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Acetylcholinesterase histochemistry: A very useful technique in the diagnosis of Hirschsprung's disease

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ABSTRACT

Hirschsprung's disease (HD) is a common congenital paediatric disorder. The gold standard for its diagnosis is the demonstration of the absence of ganglion cells on H&E stained sections from a spastic segment of the bowel, still at times; conclusive diagnosis of HD is difficult. This study was done to assess the accuracy of rapid Hematoxylin & Eosin (H&E) staining and AChE histochemistry in combination for the diagnosis of HD, especially in frozen section specimens. Samples from 36 clinically suspected HD cases were evaluated for the presence or absence of ganglion cells on H&E staining on both fresh-frozen and paraffin-embedded sections, whereas AChE staining was done on frozen sections only. Of the 36 cases of frozen section specimens from the spastic region of the colon, GCs were absent in 28 cases on H&E staining while positive staining patterns on AChE histochemistry were observed in 29 cases. Formalin-fixed paraffin-embedded sections showed the absence of GC on H&E staining in 28 cases. The sensitivity and specificity of frozen sections with rapid H&E were 77.78% and 81.82% while the sensitivity and specificity of AChE histochemistry were 80.56% and 81.82% respectively in the diagnosis of HD. Application of AChE histochemistry as an ancillary technique on frozen sections specimens of HD in combination with rapid H&E staining is very useful for definitive diagnosis of HD.

INTRODUCTION

Hirschsprung's disease (HD) is a common congenital paediatric disorder characterized by the absence of enteric neurons or ganglion cells (GC) in the myenteric and submucosal plexuses of the colon causing intestinal obstruction. It results from the failure of GC to migrate caudally during embryonic life. The loss of GC extends for a variable distance above the anorectal junction.[1]

Aganglionosis in the neural plexus is noted in all the layers of the colon together with hypertrophied nerve bundle (HTNB). There is increased cholinergic activity as demonstrated by increased Acetylcholinesterase (AChE) staining as well as the absence of non-adrenergic inhibitory nerves, which are required for normal bowel relaxation. Current criteria for histochemical diagnosis of HD are failure to demonstrate GC and positive staining for AChE in nerve fibers within lamina propria (LP), muscularis mucosa (MM), submucosa (SM), Muscularis propria (MP), and Myenteric plexus (MyP) [2], which is based on the fact that the cholinergic nerve fibers of the aganglionic segment contain an increased amount of AChE and hence are prominent. Therefore, the need for AChE histochemistry on the frozen section is considered a reliable ancillary technique for diagnosis. The aim of the present study was to assess the accuracy of rapid Hematoxylin & Eosin (H&E) staining and AChE histochemistry in combination for the diagnosis of HD, especially in frozen section specimens.

METHODS

This study was carried out in the Department of Pathology in collaboration with the Department of Paediatric Surgery, from March 2012 to February 2015. The Institutional Ethics Committee approval was taken. The patients attending the Outpatient Department of Paediatric Surgery with constipation, abdominal distension, vomiting, and dehydration during this period underwent clinical screening for HD which included detailed history, clinical

examination, routine investigation, X-ray abdomen, and barium enema. Patients suspected or clinically diagnosed as HD underwent diagnostic biopsy and/or pull through.

All types of biopsies including seromuscular, submucosal, and excised specimens (either fresh or formalin-fixed) from the suspected HD cases were included in the study. Fresh tissues were collected in saline-soaked gauze pieces. Representative sections were taken from proximal (colostomy), transitional and spastic sites from the freshly excised specimens. The remaining tissue was fixed in 10% formalin and processed for routine histopathological examination (HPE).

Rapid H&E on frozen section

Fresh small biopsies and sections from fresh resected specimens were processed immediately for frozen section and further histochemistry. Fresh tissue was properly oriented (mucosal surface kept outside) and a small amount of freezing gel poured on and was kept on the cold cryostat. When the tissue was frozen completely, 10 micron thick sections were cut. Multiple sections from each region (Proximal, transition, and spastic) were taken on 5 to 8 slides. Air-dried sections were dipped in Hematoxylin for 15 seconds and were washed in running water followed by a dip in ammonia water, washed in running water then stained in eosin for 30-40 seconds. Sections were dehydrated with three graded alcohol. Cleared the slides with xylene and mounted them. Sections were analysed for the presence or absence of GC and HTNB under the light microscope. If GC were absent on initial evaluation serial sections were assessed for the presence of GC.

