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Application of toluidine blue stain and neuron specific enolase immunohistochemical stain in the diagnosis of hirschsprung disease

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Abstract

Hirschsprung disease is one of the most common and problematic infancy and childhood maladies. Early and accurate diagnosis is a fundamental step in proper management and prevention of complications. The most reliable method for diagnosis is the histopathological analysis of colorectal biopsies and the typical finding of Hirschsprung disease is the absence of ganglion cells. Toluidine blue stain can act as a double check along with conventional H&E stain for ganglion cell detection. Neuron-specific enolase is an immune-histochemical marker that can also aid in better identifying ganglion cells, especially for small and immature ones. This study aimed to evaluate Toluidine blue stain and Neuron specific enolase immunostain along with conventional H&E stain as a panel for the diagnosis of Hirschsprung disease. This cross-sectional study was conducted from September 2019 to August 2021, involving 55 clinically suspected Hirschsprung disease cases. Paraffin blocks of colorectal biopsy samples were collected from the Department of Pathology, BSMMU. Hematoxylin & Eosin, Toluidine blue stain, and Neuron specific enolase immunohistochemical stain for Hirschsprung disease detection were performed on the sections from the paraffin blocks. Then the sections were examined and an evaluation of the stains was done. Statistical analysis was performed on the tabulated data by chi-square test. Among 55 cases, conventional H&E stain detected ganglion cells in 31 cases, that is 56.4%. Later, Toluidine blue stain and Neuron specific enolase immunohistochemical stain detected ganglion cells in 35 cases, that is 63.6%. So, these two additional stains were able to detect ganglion cells in four more cases compared to conventional H&E stain. In conclusion, conventional H&E stain, Toluidine blue stain, and NSE immunohistochemical stain can improve the diagnostic accuracy of Hirschsprung disease.

Introduction

Hirschsprung disease (HD) or congenital intestinal aganglionosis is a malformation of the enteric nervous system (ENS). It is characterized by the absence of parasympathetic intrinsic ganglion cells in the myenteric plexus and submucosal plexus.[1] In HD the absence of ganglion cells is accompanied by hypertrophy of nerve bundle in the submucosa and muscularis propria.[2] The incidence of HD is estimated to be one in 5000 newborns. In Asian people, the incidence ratio is 2.8 per 10,000 live births and there is a male predominance with male to female ratio 4:1.[3] Hirschsprung disease, being a congenital disease, is usually diagnosed in the neonatal period or early childhood. It may also present in adolescence or adulthood.[4] HD may be associated with Down syndrome in 10% patients and another 5% have other serious neurologic abnormalities.[5]

Diagnostic tests including contrast enema and anorectal manometry may be used as diagnostic screens for HD detection, but diagnosis ultimately depends upon histopathological evaluation of colorectal biopsy.[6] Histopathological examination of colorectal biopsy specimen is regarded gold standard for HD diagnosis.[7] Failure of correct histopathological diagnosis can lead to unnecessary operative procedure which may be catastrophic for the patient.[8]

Typical histologic features of HD include the total absence of ganglion cells (GC) in both submucosal and myenteric nerve plexuses. Presence of increased number of hypertrophic

nerve bundles is an additional positive diagnostic criterion.[9] Conventional Hematoxyline and Eosin (H&E) stain is commonly used in the diagnosis of HD. However, diagnosis is not always possible with H&E stain alone, because staining has limitations in the diagnosis of immature ganglion cells and the submucosal area where ganglion cells are small and irregularly distributed.[10]

Among several rapid stains available to identify ganglion cells and hypertrophic nerve trunk for HD detection in colorectal biopsy samples Toludine blue (Tb) stain is a common one. Toluidine blue is an acidophilic metachromatic dye that has an affinity for nucleic acids, and therefore binds to nuclear material with a high DNA and RNA content. It selectively stains nucleus blue, cytoplasm light blue and other acidic tissue components are stained in shades of blue.[11] Tb staining method is also a better method to identify ganglion cells in frozen section of colorectal biopsies.[12] Among various immunohistochemical marker used in the diagnosis of HD, Neuron-specific enolase (NSE) is used commonly. NSE can be demonstrated in mature and immature ganglion cells, normal nerve fibers and in the enlarged nerve bundles which are characteristic of HD.[2] Along with routine H&E stain, another stain like NSE can be used for the purpose of diagnosis of HD.[13] This study was conducted to see the expression of a special stain (Tb) and an immunostain (NSE) along with H&E stain as a panel for the diagnosis of Hirschsprung disease.

Methods

The cross-sectional observational study was conducted among 55 clinically suspected cases of Hirschsprung disease at the Department of Pathology of BSMMU from September 2019 to August 2021. After getting permission from the Institutional Review Board (IRB) of BSMMU, paraffin blocks of colorectal biopsy samples from suspected cases of Hirschsprung disease were collected for the study by inclusion and exclusion criteria. Then sections were cut and stained with H&E stain, Toluidine blue stain and NSE immune-stain. Sections were examined to see the presence or absence of ganglion cells and hypertrophied nerve bundles. Findings were recorded in the performed data sheet. The statistical analysis was carried out using the Statistical Package for Social Sciences version 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Descriptive statistics (frequencies and percentages) were used to summarize the patients' demographic characteristics and presented in Tables, Figures and Charts. The frequencies of different entities were expressed as a percentage. A p-value <.05 was considered statistically significant.

