Original Article

Efficacy of Toluidine Blue Staining in Rectal Biopsy of Patients with Hirschsprung Disease

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Abstract

Background: *The diagnosis of Hirschsprung disease (HD) is dependent on the histological study of rectal biopsy for ganglion cells and nerve fibers. The diagnosis is not possible with routine H&E staining in every times, because staining has limitations in the diagnosis of immature ganglion cells in neonates and in the submucosal area where the ganglion cells are small in number. Using Toluidine blue (Tb) stain it has become easier to identify ganglion cells.*

Objective: *To find out the efficacy of toluidine blue staining in the diagnosis of Hirschsprung disease.*

Materials and Methods: *A study was carried out with the histological findings of 50 rectal punch biopsies and 60 full thickness rectal biopsies from corresponding same patients from March 2018 to February 2020. After paraffin embedding, slides were stained with H&E and Tb stains. Then stained sections were examined for the presence or absence of ganglion cells and hypertrophic nerve fibers in the submucosa and in between muscle layers.*

Results: *Out of 50 punch biopsy cases, 33(66.0%) cases with H&E stain and 35(70.0%) cases with Tb stain showed presence of hypertrophic nerves (HN). In Non-HD cases, with H&E stain 11(22.0%) cases showed somewhat easy to identify ganglion cells. By using Tb stain ganglion cells were easy to identify in 4(8.0%) cases and somewhat easy to identify in 8(16.0%) cases. Out of 60 full thickness rectal biopsy 38(63.30%) cases showed presence of hypertrophic nerves in both H&E and Tb stains. In Non-HD cases with H&E stain ganglion cells somewhat easy to identify in 8(13.3%) cases; easy to identify in 9(15.0%) cases and very easy to identify in 2(3.3%) cases. By using Tb stain, the identification of ganglion cells were 1(1.7%), 9(15.0%) and 9(15.0%) respectively. Study showed Tb stain was superior to H&E in the identification of ganglion cells and hypertrophic nerves.*

Conclusion: *Toluidine blue stains should be used as the routine stain in adjunct to H&E stain to highlight ganglion cells in suspected Hirschsprung disease.*

Key words: *Ganglion Cells, Hematoxylin and Eosin, Hirschsprung disease, Hypertrophic nerves, Toluidine blue*

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Introduction

Hirschsprung disease is a developmental disorder of the enteric nervous system occurs in about one in 5000 births.[1] Histologically, it is characterized by absence of ganglion cells in the myenteric and meissner plexuses and presence of hypertrophied nerve terminal along variable portion of the distal intestine.[2] The causes of Hirschsprung disease are defect in the craniocaudal migration of neuroblasts originating from the neural crest, defects in the differentiation of neuroblasts into ganglion cells or accelerated ganglion cell destruction within the intestine.[3,4] These most commonly occur during the first 12 weeks of fetal development.

Rectal biopsy is the gold standard for diagnosis of HD.[5] Hematoxylin and Eosin (H&E) staining is commonly used in the diagnosis of Hirschsprung disease. However, diagnosis is not always possible with H&E staining, because staining has limitations in the diagnosis of immature ganglion cells in neonates and in the submucosal area where the ganglion cells are small in number (three to five cells per ganglion) and irregularly distributed. So their identification is difficult and requires high expertise.[6]

Toluidine blue stain (Tb) is a synthetic, acidophilic metachro

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matic dye that has an affinity for nucleic acids, and therefore binds to nuclear material with a high DNA and RNA content, in chromatin or Nissl substance and selectively stains nucleus blue and cytoplasm light blue. Other acidic tissue components (sulfates, carboxylates, and phosphate radicals) are stained in shades of blue. Toluidine blue stains mast cells metachromatic violet (with histamine and heparin metachromatic granules). Metachromasia is attributed to stacking of dye cations at the sites of high density of anionic groups in the tissue. Stacking shortens the wavelength of maximum absorption, a hypochromic shift, so that the maximum wavelength in the spectrum of the transmitted light is longer making the observed color red instead of blue.[7] In previous many studies regarding nerve cells and its identification toluidine blue was found superior to routine H&E stain. [8-13]

Toluidine blue stain is mainly used to highlight the ganglion cells in suspected Hirschsprung disease by the principle of metachromasia and it shows superior to H&E for identification of ganglion cells in rectal specimens.[13] Hence rectal biopsy with Toluidine blue stain becomes the initial and definitive modality used to evaluate the patient with constipation specially where immunohistochemistry are not available. In this study we find out the efficacy of toluidine blue staining in the diagnosis of Hirschsprung disease.