AChE stain on frozen section & its interpretation: For AChE stain, the modified AChE staining technique of Kini et al., 2010 was used.[3] AChE stained fresh frozen sections were assessed for the presence of stained nerve fibers in Mucosa, LP, MM, SM (plexus/upper/deep), MyP & MP (inner & outer).

Interpretation: Nerve fibers and the cytoplasm of GC stained positive (black). The unstained large nucleus of GC appeared as a negative shadow on a black background. Patterns of staining were formulated by utilizing criteria similar to those initially described by Challa et al., 1987.[4]

Pattern A: A Positive staining of nerve fibers was observed in the MM, SM, and in between the crypts in the LP. The nerves travel in between the crypts in the LP and may reach the surface. This is also called a 'mature pattern' and is usually seen in infants above 3-6 months of age. Pattern B: A positive staining of nerve fibers is observed in the MM, SM, and only at the base of the crypts in the LP. Nerves fibers going up along the crypts are not observed. This pattern is also called an 'immature pattern' and is usually seen in neonates and infants below 3 months of age.

Equivocal Pattern: The positive staining of nerve fibers is observed only in the SM. It can be either associated with or without HTNB in the SM. An equivocal pattern associated with HTNB is suggestive of HD whereas an equivocal pattern without HTNB is truly equivocal and cannot be suggestive of HD.

Negative Pattern: In the negative pattern, MM and LP lack stained nerve fibers, but small branches are seen in the SM. The submucosal GC stains positive with negative darkness of the nuclei, which if present, strongly suggests the presence of GC and negates the diagnosis of HD.

Formalin-fixed paraffin-embedded (FFPE) biopsies and sections from spastic, transition, and proximal parts of remaining resected specimens were stained with H&E (Kiernan et al, 2008) [5], and were assessed for the presence or absence of GC and presence of HTNB under the light microscope. A diagnosis of HD was made when no GCs were demonstrated on the FFPE section & a positive AChE pattern was recorded. SPSS software version 16.0 was used for the statistical analysis.

RESULTS

Present study included 36 patients with clinically suspected HD and 11 patients with intestinal obstruction (working control). Of these 36 specimens, 2 were mucosal biopsy, 3 seromuscular biopsy, and 31 resected specimens. The age at presentation ranged from 3 days to 9 years, with 70% (25) patients below 2 years of age. 18 (50%) cases aged from 1 day to 1 year. Male to female ratio was 6.2:1 (31 male and 5 female).

Interpretation of rapid H&E stain frozen section

Microscopic examination of frozen sections from spastic, transition and proximal sites of 36 clinical HD cases stained with rapid H&E showed results as mentioned in table 1.

Interpretation of AChE histochemistry

Sections from the spastic site (n=36) revealed, that AChE stained nerve fibers in 29 cases; however, their distribution was variable in various parts of the intestinal wall (Table 1). Accordingly, staining patterns observed in 29 cases were categorized as Pattern A in 19 cases, Pattern B in 5 cases, equivocal pattern favouring HD in 2 cases, and Mixed pattern in 3 cases (Figure 1). In one case in addition to positive stained nerve fibers, the unstained nucleus of GCs which appeared as a negative shadow on a black background was also noted. Seven specimens were negative for AChE staining. At transition zone (n=28) and proximal resected site (n=36), AChE staining

results are as mentioned in table 1. AChE staining was negative in all the 11 control specimen sections.