Results

The study included 55 clinically suspected cases of Hirschsprung disease, received at the department of Pathology, BSMMU. Mean age of the patients was 2.2±0.6 years (Table - I).

Table-I

Distribution of the patients by Age group (n=55)

Age group (years)	Frequency (n)	Percentage (%)
< 1	5	9.1
1-5	36	65.5
6-10	12	21.8
>10	2	3.6
Mean ± SD	2.2±0.6	
Total	55	100

Among all, 36 (65.5%) of the patients were male and 19 (34.5%) were female. Male and Female ratio was 1.8:1. Among the study patients, 81.8% had history of constipation, 47.3% had abdominal distension and 20% had history of vomiting. In H & E-stained sections, 56.4% cases had presence of ganglion cells and 43.6% had absence of ganglion cell. Besides, 56.4% had hypertrophic nerve trunk and 43.6% had no hypertrophic nerve trunk. 14.5% had presence of both ganglion cells and hypertrophic nerve trunk and 1.9% had absence of both ganglion cells and hypertrophic nerve trunk (Table - II).

Table-II

Histopathological findings in H and E (Hematoxylin and Eosin) stained sections of study cases (n=55)

Stain findings	Frequency (n)	Percentage (%)
Ganglion cell		
Present	31	56.4
Absent	24	43.6
Hypertrophic nerve bundle		
Present	31	56.4
Absent	24	43.6
Both Ganglion cell and Hypertrophic nerve bundles		
Present	8	14.5
Absent	1	1.9

In both Toluidine blue and NSE stained sections, 63.6% patients had presence of ganglion cell and 36.4% had absence of ganglion cell. About 56.4% patients had presence of hypertrophic nerve trunk and rest of the patients (43.6%) had absence of hypertrophic nerve trunk. Twenty percent (20%) patients had both ganglion cell and hypertonic nerve trunk (Table - III).

Table-III

Toluidine blue stain and NSE stain findings in study cases (n=55)

Stain findings	Frequency (n)	Percentage (%)
Ganglion cell		
Present	35	63.6
Absent	20	36.4
Hypertrophic nerve bundle		
Present	31	56.4
Absent	24	43.6
Both Ganglion cell and Hypertrophic nerve bundle		
Present		11 20

p value was determined by Chi-square test for comparison of H&E staining with Toluidine blue stain for ganglion cell detection and it was **<0.01** which is statistically significant (Table - IV). Same results were obtained in NSE immunostain when compared to H & E stain for detection of ganglion cell and p value was found statistically significant.

In this study, presence of ganglion cell was detected by H&E stain in 31 cases. But Toluidine blue stain and NSE immune-stain could detected presence of ganglion cell in 35 cases. Four cases were more detected by both of these stains. In case no. 4, where H&E stain was failed to detect ganglion cell but later Toludine blue stain and NSE immune stain were able to detect the ganglion cell (Figure – 1).

Table-IV

Comparison of H & E stain with Toluidine blue for the detection of Ganglion cells (n=55)

H & E stain findings	Toluidine blue stain		Total	P value*
	Ganglion cell present	**Ganglion cell absent**		
Ganglion cell present	31 (88.6)	0 (0)	31	
Ganglion cell absent	4 (11.4)	20 (100)	24 -	**<0.01**
Total	35	20	Total sample =55	

*p value was determined by Chi-square test.

H & E= Hematoxylin and Eosin

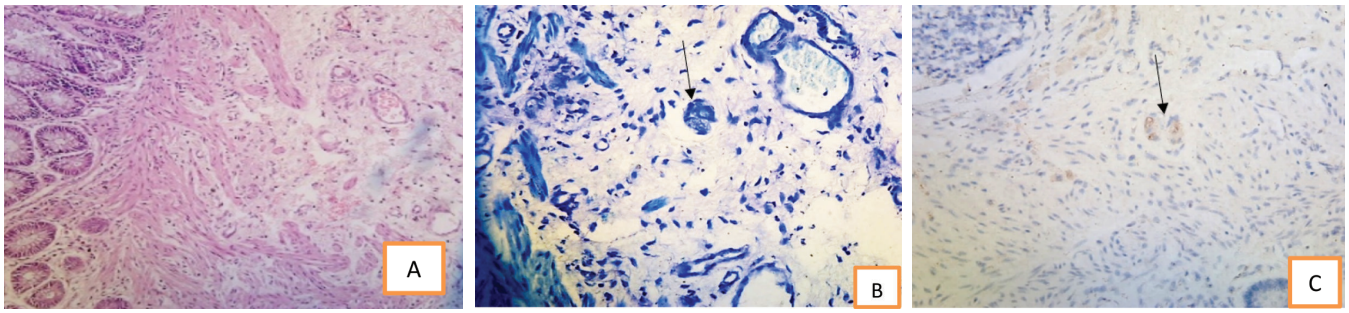

Figure -1: Ganglion cells in submucous plexus of colorectal biopsy, A (Case-4, H&E stain, X200), B (Case-4, Toluidine blue stain, X200), C (Case-4, NSE immune stain, X200).