Materials and Methods

This descriptive cross-sectional study was carried out from March 2018 to February 2020, in the Department of Pathology of Mymensingh Medical College, Mymensingh, Bangladesh. All specimens of rectal punch biopsy and full thickness rectal biopsy from children suspected to be having HD were studied. The specimens were received in 10% formalin solution and processed for paraffin embedding. Multiple serial sections were cut at 3–5 µm thickness. About 4–6 sections per slide were mounted and two slides were made and were stained with H&E and Tb, then examined under light microscope. H&E and Tb stained sections were examined for the presence or absence of ganglion cells and hypertrophic nerve fibers in the submucosa and in between the muscle layers.

In H&E and Tb special stains, all cases of rectal biopsies were graded as follows:

1 + Somewhat easy to identify;
2 + Easy to identify;
3 + Very easy to identify;
- No ganglion cells seen.

Results

A total 50 rectal punch biopsy specimens and 60 rectal full thickness biopsies were studied. Analysis of rectal punch biopsies revealed 38 (63.3%) were aganglionic (HD), 12 (20.0%) were ganglionic (Non-HD).

Table I: Distribution of the study population according to identification of ganglion cells (GC) by H&E and Tb stain in punch biopsy (n=50)

Tb	H&E		p value*
	Present	Absent	
GC			
○ Present (n=12)	11 (100.0)	1 (2.6)	<0.001
○ Absent (n=38)	0 (0.0)	38 (97.4)	
Total (n=50)	**11 (100.0)**	**39 (100.0)**	

*Fisher's Exact test was done to measure the level of significance.
Figure within parenthesis indicates the percentage.

Table II: Distribution of the study population according to the identification of hypertrophic nerve (HN) by H&E and Tb stain in punch biopsy (n=50)

Tb	H&E		p value *
	Present	Absent	
HN			
○ Absent (n=15)	1 (3.0)	14 (82.4)	<0.001
○ Present (n=35)	32 (96.9)	3 (17.6)	
Total (n=50)	**33 (100.0)**	**17 (100.0)**	

*Fisher's Exact test was done to measure the level of significance.
Figure within parenthesis indicates the percentage.

Table III: Distribution of the study population according to identification and categorization of GC with H&E and Tb stain in punch biopsy (n=50)

Punch biopsy	H&E		Tb	
	Frequency	Percent	Frequency	Percent
HD				
No ganglion cells seen	39	78.0%	38	76.0%
Non-HD				
Somewhat easy to identify	11	22.0%	8	16.0%
Easy to identify	-	-	4	8.0%
Very easy to identify	-	-	-	-

Table IV: Distribution of the study population according to the identification of ganglion cells (GC) by H&E and Tb stains in full-thickness biopsy (n=60)

H&E		Tb	Total
Present		**Absent**	
GC			
Present (n=19)	19 (100.0)	0 (.0)	19 (31.7)
Absent (n=41)	0 (.0)	41 (100.0)	41 (68.3%)
Total (n=60)	**19 (100.0)**	**41 (100.0)**	**60 (100.0)**

Figure within parenthesis indicates the percentage

Table V: Distribution of the patients according to identification of HN by H&E and Tb stain in full thickness biopsy (n=60)

H&E	Present	Tb Absent	Total
HN			
Present (n=38)	38 (100.0)	0 (.0)	38 (63.3)
Absent (n=22)	0 (.0)	22 (100.0)	22 (36.7)
Total (n=60)	**38 (100.0)**	**22 (100.0)**	**60 (100.0)**

Figure within parenthesis indicates the percentage

Table VI: Distribution of the study population according to identification and categorization of GC with H&E and Tb staining in full thickness biopsy (n=60).

