	**7**	2	2	2	**6**
71	2	3	3	**8**	2	1	1	**4**
72	2	3	3	**8**	2	2	1	**5**
73	3	3	3	**9**	2	1	1	**4**
74	2	3	3	**8**	2	2	2	**6**
75	3	2	3	**8**	2	2	1	**5**
76	3	3	2	**8**	2	1	1	**4**
77	3	2	3	**8**	2	1	1	**4**
78	3	3	3	**9**	2	2	2	**6**
79	3	2	2	**7**	2	2	2	**6**

80	2	3	3	**8**	2	2	1	**5**
81	2	2	1	**5**	2	2	1	**5**
82	2	2	2	**6**	3	2	1	**6**
83	2	2	1	**5**	2	2	2	**6**
84	3	3	3	**9**	3	2	1	**6**
85	1	3	3	**7**	2	2	1	**5**
86	1	3	3	**7**	2	2	1	**5**
87	3	2	2	**7**	3	2	1	**6**
88	2	3	3	**8**	1	2	2	**5**
89	3	2	3	**8**	1	2	2	**5**
90	3	3	3	**9**	2	2	2	**6**
91	3	3	3	**9**	2	1	1	**4**
92	3	3	2	**8**	2	1	1	**4**
93	3	2	3	**8**	2	2	1	**5**
94	3	2	3	**8**	2	2	2	**6**
95	3	3	3	**9**	2	2	1	**5**
96	1	3	3	**7**	3	2	1	**6**
97	3	2	3	**8**	2	2	2	**6**
98	2	3	3	**8**	2	2	1	**5**
99	3	2	3	**8**	3	2	1	**6**
100	3	3	3	**9**	2	2	1	**5**
101	3	2	3	**8**	2	2	1	**5**
102	3	2	2	**7**	3	2	1	**6**
103	3	3	2	**8**	3	2	1	**6**
104	3	2	3	**8**	1	2	2	**5**
105	2	3	3	**8**	2	2	2	**6**
106	3	3	2	**8**	2	1	1	**4**
107	3	2	2	**7**	2	2	1	**5**
108	1	3	3	**7**	2	2	1	**5**
109	3	2	2	**7**	2	1	1	**4**
110	2	2	1	**5**	2	2	2	**6**
111	3	2	2	**7**	2	1	1	**4**
112	2	2	1	**5**	3	2	1	**6**
113	3	3	3	**9**	1	2	2	**5**
114	2	2	2	**6**	1	2	2	**5**
115	2	2	2	**6**	3	2	1	**6**
116	2	3	3	**8**	1	2	2	**5**
117	3	2	3	**8**	2	1	1	**4**
118	3	3	3	**9**	2	2	1	**5**
119	3	2	2	**7**	2	2	2	**6**
120	2	2	2	**6**	2	2	1	**5**
121	3	3	2	**8**	2	2	2	**6**
122	3	3	2	**8**	3	2	1	**6**
123	3	3	3	**9**	3	2	1	**6**
124	2	2	1	**5**	2	2	1	**5**
125	3	3	3	**9**	1	2	2	**5**
126	3	2	3	**8**	2	2	2	**6**
127	3	3	3	**9**	2	2	1	**5**
128	2	2	2	**6**	3	2	1	**6**
129	2	2	1	**5**	2	2	1	**5**
130	2	3	3	**8**	2	2	1	**5**
Mean	**2.5**	**2.5**	**2.5**	**7.5**	**2.1**	**1.8**	**1.3**	**5.2**
SD				**1.204**				**0.748**

Table 1. Evaluation of the Two Staining Modalities, each Parameter was Graded from 1 to 3. (n = 130)

Difference	-2.3
Standard error	0.124
95% CI	-2.5448 to -2.0552
t-statistic	-18.501
DF	258
Significance level	P < 0.0001

Table 2

- The man score of the DAB method was 7.5 with a SD of 1.204 and the values for MPO by old method were 5.2 and 0.748 respectively. A paired T-test was performed which showed a P-value of <0.0001 indicative of the fact that the new method was significantly better on all the three parameters assessed.

Figure 1. By Old Benzidine Method

Figure 2. By DAB Method

DISCUSSION

Subtyping of leukaemia by cytochemical staining dates back to 1950 to 1960. MPO staining was the backbone of cytochemical staining as distinguishing AML from ALL is the basic.[3] The disadvantages of the routine MPO staining was not only its carcinogenicity but also it is cumbersome and time consuming and with a background staining. The method evaluated in this study using 3, 3 Diaminobenzidine as the substitute for benzidine with uncertain carcinogenic potentiality, is liquid stable and is recommended by the International Committee for Standardisation in Haematology (ICHS) is sensitive, quick and easy to perform and also has a strong clear resolution product that is stable for storage.

CONCLUSION

The technique described using 3-3 diaminobenzidine for myeloperoxidase staining is rapid simple and relatively safe method for demonstration of MPO activity.

REFERENCES

[1] Fischel R. Der histochemisch Nachweis der Peroxydase. Wiener Klin Wschr 1910;223:1557-1558.
[2] Sato A, Sekiya S. A simple method for differentiation of myeloic and lymphatic leukocytes of the human blood. Tohoku J Exp Med 1926;7:111-115.
[3] Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol 1976;33(4):451-458.
[4] Dacie JV, Lewis SM. Practical haematology. 7th edn. Edinburg: Churchill Livingstone 1991:137-138.
[5] Goodpasture EW. A peroxidase reaction with sodium nitroprusside and benzidine in blood smears and tissues. Journal of Laboratory and Clinical Medicine 1919;4(7):442-444.
[6] Griswold DP, Casey AE, Weisburger EK, et al. On the carcinogenicity of a single intragastric dose of hydrocarbons, nitrosamines, aromatic amines, dyes, coumarins, and miscellaneous chemicals in female Sprague-Dawley rats. Cancer Res 1966;26(4):619-625.
[7] Kaplow LS. Simplified myeloperoxidase stain using benzidine dihydrochloride. Blood 1965;26:215-219.