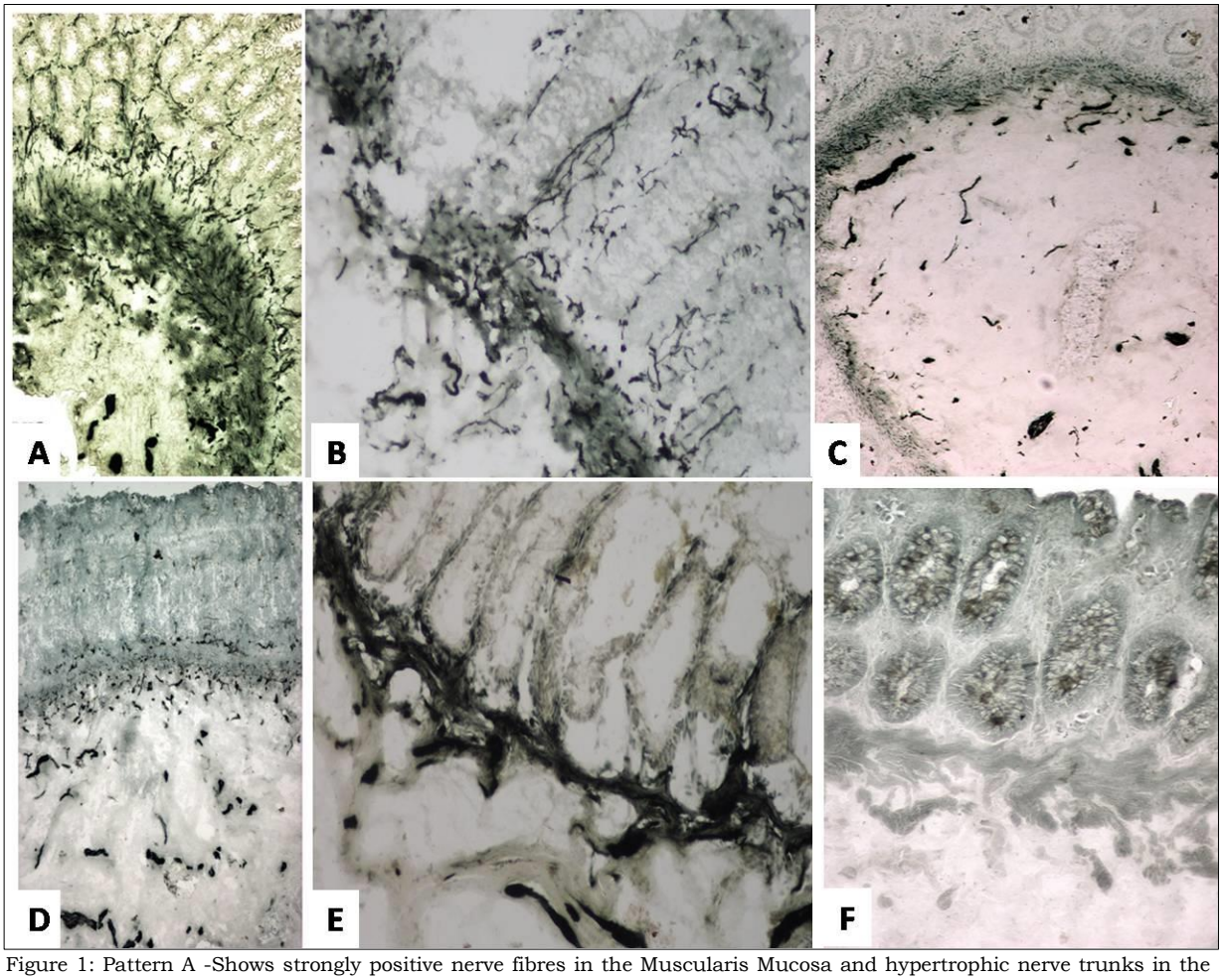

Figure 1: Pattern A -Shows strongly positive nerve fibres in the Muscularis Mucosa and hypertrophic nerve trunks in the Submucosa. Positive nerve fibers are going up into the Lamina Propria along with the crypts (A and B). Pattern B – Shows positively stained nerve fibers in the Muscularis Mucosa and Submucosa. No nerve fibers are seen in the Lamina Propria (C and D). Equivocal Pattern-Nerve fibers in the Sub-mucosa showed only positive staining with hypertrophied nerve trunk suggestive HD (E). No staining in Non- HD cases (F).

Table 1: Distribution of Positively stained nerve fibers on H&E, AChE fresh frozen stained section, and formalin fixed H&E stained section of suspected cases of HD (n=36)

Site	Frozen section H&E			Formalin fixed H&E			Staining of AChE							Interpretation
	Ganglion cells		Presence of HTNB	Ganglion cells		Presence of HTNB	Lamina	MM	SM (U)	SM (L)	MP (I)	MyP	MP (O)	Positive (Negative)
	Absent	Present		Absent	Present									
Spastic (n=36)	28	8	13	28	8	25	23	21	22	19	28	29	23	29 (7)
Transition (n=28)	8	20	4	8	20	20	2	6	12	12	4	6	4	8 (20)
Colostomy (n=36)	0	36	2	0	36	2	4	3	11	8	6	5	4	0 (36)
Control (n=11)	0	11	0	0	11	0	-	-	-	-	-	-	-	0 (11)

AChE: Acetylcholinesterase, U: upper, L: Lower, I: Inner, O: Outer, HTNB: Hypertrophied Nerve Bundle, H&E: Hematoxylin & Eosin, MM: Muscularis, Mucosae, SM: Sub-mucosa, MP: Muscularis Propria, MyP: Myenteric Plexus.