Full thickness biopsy	H&E		Tb	
	Frequency	Percent	Frequency	Percent
HD				
No ganglion cells seen	41	68.3%	41	68.3%
Non-HD				
Somewhat easy to identify	8	13.3%	1	1.7%
Easy to identify	9	15.0%	9	15.0%
Very easy toidentify	2	3.3%	9	15.0%

Discussion

Evaluation of rectal punch biopsies for the presence of ganglion cells remains very challenging, especially in negative or equivocal cases.[8] Most of cases biopsies are performed on newborn infants, in which the immature ganglion cells can be confused with endothelial cells or fibroblasts. As a result of these difficulties, a large number of histochemical and immunohistochemical stains have been proposed to assist in the identification of ganglion cells, or to delineate the nature of the nerve fibers in rectal punch biopsies.[9]

Toluidine blue stain is mainly used to highlight the ganglion cells in suspected Hirschsprung disease by the principle of metachromasia. It is superior to H&E for identification of ganglion cells in rectal specimens with statistically no significant difference between two stains.[9-12]

Canil et al who used Tb stain and found that Toluidine blue method is a reproducible and reliable way of demonstrating ganglion cells in frozen rectal biopsies.[13] The toluidine blue stains the neural cytoplasm of the ganglion, allowing for easy identification of the ganglion cells. This method provides faster and easier identification of ganglion cells than with H&E staining found by many other authors[13-15] and correlate with our study.

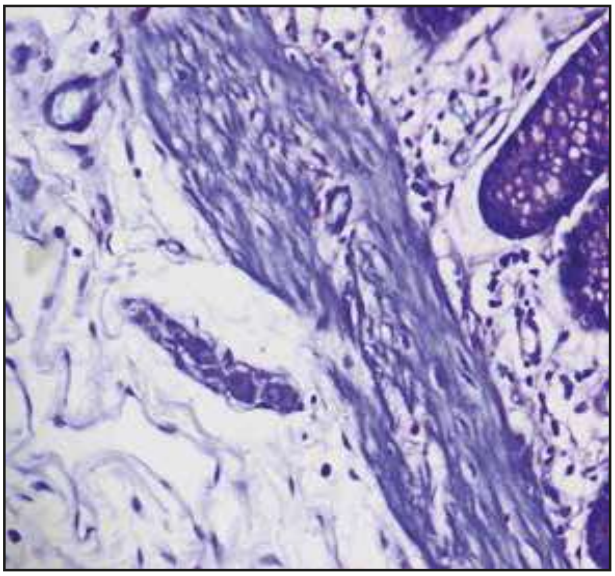

Figure 1: Photomicrograph shows ganglion cells in submucosal layer. (Tb stain 400x)

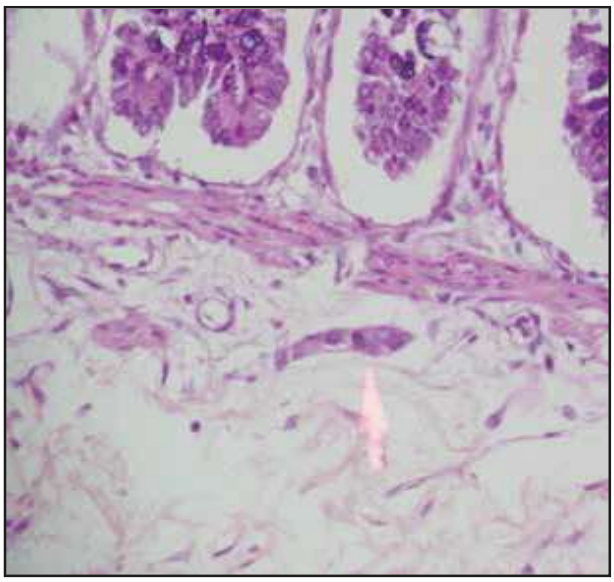

Figure 2: Photomicrograph shows ganglion cells in submucosal layer. (H&E stain 400x)

Tb stain is fast, easy, cheap, do not need a counter stain and can be performed in any laboratory with an adjunct to routine stain H&E in suspected HD cases.

Babu et al used H&E-and Tb and AChE-stained sections in their study to measure the thickness of nerve trunks in the submucosa using the Leitz oculometer.[16]

The hypertrophic nerve bundles, unlike the random arrangement of the round nuclei of Schwann cells in normal neuronal plexuses, had a parallel, longitudinal pattern of elongated Schwann cell nuclei in the nerve trunks. Collagen and a distinct perineurium were clearly seen in the hypertrophic nerve bundles but not in normal plexuses. A significant correlation between the presence of submucosal nerve bundle hypertrophy and HD cases is found in this study and confirmed by Toluidine blue.

Conclusion

Considering simplicity, feasibility, easy and faster identification of ganglion cell with toluidine blue stain; it should be used as an adjunct to the routine stain (H&E) to highlight the ganglion cells in suspected Hirschsprung disease cases in tertiary level hospitals where immunohistochemistry is yet to be practiced.

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