Discussion

Hirschsprung disease is a common congenital anomaly which affect the pediatric age group. Delayed diagnosis of this disease leads to significant morbidity, for example failure to thrive, anemia and acute enterocolitis.[7] Despite various investigations, the only unequivocal criterion for diagnosis of this disease is the histological demonstration of complete absence of ganglion cells in the distal bowel.[14] Total 55 patients of suspected Hirschsprung disease were enrolled in this study. Age of the patients varied between 3 months to 11.2 years. Toluidine blue and Neuron specific enolase immuno stained sections of colorectal biopsies were used for the detection of ganglion cells and hypertrophic nerve bundles. Findings of these stains were compared to routine Hematoxylin and Eosin stain.

In the present study, majority (65.5%, n=55) of the patients were aged between 1-5 years with a mean age of was 2.2±0.6 years (SD). Male predominance was observed with 65.5% of total patient. In another study[15] conducted on 216 patients, male patients were 143 and female were 73. Ninety percent

children of their study were within one year of age (age range 03 day - 03 years). In the study by Yasseen et al[10], age of the patients ranged from 6 months to 84 months and the mean age was 19.4 months. In current study age range was 4 days to 12 years. As failure to pass meconium in 1st 48 hours is the key suspicion for Hirschsprung disease diagnosis, so samples of first 3 days were not taken. The children in this study are found slightly older than other studies.

Fifty-five cases were summarized in four groups according to findings of H&E-stained sections. Group A - had presence of ganglion cells and absence of hypertrophic nerve bundles (41.8%), Group B - had absence of ganglion cells and presence of hypertrophic nerve bundle (41.8%), Group C had presence of both ganglion cells and hypertrophic nerve bundle (14.5%) and Group D had absence of both ganglion cells and hypertrophic nerve bundle (1.9%). Ganglion cells were identified in 31 cases by routine H&E stain in the present study. Toluidine blue stain and NSE stain have detected ganglion cells in four more cases in comparison with H&E stain.

Memarzadeh et al.[10]conducted a study which included 54 rectal biopsy specimens of suspected Hirschsprung disease cases. Conventional H&E stain has detected absence of ganglion cells (negative) in 30 cases (55.5%), presence of ganglion cells (positive) in 17 cases (31.04%), and suspected presence of ganglion cells was observed in seven cases (12.9%). Later these findings were compared with IHC stained section findings which included S100, NSE, CD56 and Cathepsin D immunohistochemistry. Ganglion cells were detected in seven more cases which were absent in H&E stain. Yasseen et al[11]have divided their patients into two groups based on the findings of H &E stained sections of rectal biopsies. HD included 20 cases (40%) and non-HD included 30 cases (60%). By using Toluidine blue stain, they detected ganglion cells in four more cases which were undetected by H&E stain. In a retrospective observational study conducted in Department of Pathology, BSMMU, H&E stain was found quite effective in detecting presence or absence of ganglion cells in frozen section biopsy of suspected HD.[16]

In present study, Toluidine blue and NSE stain findings were summarized in three groups; Group A - 43.6% patients have presence of ganglion cells and absent of hypertrophic nerve bundle, Group B - 36.4% patients have absence of ganglion cells and presence of hypertrophic nerve bundle, and Group C - 20% patients have presence of both ganglion cells and hypertrophic nerve bundle. No group having absence of both Ganglion cells and hypertrophic nerve bundles were seen. Ganglion cells were detected in 35 cases by Toluidine blue stain. These findings correlate with the observation of Canil et al[12] as they concluded that Toluidine blue stain offered superior histological detail for identification of ganglion cells on rectal frozen sections compared to H&E stain. These findings also correlate with the observation of Yaseen et al[11] who had showed that Toluidine blue stain was superior to H&E for identification of ganglion cells. Zaidoon A. Musa et al[17] conducted a study which included 48 clinically suspected cases of HD and showed that sensitivity, specificity, positive predictive value and negative predictive value were all 100 % for NSE in the confirmation of HD. In this study Ganglion cells detection by NSE stain was quite easy as it strongly and diffusely stained their cytoplasm. In this study, Toluidine blue and NSE stains detected ganglion cell in four more cases than H&E stain.

Conclusion

Both Toluidine blue stain and Neuron specific enolase immuno stain detected ganglion cells in 63.6% patients whereas H&E stain only could detect ganglion cells in 56.4% patients of suspected Hirschsprung disease. To conclude, Toluidine blue stain can easily be done along with conventional H&E stain during colorectal biopsy for diagnosis of Hirschsprung disease. Immunohistochemical marker NSE can be used for suspicious cases as well for detection of hypertrophic nerve bundles which may aid in the diagnosis of Hirschsprung disease.

Conflicts of interest*:* The authors declare that they have no conflict of interest.

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