Diagnosis on fresh tissue based on AChE staining pattern and rapid H&E

Out of 36 cases, 29 stained positive with AChE while 7 cases stained negative. Of these 29 cases, ganglion cells were not detected in 28 cases even after a diligent search, whereas one case showed the presence of ganglion cells on rapid H&E on frozen sections. Thus the diagnosis of HD was offered in 28 cases. Whereas the diagnosis of HD was ruled out in the case which showed the presence of ganglion cells in addition to positive staining for AChE histochemistry.

Diagnosis on Formalin-fixed paraffin-embedded (FFPE) sections

Microscopic examination of H&E stained FFPE sections from the spastic site of 28 cases showed absence of GC. HTNB were also observed in the spastic segment in 25 cases only whereas it was absent in three of the cases. Thus the Diagnosis of HD was confirmed in 28 cases.

Intestinal neuronal dysplasia (IND): In the present study, one patient was clinically diagnosed as a case of HD and underwent colostomy; biopsy showed the presence of ganglion cells while AChE histochemistry revealed Pattern A staining. Paraffin-embedded sections from this case revealed Giant ganglion. Therefore a diagnosis of intestinal neuronal dysplasia (IND) was offered in this case.

The Colostomy (Proximal) site of all the specimens (n=36) showed the presence of GC & HTNB in 2 cases. Sections from the Transition Zone showed the absence of GCs in 8 out of 28 cases & presence of HTNB was noted in 20 out of 28 cases.

Sensitivity and specificity

Sensitivity and specificity of rapid H&E on frozen sections and AChE histochemistry in the diagnosis of HD were calculated by taking paraffin-embedded H&E stained sections as the gold standard. The sensitivity of rapid H&E on the frozen section in the diagnosis of HD is 77.78 % and specificity is 81.82 % ($p<0.0001$). The positive and negative predictive value is 93.33% and 52.94% respectively ($p<0.0001$). The sensitivity of AChE histochemistry in the diagnosis of HD is 80.56 % and the specificity is 81.82 %. The negative and positive predictive value was 56.25% and 93.55% respectively ($p<0.0001$).

DISCUSSION

Diagnostic modalities for HD are barium enema, anorectal manometry, and histopathology. Histopathology is the gold standard for diagnosis and is mandatory before surgery in patients with HD. Histologically HD is characterized by the absence of colonic GC. The reliability of this method depends on the observer's ability to accurately detect a GC based on its morphology on H&E stained sections. Different approaches have evolved to identify GC. Most paediatric pathology laboratories, evaluate numerous H&E stained levels from each paraffin-embedded biopsy to confirm the absence of GCs.[6]

Although the absence of GC in the nerve plexuses is a key finding of HD, other abnormalities of innervation have also been demonstrated with the aid of various neural markers.[7] HD is characterized by profuse nerve plexuses in the SM, MM, and nerve fibers running transversely and forming a network of thin terminal nerve fibers in the LP. All these nerve plexuses have very intense AChE activity. The development of the rectal suction biopsy technique by Noblett in 1969 [9] and the introduction of AChE histochemistry by Meier-Ruge et al, 1972, were major landmarks for HD diagnosis. AChE histochemical staining has demonstrated hypertrophic submucosal plexuses and nerve fibers, which have been used as a useful ancillary diagnostic tool for the interpretation of suction biopsies and have gradually replaced conventional full-thickness biopsy at many institutions.[8,10,11]

FFPE H&E stained section along with AChE is a perfect combination for making a conclusive diagnosis.

The present study showed male preponderance, similar to reports by Agrawal et al. [2.5:1] and Schofield DE et al [4:1].[1,12]

We demonstrated sensitivity of 77.8% & specificity of 81.82% for the diagnosis of HD on frozen section H&E. Agrawal et al. evaluated 72 samples and reported sensitivity of 57.57% on rapid H&E on frozen section and specificity of 79.10% while Mohsen et al. documented sensitivity of 85.8% and specificity 90.2% with H&E staining on frozen section and reported a positive and negative predictive value of 93.9% and 78.3% respectively.[1] The slightly low rates in the current study may have been due to the small sample size.

AChE histochemistry showed positive staining in 29 cases while 7 cases stained negative. Among these, a final diagnosis of HD was offered in 28 cases while one case was diagnosed as IND. Out of 29 cases, Pattern A was observed in 19 cases, and 10 of these were aged more than 6 months. Pattern B was seen in 5 cases, with equivocal and mixed patterns favouring HD in 2 and 3 cases respectively. Agrawal et al.,2015 reported 17 samples (out of 72) as either A (10 cases) or B (7 cases) patterns with AChE histochemistry. One case showed a mixed pattern, which was predominantly pattern B. 5 samples showed an equivocal pattern without HTNB, which was considered to be negative.[1] Schofield et al., 1990 found Pattern A in 33 cases and Pattern B in 25 cases in a study of 60 cases.[12] In the present study, the

sensitivity of AChE histochemistry staining was 80.56% and specificity was 81.82% (p<0.0001). Agrawal et al and Nakao et al. found a sensitivity and specificity of 90.47% & 96.36% and 91% & 100% respectively for AChE staining.[1,13] The lower values in the current study may have been due to the small sample size. Bagdzevičius et al [14] documented the sensitivity and specificity of the AChE staining in mucosal-submucosal rectal biopsies as 40% and 100% respectively in neonates (Table 2). Thus, AChE histochemistry is an excellent technique for confirming the presence or absence of GC.

Table 2: Comparison of sensitivity, specificity, positive predictive and negative predictive value of previous studies on Frozen H&E staining

Authors	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Nakao M et al [13]	91%	100%	-	-
de Lorijn F et al [18]	96%	98%	-	-
Haricharan RN et al [19]	97-100%	99-100%	-	-
Mohsen R et al [11]	85.8%	90.2%	93.9%	78.3%
Agrawal et al [1]	90.47%	96.36%	-	-
Present Study	77.78%	81.82%	93.33%	52.94%

False-positive and negative results have been reported by many authors. The age of the patient is a very important factor for false-negative results.[15,16] Possible causes for false-negative test results include variability in the biopsy site. Superficial biopsy lacks MM. Other important causes of false-negative results are immaturity of the enzyme system, technical variations in the performance of staining, and the experience of individual pathologists.[17]

Colonic aganglionosis along with positive or high AChE staining is a diagnostic hallmark of HD. Diagnosis of HD remains difficult due to the spectrum of its clinical presentation and the reluctance of pathologists to label a case as HD because of the negative nature of diagnosis on histopathology. In neonates, diagnosis of HD is difficult due to paucity, scarcity, and immature development of ganglion cells, and therefore categorically labelling an index cell as a GC is very difficult at times. Furthermore, distinguishing GC from endothelial or other submucosal cells may be difficult. In addition to the immature sub-mucosal GCs in the very young age group (neonates & infants), HTNB in the LP and MM are not always detectable.

Most authors report that AChE staining of rectal mucosal biopsy specimens is essential for a definite diagnosis of HD, especially when barium enema and anorectal-manometry tests are not informative due to total colonic aganglionosis (TCA), short-segment HD (SSHD), neonatal age, or presence of colostomies. The introduction of the AChE histochemical method is considered as a path breaker in difficult cases. The requirement of a fresh frozen section and a high level of expertise are the main disadvantages. Due to the complex technology of preparation and lack of experience, AChE histochemistry seems cumbersome and thus is used in specialized Pathology Centres only. But once the technique is mastered, there is no looking back. However, modern commercial diagnostic sets, using modified lyophilized media are in the market and they will no doubt, increase the number of laboratories using AChE histochemistry for both preoperative diagnosis and in aiding the intraoperative procedures of HD.

Apart from these technical complexities, interpretation of AChE staining needs several other considerations. In neonates, especially within the first 3 weeks of life, an increase in AChE reaction is not detected in patients with HD. In the neonatal age group, the enzyme system is immature and MM is not well developed, which further limits the use of AChE. Due to this drawback, the requirement for more specific neural markers needs to be considered. Several immunohistochemical markers have been tried to look for GC in paraffin-embedded tissues. Recently two new markers, calretinin and PGP9.5 [20] have been used as an adjunct in the diagnosis of HD, but these cannot be used in the intraoperative setting.

CONCLUSION

The application of AChE histochemistry as an ancillary technique on frozen sections specimens of HD in combination with rapid H&E staining is a very useful technique for definitive diagnosis of HD, especially in difficult cases.

List of Abbreviations

AChE: Acetylcholinesterase, GC: ganglion cells, HD: Hirschsprung's disease HTNB: hypertrophied nerve bundle, LP: lamina propria, MM: muscularis mucosa, MP; Muscularis propria, Myp; Myenteric plexus, PGP 9.5: Protein Gene Product 9.5, SM submucosa, SSHD: short-segment HD. TCA: total colonic aganglionosis, FFPE: Formalin-fixed paraffin-embedded, HPE; Histopathological Examination.

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